Thèse de Doctorat à l'Université Paris-Est Créteil

Ecole Doctorale SIE:

Sciences, Ingénierie, Environnement

Macro and micro-evolutionary processes within a complex of species, case study of the tropical invasive earthworm; *Pontoscolex corethrurus*

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Date de Soutenance : 6 mars 2018

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Remarque préliminaire : Cette thèse est rédigée en anglais, cependant, étant donné qu'elle a été réalisée en France, un résumé en français est également fourni à la fin du manuscrit.

Acknowledgments

First of all, I would like to express my gratitude to Kurdish institute of Paris and Doctoral school of Science, Engineering and Environment (SIE) for the financial support which permitted me to do my PhD during three years.

I want to express my gratitude to the revisers of my manuscript Jorge Domínguez, Myriam Valero, Thiabud Decaëns, Rodolphe Rougerie and Virginie Roy.

I am deeply grateful to Lise Dupont, my thesis supervisor, with whom I have worked since my masters' internship. Her trust in me when I started working with her meant a lot to me. I would like to thank her for her constant availability and all the things she taught me during these years to make me a better student and a future researcher in the field of evolutionary biology.

I would like to thank members of BioDis group, for their support during these three years. Particularly, I would like to thank Naoise Nunan whom without his support I couldn't participate the international conference on soil zoology in Japan.

During my thesis, I greatly benefited from vast collaborations all over the world and more precisely I would like to thank; Céline Pelosi whom was my co-supervisor during masters' internship, and during chapter one, I benefited from her guides and useful discussions. George Brown, Luis Cunha and Elodie Da Silva for my two month stay in Curitiba, Brazil, and the collaboration for samplings in Brazil and Azores Island. Jean-Pierre Coutanceau and Chantal Guidi-Rontani for the cytogenetics experimentations. I am also grateful to Patrick Lavelle for his encouragement and enthusiast for the results I gained, and nice conversations we had during Japan's conference. And finally, I would like to thank Thiabud Decaëns, for the samples he provided me from all over the world.

I was so lucky to have the support of my friends whom their presence meant a lot to me in different stages of this adventure; Valentina Cima, Matt Cabaret, Mahsa Dehghan, and also other PhD students with whom we shared constructive comments and warm encouragements; Romain Fougeyrollas and Charlotte Pruvost. I would like to thank particularly Maeva Hirsch, who helped me a lot in the redaction of French parts and was always present when I needed a friend.

I would like to express my gratitude to Hélène Audusseau and Marine Zwicke, two precedent ATERs of BioDis group. They were my colleagues at University, but eventually became friends and their experiences and suggestions helped me through my thesis.

I would like to show my greatest appreciation to my sister and brother in law, Parisa and Reza, for their constant heartwarming presence, even though from far.

My heartfelt appreciation goes to Milad for his unconditional love, constant presence in difficult moments, and his informatic helps and suggestions, all the way through this journey.

Finally, my deepest gratitude goes to my parents, to whom I would like to dedicate this doctoral thesis of mine which I have written during long days and nights without sleep but with lots of love. To my angels without whom I wouldn’t be ‘’who I am’’.

بزرگترین قدردانی ها نثار پدر ومادر بزرگوار ومهربانم، که این نوشتار را به آنها تقدیم می کنم. دو فرشته ای که "وجودم" از وجود آنهاست.

بی عشق نشاط وطرب افزون نشود
بی عشق وجود خوب وموزون نشود
صد قطره ز ابر اگر به دریا بارد
بی جنبش عشق در مکنون نشود
(مولوی)

I. Introduction

I.1. Biological invasion

I.1.1. General definition

Invasive species are often defined as those who overcome a geographical barrier and are found in areas out of their native zone. Another criterion used by scientists to determine that a species is invasive is based on its negative impacts on the ecosystem. But the question is; what is the threshold for a species to be considered as having negative impacts? One general definition that will be used in this thesis is : "a biological invasion consists of a species' acquiring a competitive advantage following the disappearance of natural obstacles to its proliferation, which allows it to spread rapidly and to conquer novel areas within recipient ecosystems in which it becomes a dominant population" (Valéry et al., 2008).

Negative impacts of invasive species on indigenous biodiversity, ecosystem functions and services, and agricultural productivity (review in Anderson et al., 2017), may result in severe economic impacts which could range from millions to billions of dollars annually (Sakai et al., 2001). For instance, the cost of the invasion by the neotropical termite *Nasutitermes corniger* in Florida, was estimated between $6.9 and $9.9 million within ten years (Alvarez, 2016). Although some biological invasions could result in severe economic losses, there are a large number of non-native species that result in overall gains for society, or in no deleterious impacts at all. For instance, it has been estimated that 98% of food production in the United States relies on non-native crops and livestock (review in Alvarez, 2016). Therefore, reliable information on the potential negative impacts of invasive species is primordial for good policy decision-making programs since in some cases the costs of control, eradication and mitigation measures may exceed their benefits (Alvarez, 2016).

I.1.2. Steps of the biological invasion process

There are three main stages for successful introduction and following invasion of a species (Figure 1); 1) introduction of a new species in a new habitat, 2) species initial colonization and successful establishment, and 3) afterwards dispersal and secondary spread in new habitats (review in Sakai et al., 2001). In each stage, several factors impact the invasion process, these factors are related to the species characteristics, the environment it has invaded and propagule pressure (i.e., propagule number, size, and temporal and spatial patterns of propagule arrival) (Simberloff, 2009). The impact of propagule number is mainly related to decreasing the impacts of environmental stochasticity, while increasing propagule size enhances establishment probability by decreasing the effects of demographic stochasticity. For instance, Ahlroth et al. (2003) introduced propagules from 2 to 16 mated females of waterstriders (*Aquarius najas*) into 90 Finnish streams and found that the probability of population establishment increased with propagule size (Simberloff, 2009).

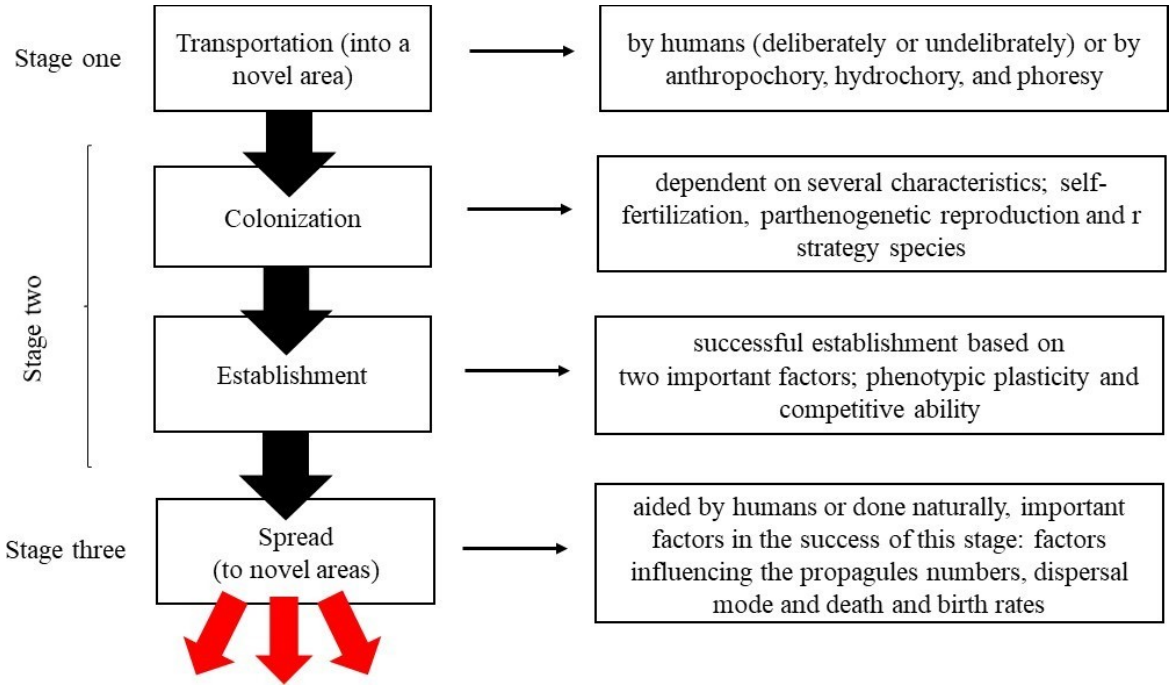


Figure 1 : Different stages of invasion and the factors influencing the success of each stage based on Sakai et al. (2001).

The phase one of the invasion is the introduction of a species into a new habitat which could be done deliberately or undeliberately by anthropochory, hydrochory, and phoresy i.e., dispersal by humans, water, and a nonparasitic relationship in which one species is carried by another, respectively (Sakai et al., 2001). Once the species is introduced in a new environment, the second phase of invasion starts. This phase is divided into two parts; initial colonization and establishment. The success of an invader in initial colonization depends on several characteristics. Self-fertilization or 'selfing' and parthenogenetic reproduction (types of uniparental reproduction that allow the establishment of a new population without the necessity of having a mating partner), are considered as one of the most important qualities of a species when introduced in a new habitat (Sakai et al., 2001). One of the reasons of flash invasion (i.e., recently initiated invasion by a very efficient clone) of *Pseudosuccinea columella*, an invasive hermaphroditic freshwater snail, from north America into the world is selfing (Lounnas et al., 2017). These results were inferred by population genetics analyses of eight nuclear microsatellite markers and two mitochondrial genes, where a unique haplotype/genotype was found in invaded populations. Among the successful parthenogenetic invasive species is the hemlock woolly adelgid, an insect that has spread across a large geographic temperature gradient in its introduced range (this insect is native to Asia as well as to the Pacific Northwest of the United States but now invasive in almost all of United States) (Lombardo and Elkinton, 2017). Another important quality of an invasive species in the second phase of invasion (initial colonization), is the r strategy of species (i.e., use of pioneer habit, short generation time, high fecundity, and high growth rates) (Sakai et al., 2001). The other stage of second phase of invasion is establishment. The success of an invasive species during this stage is closely linked to phenotypic plasticity and competitive ability. These abilities are important

characteristics in order to cope with different environmental conditions and to compete with other species, especially with native ones (Sakai et al., 2001). An example of competitive ability between species could be the case of *Prosopis juliflora*, an aggressive invasive plant species, which releases L-tryptophan amino acid that inhibits the growth of other plants (Mukherjee et al., 2017). This allelopathic ability likely helped the establishment of *P. juliflora* as an invasive species. Once the invasive species is established it goes through the last phase of invasion which is the spread and dispersal in other habitats which are done naturally or aided by humans. Several factors are important in the success of this stage; such as factors influencing the propagules numbers, dispersal mode and death and birth rates (review in Sakai et al., 2001).

Most of the introduced species fail at the first stages of invasion process e.g., during establishment phase (Facon et al., 2003) and cannot carry on to other steps due to numerous reasons. One of the explanations could be related to founder effect or bottleneck effect (i.e., a sharp reduction in population size due to environmental events, which in turn causes big losses of genetic variations due to genetic drift). Therefore, scientists wondered how invasive species overcome this challenge of low genetic diversity while introduced into a new environment. Reduced genetic diversity can have two consequences; one is inbreeding depression that constrains the population growth and decreases the chance of population's persistence. Another one is the limited ability to evolve in a new environment. However, consequences of genetic diversity reduction could be less visible by the increase of genetic variation through multiple introductions that could defeat inbreeding depression and drift (Sakai et al., 2001; Sax et al., 2007; Simberloff, 2009). These populations, due to multiple introductions from different locations in the world, could have new genotypes which could be better adapted to the environment, thus increasing the chance of persistence and spread (Sax et al., 2007; Simberloff, 2009). Less genetic diversity could also be due to parthenogenetic reproduction which is one of the characteristics that help invasive species in establishment phase (as mentioned above). Hence, how could a parthenogenetic invasive species have a successful invasion into new areas with weak genetic diversity in comparison with origin areas?

I.1.3. Parthenogenesis, a means of successful invasion

Parthenogenesis is a unisexual type of reproduction where the development of an egg occurs without fertilization, usually resulting in the production of female offspring (Simon et al., 2003). Different types of parthenogenetic reproduction are described in Box 1. Eight types of parthenogenetic reproduction exists; tychoparthenogenesis (or facultative), apomictic, automictic, gynogenesis, hybridogenesis, arrhenotoky, thelytoky, and deuterotoky, or amphitoky. The main difference between apomictic and automictic parthenogenesis is the meiosis retention in the latter. In most cases, parthenogenesis is thelytokous where asexually produced progenies are females (Lynch, 1984). However, haplodiploid insects utilize an arrhenotokous parthenogenetic system

i.e., unfertilized eggs develop into males, whereas fertilized eggs give rise to diploid females (Lynch, 1984).

Box 1: Different modes of parthenogenetic reproduction (Simon et al., 2003; Suomalainen et al., 1987)

Tychoparthenogenesis (or facultative): occasional, spontaneous production of eggs without fertilization. This type of parthenogenesis is specially known for at least 10 insect orders from which in 6 genera obligatory parthenogens exist. The cytological mechanism is similar to apomictic and automictic parthenogenesis.

Apomictic parthenogenesis: suppression of meiosis and the fertilization of eggs from a mitosis like cell division, thus the offspring are genetically similar to their mothers except mutations. Apomixis is commonly found in invertebrates such as rotifers and all major groups of arthropods.

Automictic parthenogenesis: in contrast to apomixes, in this type of parthenogenesis meiosis is retained with restoration of diploidy by duplication or fusion of the gametes produced by the female parent. Generally, it leads rapidly to complete homozygosity of offspring. This type is common in many parthenogenetic stick insects and some weevils. However, one form found in unisexual lizards and Australian grasshoppers, known as endomeiotic doubling which involves replication of chromosomes prior to meiosis, that restores diploidy for the offspring. As the replicated chromosomes pair prior to meiosis I, the offspring are genetically identical to mothers and the heterozygosity is maintained.

Gynogenesis: a form of parthenogenesis in which sperm should be inoculation form a related bisexual species to stimulate egg development, without genetic contribution to the offspring.

Hybridogenesis: it's a hemiclonal type of parthenogenesis in which half of the genome is transmitted sexually and the other half clonally. In this type sperm and egg nuclei fuse, in the offspring paternal genes are expressed but only maternal genome is transmitted to the next generation. Both gynogenesis and hybridogenesis are reported for a unisexual fish species in the genus *Poeciliopsis.*

Arrhenotoky: unfertilized eggs producing only male descendants

Thelytoky: unfertilized eggs producing only female descendants

Deuterotoky, or amphitoky: unfertilized eggs producing descendants of both sexes

A parthenogenetic species, due to lack or limited rate of meiosis, have less genetic diversity than sexual species. In case of parthenogenetic invasive species, a drastic loss of variation is thus expected in the invaded areas in comparison with the area of origin, because a single clone has

invaded an extensive area (review in Facon et al., 2003). This could be studied through genetic markers which help us to identify the pathways of invasions and to count the number of introductions. For instance, through a phylogeography study, the invasion by a parthenogenetic snail, *Melanoides tuberculate*, was studied to evaluate the pathways and number of invasions from the Old World (from Africa to southeastern Asia) into the Neotropical area (from northern Argentina to Florida) (Facon et al., 2003). Based on two mitochondrial genes (12S and 16S), results showed that several clones have independently invaded the Neotropical area. Thus, though parthenogenesis promotes genetic uniformity in the short-term, new morphs can appear as a result of sporadic changes in ploidy and/or rare events of sexual reproduction, which help them invading new areas.

Population genetics and phylogeography approaches are useful tools to study the genetic diversity in both source and invading populations and to elucidate the mechanisms driving the success of invasive species (review in Lounnas et al., 2017).

I.1.4. Usefulness of population genetics and phylogeography approaches for a better understanding of biological invasions

Comparing the distribution of genetic variability within and among populations between the origin and invaded areas, could be useful to understand the routes of invasions and the genetic variations of invasive species in these populations (Lounnas et al., 2017). For many years, mitochondrial DNA (mtDNA) was used as the molecular marker of choice in phylogeographical studies in animals due to its lack of recombination, putative neutrality, and smaller effective population size, and consequently a shorter expected time to reciprocal monophyly between geographic regions. However, single-locus markers of mtDNA are often inadequate for accurate calculations of parameter estimates, and multi-locus data could intensely improve the performance of analytical methods (review in Hickerson et al., 2010).

The current molecular approaches for phylogeographic studies include several techniques for detecting and analyzing variation of mtDNA, and nuclear DNA (nDNA). These approaches include; Amplified Fragment Length Polymorphism (AFLP), Simple Sequence repeats (SSR) or microsatellites, Random Amplified Polymorphic DNA (RAPD), restriction site-associated DNA sequencing (RADseq.) and Single-Nucleotide Polymorphism (SNP). Based on the study, question, and information required, each of these approaches has advantages and disadvantages which are listed below in Table 1.

Table 1: Advantages and disadvantages of the most-used PCR based molecular approaches in phylogeography and population genetic studies (Aguirre-dugua and González-rodríguez, 2016; Andrews et al., 2016; Miah et al., 2013)

Method	Advantages	Disadvantages
AFLP (dominant marker)	-fast and simple -cheap -genomic abundance high -low to moderate polymorphism -mostly from non-coding regions -reliable information for hundreds of loci -no need for sequence data -can be used across species	-reproducibility is low -difficult for assessing the homology among markers of the same molecular size -the state of 'present allele' could correspond equally to an individual which is homozygous or heterozygous -possible experimentations failure, due to changes in materials used
RAPD (dominant marker)	-fast -cheap -genomic abundance high -low to moderate polymorphism -no need for sequence data	-not reproducible -low accuracy -not suitable across species
microsatellites (co-dominant marker)	-genomic abundance high -highly polymorphic -found in both coding and non-coding regions -highly reproducible -usable for fluorescent techniques	-high mutation rates -high development costs -technical challenges during the construction of enriched libraries and species-specific primers -sequence information needed
SNP (co-dominant marker)	-a suite of unlinked nuclear genetic loci that capture a genome-wide picture of population history -highly polymorphic -low mutation rates -high number of SNP loci usually available -high accuracy	-a maximum of only four characters states (A, T, G, C) making them less informative than microsatellites -sequence information needed

Dominant markers are useful for species with unknown ploidy degree and for which we suspect polyploidy. In case of polyploidy, co-dominant markers are not advisable (e.g., microsatellites, RFLP, SNP and RADseq.), because it is difficult to determine the number of copies of each of the alleles present. Among dominant markers, AFLP is highly valuable and its efficacy for having information on the genome has been reported in several studies. For instance, sympatric speciation (the evolution of reproductive isolation without geographic barriers) of two endemic palm species *Howea forsteriana* and *H. belmoreana* on Lord Howe Island was shown by divergence of four loci out of 274 AFLP markers between the two species, involving disruptive/divergent selection (Savolainen et al., 2006). AFLP method is also very useful for non-model species for which we don't have much genomic information. With this method, we can have information about genetic variations relatively fast and easily, without the need to have sequence data.

The use of dominant markers such as AFLP is thus interesting for studying the genetic structure of soil living organisms for which very poor genomic data are available. For these taxa, molecular techniques have been rarely used but these methods have allowed to describe the spread of a few invasive earthworm species (Dupont, 2009).

I.1.5. Invasive earthworm species

Underground invasions have attracted less attention than above ground invasions, mostly due to the cryptic nature of soil environment and less apparent impacts of invasions (Bohlen, 2006; Fernández et al., 2016; Hendrix et al., 2008). Earthworms are known as ecosystem engineers (Box 2) thus, they can impact the soil ecosystem by their activities. When non-native earthworm species are introduced in a new habitat, they can alter soil structure, organic matter and nutrient cycling, and plant and animal communities below and above ground and replace or out-compete native earthworm species (Fernández et al., 2016; Hendrix et al., 2008).

Box 2: Earthworms as ecosystem engineers

Ecosystem engineers are organisms that directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic materials (Jones et al., 1994). Earthworms have been recognized as typical ecosystem engineers and represent an excellent potential partner for humans in managing ecosystem services. Earthworms have important roles on biological (interactions with plants and other soil organisms), chemical (mineralization of organic matters of the soil) and physical (change in soil structure by burrowing activity) aspects of the soil (Blouin et al., 2013). Earthworms are especially useful for farming and agricultural practices. For instance, by their activities they release assimilable nutrients which increase soil fertility (Lavelle et al., 1992) and by their burrowing activity (Lavelle et al., 1997) they increase soil aeration and water absorption which are in interaction with plant species.

Earthworms dispersion in the world is facilitated mainly by humans through global commerce; unintentionally (e.g., in agricultural and horticultural products) or intentionally (e.g., for waste management and land bioremediation) (Hendrix et al., 2008). To be transported successfully to other regions in the world, the earthworms or cocoons should be highly tolerant to different soil conditions and structures (Fernández et al., 2016). Most peregrine (i.e., widely ranging species out of their native zone, often owing to human action) earthworms can be grouped in three families: Lumbricidae, Megascolecidae and Rhinodrilidae (Fernández et al., 2016). The majority of known peregrine species belong to the Lumbricidae family with 30 species. These species are found in the genera; *Lumbricus*, *Allolobophora*, *Octolasion*, *Aporrectodea*, *Dendrobaena*, *Eisenia* or *Eiseniella* (Fernández et al., 2016). These species are mostly studied in temperate region and as Hendrix et al. (2008) stated, despite the existence of higher number of species in tropical zone, the proportion of our knowledge on the existence of peregrine earthworm species in temperate zone is much higher than those in tropical region. It has been estimated that out of several thousand of earthworm species in tropical zone, only 51 peregrine species are known, while in temperate zone 45 peregrine species are known out of 500-600 estimated species.

To date, only three studies have investigated the phylogeography and population genetics of peregrine earthworm species i.e., *Aporrectodea trapezoids* (Fernández et al., 2016, 2011), *Aporrectodea rosea* (Fernández et al., 2016) and *Pontoscolex corethrurus* (Dupont et al., 2012). A common point of these studies is a high genetic diversity within samples of different locations. Using AFLP markers to investigate *P. corethrurus* population genetics in French Guiana, Dupont et al. (2012), showed that higher genetic diversity was observed in disturbed areas due to human activities which supplied these populations with migrants thus new genotypes. By comparing sequenced haplotypes in the ancestral distribution areas and the colonized lands, we can trace back roads of biological invasions and understand the success of peregrine species. For instance, two phylogeographic analyses using COI and COII genes revealed that *Aporrectodea trapezoids* and *Aporrectodea rosea* peregrine earthworm species have undergone a similar pattern of diversification (Fernández et al., 2016).

With the advent of molecular approaches in species taxonomy, several cryptic species (i.e., morphologically undistinguishable species) were revealed for earthworms (King et al., 2008; Novo et al., 2010, 2009; Pérez-Losada et al., 2009). Among these cryptic species, some are peregrine. Hence, one question remains to be answered; could a peregrine earthworm morphospecies, be a complex of several species?

I.2. Speciation and cryptic species

I.2.1. Processes of speciation and 'grey zone'

Species concept has been controversial for scientists for a long time. This concept varies based on different scientific fields to define it. For instance, intrinsic reproductive isolation in the case

of the biological species concept, occupation of a distinct niche or adaptive zone in the case of the ecological species concept, fixed character state differences in the case of the phylogenetic concept, and different evolutionary role, tendencies, and historical fates in the case of evolutionary concept of species (De Queiroz, 2007). De Queiroz (2007) suggested that when there are debates on the existence of one species versus two species, we are in the 'grey zone' of speciation process (a process by which one species splits into two species), that's where scientists in different disciplines come into conflict, but before and after the 'grey zone' they all agree that there is one species or two species (Figure 2).

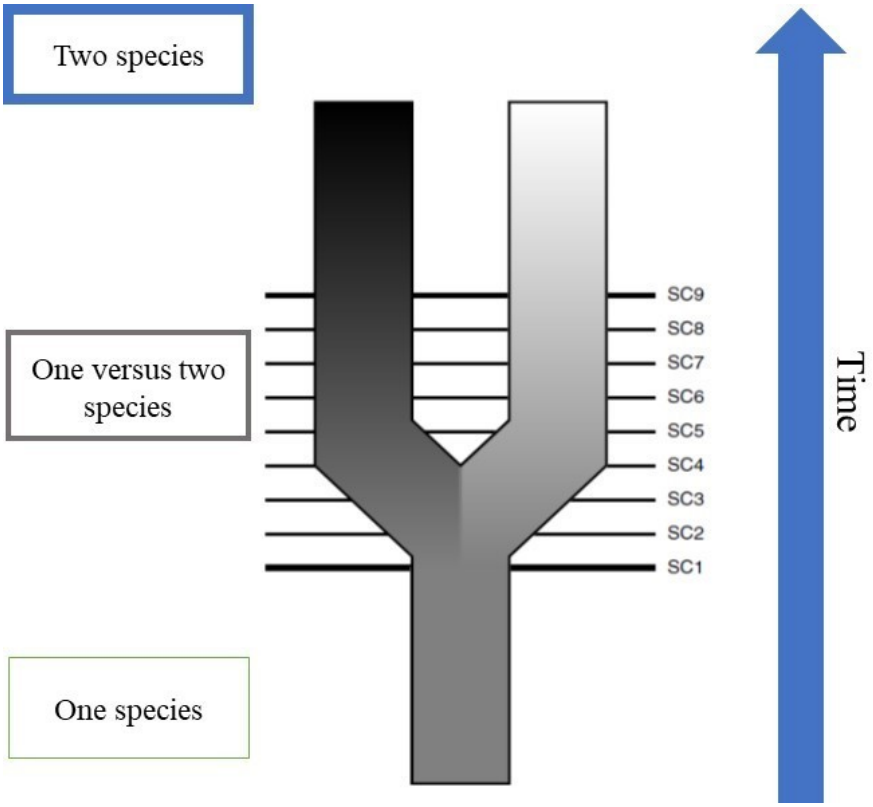


Figure 2 : A simple illustration of speciation process after De Queiroz (2007). At first one species exists and with time it splits into two species. The SC1 to SC9 correspond to the progression of time.

Integrative taxonomy is an approach that highlights congruence among different disciplines and gives evolutionary explanations for incongruence among them, acknowledging that these different methods reflect different stages of the speciation process in the 'grey zone' (Dejaco et al., 2016). Integrative systematics require multifaceted skills in taxonomic, phylogenomic, and bio-informatic skills (Wen et al., 2017). Nowadays, species delimitations based on molecular approaches and bio-informatic software are used more and more.

I.2.2. Phylogenetics/phylogenomics and species delimitation methods

Mitochondrial markers are widely used for species delimitations and cryptic species discovery studies. However, these markers are considered as a 'double-edged sword' due to their accelerated evolutionary rates (Akihito et al., 2016). Meaning that in one hand, significant amounts of sequence variation could be observed in closely related species and as maternally transmitted, interpretation of species identification is simplified (review in Yang et al., 2014). But at the other hand, because of the reduced N_e (effective population size) of mitochondrial genes compared to nuclear genes, they may facilitate fixation of an introgressed haplotype lineage, even at low levels of introgression in a foreign population (review in Akihito et al., 2016). Hence, combining mtDNA and nDNA markers improves the power of genetic data for systematic studies (Song and Ahn, 2014). Meanwhile, evolutionary inference from single-locus data (nuclear or mitochondrial markers) has limitations including; lower power for detecting independent evolutions compared with multi-locus approaches, the potential discordance between gene trees and species trees and the lack of information on adaptive divergences (review in Fujisawa and Barraclough, 2013). Thus, single-locus analysis could lead us to an under or over estimation of existing species (Meyer and Paulay, 2005; Will and Rubinoff, 2004). With the decrease in the cost of high-throughput Sanger sequencing and the advent of Next Generation Sequencing (White et al., 2014), more and more studies are using multi-locus analysis. Table 2 shows some of the molecular species delimitation methods and they are organized based on single-locus and multi-locus types of input data for these methods. Among these methods, two are species validation approaches which require the user to assign samples to putative phylogenetics/phylogenomics lineages (i.e., BPP and SpedeSTEM), while the other approaches assign samples to groups without a priori information for discovering species (review in Carstens et al., 2013).

Table 2: Common single and multi-locus species delimitation methods based on Carstens et al. (2013) review

Single-locus /multi-locus	Delimitation method	Reference
Single-locus	Generalized Mixed Yule Coalescent (GMYC)	(Pons et al., 2006)
	Automatic Barcode Gap Discovery (ABGD)	(Puillandre et al., 2012)
	Bayesian Poisson tree processes (bPTP)	(Zhang et al., 2013)
	Multi-rate Poisson tree processes (mPTP)	(Kapli et al., 2017)
Multi-locus	Structure	(Pritchard et al., 2000)
	Gaussian Clustering	(Hausdorf and Hennig, 2010)
	O'Meara's	(O'Meara, 2010)
	Species delimitation using species trees (SpedeSTEM)	(Carstens and Dewey, 2010)
	Structurama	(Huelsenbeck et al., 2011)
	Bayesian Phylogenetics and Phylogeography (BPP)	(Ziheng, 2015)

Phylogenetics and phylogenomics permit us to visualize the relationships among species. In phylogenetics the sequences of a small number of genes are analyzed, while with phylogenomics entire genomes, or at least large portions of genomes are compared (Divakar and Crespo, 2015). Sequence capture and restriction site associated DNA sequencing (RADseq.) are emerging as two of the most useful reduced-representation genome sequencing methods for phylogenomics (Leaché et al., 2015). Sequence capture methods use short probes (60–120 nucleotides) to hybridize to specific regions of genome that are subsequently sequenced, these methods require some advanced level of knowledge of the genomes under investigation or of related species (Leaché et al., 2015). One of the sequence capture methods is Anchored Hybrid Enrichment (AHE), which is a cost-efficient (∼1% of the cost of traditional Sanger sequencing and ∼3.5% of the cost of high-throughput amplicon sequencing for projects on the scale of 500 loci × 100 individuals), and rapid (∼2 weeks of laboratory time) approach to obtaining data from hundreds of loci (Lemmon et al., 2012). Another sequence capture method is ultraconserved elements (UCEs) (Bejerano et al., 2010) which was originally described as an approach for resolving deep phylogenies (Leaché et al., 2015). Phylogenetic relationships were resolved for mammals, birds, turtles and archosaurs, fishes and squamates based on capture sequences (review in Leaché et al., 2015). At the other hand, RADseq methods (Baird et al., 2008) rely on restriction enzyme digestion of genomic DNA followed by the subsequent size-selection and sequencing of fragments that are of a certain size range, this approach requires limited to no previous knowledge of the genome (Leaché et al., 2015). This approach has been used for speciation analysis of animals with scarce genomic knowledge including mosquitos, plants, cichlids, and beetles (review in Leaché et al., 2015). Based on the scientific question, we could use one of these methods. Generally, sequence capture methods are more adapted for resolving relationships among distantly related species while RADseq. is mostly used for phylogeographic and population-level studies (Leaché et al., 2015).

In recent years, science of species delimitation has witnessed a dramatic increase in the number of available methods for delimiting species based on molecular results. Carstens et al. (2013) suggested that in order to have accurate species delimitation boundaries, researchers should apply a wide range of species delimitation analyses to their data and place their trust in delimitations that are congruent across different methods. For instance, Dejaco et al. (2016) based on congruence among different methods: using morphological examination; reciprocal monophyly on mitochondrial (COI), ribosomal DNA (ITS2) and AFLP profiles; bPTP on ITS2 and COI; and BAPS and Structure on AFLP, found six nominal species as taxonomic junior synonyms and two new species for the jumping bristletail genus *Machilis* from the European Eastern Alps (if species delimitations were done with only COI, 16 species by reciprocal monophyly or 20 by bPTP would be recorded).

I.2.3. Cryptic diversity

With the help of molecular species delimitation methods, more and more cryptic species are recognized. For instance, nine putative species were found for the terrestrial gastropod genus *Pyramidula* a case of cryptic species complex, based on mitochondrial and nuclear markers, and GMYC, ABGD, BPP and SpedeSTEM methods (Razkin et al., 2017). Differentiating cryptic species is important because they impact and respond differently to the environmental pressures e.g., in their tolerance to pollution, reproductive timing, feeding mechanisms, and ecological traits (White et al., 2014) or while used as biological models in scientific studies (e.g., ecotoxicological, bioindicator for soil or water quality, etc.). Therefore, if the experimentations are done on several species or lineages, the results could be biased. For example, in *Gammarus fossarum* (an amphipod) complex of species, different concentrations of the fungicide tebuconazole were tested on cryptic lineages A and B (Feckler et al., 2012). After seven days, cryptic lineage A showed a significantly higher overall sensitivity compared to lineage B, with an approximate 50% more pronounced decline in feeding behavior (Feckler et al., 2012). However, as this study was done on only one population for each cryptic lineage, authors suggested further studies to draw definite conclusions. One of the organisms which is very important to humans due to their impacts on farming and agricultural activities, are earthworms. These organisms are used for ecotoxicological studies, and in cases of cryptic species (as they are common among earthworm species) misleading results could be obtained.

I.2.4. Molecular studies revealed several complexes of species in earthworms

Cryptic diversity has been revealed for several earthworm morphospecies; *Hormogaster elisae* Alvarez, 1977 (Novo et al., 2010, 2009), *Metaphire paiwanna* Tsai et al., 2000 (Chang et al., 2008), *Allolobophora chlorotica* Savigny, 1826 (Dupont et al., 2011; King et al., 2008), *Aporrectodea caliginosa* Savigny, 1826 (Pérez-Losada et al., 2009), *Lumbricus terrestris* Linnaeus, 1758 (James et al., 2010), *Lumbricus rubellus* Hoffmeister, 1843 (Donnelly et al., 2013), *Pontoscolex corethrurus* Müller, 1856 (Cunha et al., 2014), *Eisenia nordenskioldi* Eisen, 1879 (Shekhovtsov et al., 2016a, 2013), and *Eisenia nordenskioldi pallida* Malevick, 1956 (Shekhovtsov et al., 2016b). Table 3 shows comprehensive information on these cryptic species studies. Only two studies have used multi-locus genomic DNA analysis by AFLP method for cryptic species discovery (Cunha et al., 2014; King et al., 2008), and other studies have used the amplification of mitochondrial and nuclear genes (with two studies using microsatellite markers; Donnelly et al., 2013; Dupont et al., 2011). These data show also that most of the species delimitations are based on the bootstrap or posterior priority values of phylogenetic trees constructed based on Bayesian Inference (BI), Maximum Parsimony (MP), and Maximum Likelihood (ML) methods, and further species delimitation methods e.g., ABGD, GMYC, mPTP

etc. have not been used. Only one study has used multi-locus species delimitation analysis (BPP) on specimens sequences (Martinsson and Erséus, 2017).

As mentioned earlier, combination of nuclear and mitochondrial markers could help to improve the construction of species relationships in phylogenetic trees. For instance Novo et al. (2009) found six cryptic lineages for *Hormogaster elisae* based on COI gene and phylogenetic trees done by BI, MP, NJ, and ML methods. One year after, results based on more intensive sampling effort and more molecular markers i.e., COI, 16S and 28S genes, showed the existence of five cryptic lineages within *H. elisae*. Amplification of two more genes, hence more information of species evolution, could explain the reason of finding five cryptic species instead of six. Both studies have been done on samples coming from central Iberian Peninsula but from different locations.

The molecular approaches and methods of species delimitation used, have important impact on the results obtained. For instance, Giska et al. (2015), didn't find cryptic species for *L. rubellus* by RADseq. which is a multi-locus nuclear genome data, despite highly divergent mitochondrial lineages, while Martinsson and Erséus (2017) found seven cryptic species for this morphospecies by H3 gene (nuclear gene) phylogenetic tree and multi-locus species delimitation method (BPP). The first study was based on samples coming from Poland while the second one was on a wider range of countries; Norway, Sweden, Czech Republic, Denmark, Germany, New Zealand and USA. Between these two studies only one lineage; *L. rubellus* A, was common thus making it impossible to compare their results. One question remains to be answered; could we find the same number of cryptic species found by Martinsson and Erséus (2017) under a multi-locus nuclear genome data e.g. RADseq.? A similar example for disaccordance among studies for cryptic diversity is the case of *Allolobophora longa.* Pérez-Losada et al. (2009) suggested the existence of two cryptic species for
A. longa morphospecies based on concatenated ML, MP and BI phylogenetic trees using one nuclear, and five mitochondrial markers (Table 3). Samples of this study came from 27 sites of western and central Europe (France, Finland, Germany, Spain, Denmark, Poland, United Kingdom, and Serbia). However, based on another study (Martinsson et al., 2015), the two divergent haplogroups based on samples from Sweden, Norway and Denmark, didn't suggest the existence of cryptic species for *A. longa*. This study was done based on one mitochondrial (COI) and two nuclear markers (ITS2 and H3). Analyses were done by distance methods, parsimony networks and Bayesian coalescent trees, and the statistical distinctness of the groups was tested on gene trees using the genealogical sorting indexes. Unfortunately, in the latter study, results of the former study are not mentioned or discussed. Therefore, we have no information if any haplotypes were shared among the two studies, and eventually if cryptic species exist for *A. longa* or not.

Cryptic species discovery on invasive peregrine species are rare, and among them is *Aporrectodea trapezoids*. Pérez-Losada et al. (2009) found two cryptic lineages within this morphospecies based on mitochondrial genes COII, 12S, 16S, ND1, tRNA and one nuclear marker 28S for samples coming from four countries i.e., Spain, France, Poland and Serbia. In another study done on *A. trapezoids* samples from Spain, France, Portugal, Italy, Greece, Turkey, Algeria, Egypt and Australia, Fernández et al. (2011) found two lineages by COI, COII, 28S rRNA and H3

concatenated data sets. They suggested that these lineages are not divergent enough to consider them differentiated evolutionary lines, thus they didn't confirm the existence of cryptic species for *A. trapezoids* morphospecies. Some of the haplotypes within the former study were found in the latter study as well. However, eventually in Fernández et al. (2016) study, existence of cryptic species for *A. trapezoids*, through samples from the previous study plus specimens from Algeria and Balearic Islands and the amplification of one more gene; 16S, was suggested.

Some of these earthworm species have been widely used as models in different studies, thus we could question the credibility of studies done on these morphospecies, because different species have diverse impacts and respond differently to environmental conditions. For instance, Kille et al., (2013) showed that cryptic lineages within *Lumbricus rubellus* (i.e., LA and LB) adapt differently to soil contaminations. The results on methylation sensitive AFLP analyses on *L. rubellus* cryptic lineages showed a clear association of methylation patterns with soil arsenic concentrations for LB. These results suggested that LB utilizes epigenetic mechanisms to adapt to the presence of contamination, while LA doesn't have this mechanism (Kille et al., 2013).

Proof of hybridization between cryptic species were found in three studies; between green and pink morphs of *A. chlorotica* and within pink morph lineages (Dupont et al., 2011), and between *L. terrestris* and *L. herculeus* and A-B and A-H lineages of *Lumbricus rubellus* complex (Martinsson and Erséus, 2017). Therefore, these cryptic species are in speciation process and reproductive isolation between them are not yet complete. Hybridization was not recorded in several cases of cryptic lineages sympatry i.e., for *Aporrectodea caliginosa*, *Eisenia nordenskioldi nordenskioldi* and *Eisenia nordenskioldi pallida* species complex.

Table 3: A summary table of studies on the cryptic species discovery for earthworms

Species	Nb of localities	Mito.[1] genes	nuclear genes or genome	Analysis Method	K2P Genetic div.[2]	Nb[3] of lineages (species)	Sympatry of lineages	Hybridization between lineages	Morph.[4] differences between cryptic species	Ploidy degree	Rep.[5] strategy	Reference
Hormogaster elisae	7 in temperate zone	COI	–	BI[6], MP[7], NJ[8], ML [9]trees	5.75% to 20.20%	6	–	–	–	?	Sexual	Novo et al., 2009
	16 in temperate zone	COI, 16S	28S	Concatenated BI, MP, ML trees	9.49% to 18.31%	5	–	–	–			Novo et al., 2010
Metaphire paiwanna	11 in tropical zone	COI, 16S	NADH	Concatenated BI, MP, NJ, ML trees	–	2	–	–	Yes	?	?	Chang et al., 2008
Allolobophora chlorotica	24 in temperate zone	COI, 16S	AFLP	Un-concatenated MP, NJ and BI trees + AFLP (NJ tree)	0.17% to 21.28%	5 by mitochondrial and 4 by AFLP trees	Yes	Yes some evidence for green morph by AFLP	No	2n	Sexual	King et al., 2008

[1] Mitochondrial
[2] Divergence
[3] Numbers
[4] Morphological
[5] Reproduction
[6] Bayesian Inference
[7] Maximum Parsimony
[8] Neighbor Joining
[9] Maximum Likelihood

	2 in temperate zone	COI	Microsatellite	NJ tree for microsatellite	No	4	Yes	Yes	No			Dupont et al., 2011
A. caliginosa	11 in tempetae zone	COII, 12S, 16S, ND1, tRNA	28S	Concatenated ML, MP, BI trees	No	2	Yes, but not precised for which ones	No	No	2n, 3n, 4n	Sexual and parthenogenetic	Pérez-Losada et al., 2009
A. trapezoides	12 in tempetae zone	COII, 12S, 16S, ND1, tRNA	28S	Concatenated ML, MP, BI trees	No	2		No	No	3n, 4n	Parthenogenetic	
A. longa	6 in tempetae zone	COII, 12S, 16S, ND1, tRNA	28S	Concatenated ML, MP, BI trees	No	2		No	No	2n	?	
Lumbricus terrestris	21 in temperate zone	COI	–	NJ tree	3.37% to 23.74%	2	No	No	Yes	2n	Sexual	James et al., 2010;
	1in temperate zone	COI	H3	Single BI gene trees, multilocus analysis BPP	No	2	No	Yes	No			Martinsson and Erséus, 2017
Lumbricus rubellus	2 in tempertae zone	COII	Microsatellite	Population genetic analyses (structure, AMOVA, Fst)	No	2	No	No	No	2n	Sexual	Donnelly et al., 2013;
	44 in temperate zone	COI	H3	Single BI gene trees, multilocus analysis BPP	No	7	No	Yes between two lineages A-B and A-H	No			Martinsson and Erséus, 2017

Pontoscolex corethrurus	3 in temperate zone	s-rRNA, NADH 2, NADH 3	AFLP	Concatenated ML and BI trees + AFLP	No	2	No	No	No	?	Parthenogenetic and sexual (?)	Cunha et al., 2014
Eisenia nordenskioldi nordenskioldi	10 in temperate and polar zones	COI	ITS2	ME and MP trees for COI + Concatenated BI tree	No	6	Yes, 4 lineages in one location	No	No	?	?	Shekhovtsov et al., 2013
	12 in temperate zone	COI	–	BI and ME tree	No	?	Yes, in one location	No	No			Shekhovtsov et al., 2016a
Eisenia nordenskioldi pallida	18 in temperate and polar zones	COI	ITS2	Un-concatenated ME, ML, BI trees	No	5	Yes, lineages 2 and 3	No	No	2n	Sexual	Shekhovtsov et al., 2016b

I.3. Reproductive isolation, polyploidy and speciation

I.3.1. Reproductive isolation

In order to know the mode and process of speciation in nature, it is necessary to determine how reduction of gene flow in the 'grey zone' occurs between species, sufficiently to allow each of them to become irrevocably committed to different evolutionary paths (Bush, 1994). Reproductive isolation is essential for the gene flow to be stopped, which is a consequence of a gradual process happening in the 'grey zone' and has two different types; pre- and postzygotic barriers (Widmer et al., 2009). Pre-zygotic barriers could be related to differences of habitat, behavioral changes, temporal differences or gametic incompatibilities between species. For instance, the recent evolution of *Senecio eboracensis* from *Senecio vulgaris*, two plant species which are interfertile and are both visited by generalist pollinators, is due to selfing of these species by which species boundaries are maintained (review in Widmer et al., 2009). Prezygotic barriers in animals are controlled by few major genes and are important during early stages of speciation, whereas postzygotic barriers are controlled by numerous genes of minor effect and accumulate more gradually (review in Widmer et al., 2009). Therefore, prezygotic barriers evolve faster than postzygotic ones in animals. Postzygotic isolation is related to hybrid sterility, hybrid inviability, and embryo mortality, and among them hybrid sterility most likely evolves faster than hybrid inviability (review in Widmer et al., 2009). For example, in a study based on data available in literature on 368 species, Price and Bouvier (2002) showed that complete loss of F1 hybrid fertility of birds takes on the order of millions of years, while loss of F1 hybrid viability occurs over longer timescales.

The evolution of complete reproductive isolation among species may take hundreds to millions of generations to occur, and during this long history populations change in size and spatial distributions, and the processes that enhance or erode barriers to gene flow including hybridization may occur (Abbott et al., 2013). Hybridization in a spatial context mostly occurs in 'hybrid zones'; which is at abrupt parapatric boundaries. In this context, the exchange of genes occurs between locally adapted populations. In a temporal context, hybridizations occur with 'secondary contacts'; which is after a period of independent evolution (Abbott et al., 2013).

In some cases, speciation is much faster i.e., polyploid speciation (the presence of three or more complete chromosome sets in an organism, in this case the organism may be immediately isolated reproductively from the other individuals of the same species). This type of speciation has been highly reported for plant species. For instance, Wood et al. (2009) showed that 15% of angiosperm and 31% of fern speciation events are accompanied by ploidy increase.

I.3.2. Mechanisms of immediate speciation

Polyploidization has been documented across a wide range of animal species (Schmid et al., 2015). Schmid et al. (2015) highlighted that in parthenogenetically reproducing animals, polyploidy is observed relatively frequently, however in sexually reproducing animals, polyploid species are rare. Possible barriers to polyploidization are the presence of sex chromosomes, prevalence of cross-fertilization, and the histological complexity of animals, which may explain why polyploidy is much rarer in animals than in plants. Polyploids originate from autopolyploidy (mutation in chromosome number) and allopolyploidy (hybridization and mutation in chromosome number) (Comai, 2005; Schmid et al., 2015). Polyploid species are reproductively isolated from other organisms because when polyploids mate with diploids, progenies with odd-numbered ploidies, such as triploids, are produced (Mallet, 2007). These offspring may be viable but typically produce sterile gametes with unbalanced chromosomal complements (aneuploidy) (Mallet, 2007). Polyploidy is thus a simple abrupt means of achieving speciation (Madlung, 2013; Mallet, 2007). For instance, allopolyploid speciation can result from somatic chromosome doubling in a diploid hybrid, followed by selfing to produce a tetraploid. This case was found in the allopolyploid *Primula kewensis* plant which arose among cultivated diploid hybrids of *Primula verticillata* and *Primula floribunda* (Mallet, 2007). Karyotyping (i.e., process of arranging pairs of chromosomes in order of size; Ferguson-Smith and Trifonov, 2007) has helped the determination of the ploidy degree of polyploid species. For instance, a pentaploid (5n= 65) froglet within Water Frog (*Pelophylax esculentus*) hybrid complexes was found.

The genetic advantageous of polyploidy are mainly related to heterosis that causes polyploids to be more vigorous, and gene redundancy that shields polyploids from deleterious mutations (Comai, 2005). Muldal (1952) suggested that because of polyploidy, parthenogenetic species could be found in a broadened span than sexually reproducing ones (Hongell and Terhivuo, 1989; Muldal, 1952; Viktorov, 1997). This fact is mainly related to high levels of heterozygosity and fit genomes which are maintained by avoiding segregation and recombination (Diaz Cosin et al., 2011), thus more tolerant to environmental fluctuations (Lynch, 1984; Viktorov, 1997). Parthenogenetic species often occur at higher altitudes, on islands or in island-like habitats, in dry environments, or in disturbed habitats compared to their sexual relatives (review in Lynch, 1984). Vandel (1928) introduced the term "geographic parthenogenesis" for referring to parthenogenetic organisms' distribution. In this pattern, the sexual forms are mostly in central part and the parthenogenetic ones in marginal or disturbed habitats around the sexual ones (Vandel, 1928). For instance, in *Taraxacum* and *Chondrilla* genera, apomicts (asexually reproducing) have a much wider distribution. In the former, the apomicts dominate the northern regions while sexual ones are found in south and smaller parts of Europe, and in the latter, apomicts dominate the western and eastern parts and the sexuals occupy small parts in the middle of this region in Europe (Dijk, 2003). As mentioned earlier, one of the mechanisms helping invasive species to establish in a new environment is parthenogenetic reproduction due to the advantage of founding a population without the need for a partner. Another reason for the invasion success of parthenogenetic species

is probably due to polyploidy of these organisms, which make them more tolerant to different environmental conditions.

Polyploidy has been observed in earthworms, especially for peregrine and invasive species.

I.3.3. Polyploidy in earthworms

Parthenogenetic reproduction is common in earthworms, which could be related to the lack of sex determining chromosomes in them (Lynch, 1984). Parthenogenetic reproduction in earthworms could result in or maintains odd or even ploidy degrees, while sexually reproducing polyploid species have even numbers of ploidy (Terhivuo and Saura, 2006). For instance, for the athecal individuals (without spermathecae) of *Amynthas catenus* the ploidy degree are; 2n, 3n, and 4n, while the sexthecals (with spermathecae) are diploid (2n) (Shen et al., 2011).

Sometimes different ploidy degrees within one morphospecies could be observed by morphological differences and ecological preferences. For instance, polyploid species; *Dendrobaena subrubicunda* (4n) and *Octolasion cyaneum* (10n), are larger specimens than their diploid forms, because of clitellum displacement in some segments (Muldal, 1952). Difference in ecological preferences has also been observed for different ploidy degrees within a species. Viktorov (1997) demonstrated niche partition for diploid and polyploid individuals in sympatry. For instance, in south Siberia diploid *Eisenia nordenskioldi f. pallida* are endogeics while octoploid *E. nordenskioldi f. typica* are anecics (Viktorov, 1997). As mentioned above parthenogenetic species are widespread in a wider range than sexual ones in a pattern called "geographic parthenogenesis" which is also observed for the earthworms. For instance, sexual forms of *Achaea trapezoids* earthworm species are found in the circum-Mediterranean areas, while parthenogenetic ones are found in a wider range in the rest of the world (De Sosa et al., 2017b).

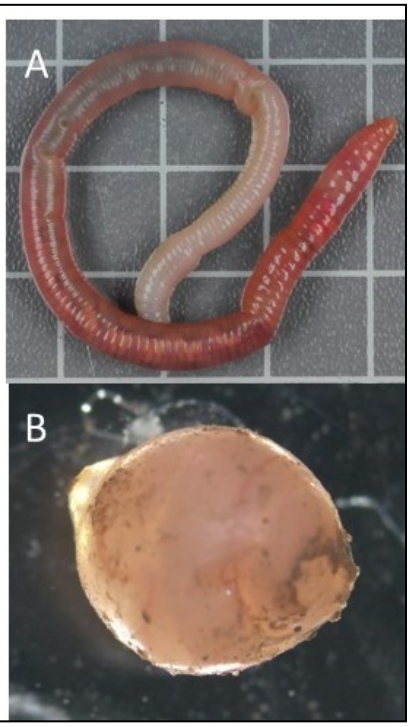
Despite the fact that more and more species complexes are found in earthworms, no studies have investigated the possible ploidy degree differences between cryptic species within a complex. This hypothesis could show the possibility of reproductive isolation among cryptic species which has occurred by polyploidization which is an abrupt speciation mechanism.

I.4. Model of study: **Pontoscolex corethrurus**

Pontoscolex corethrurus, is one the most widespread earthworm species in the tropical and sub-tropical zones (Box 3). *P. corethrurus* is also one of the most studied in soil science and high numbers of studies in different disciplines exist on this morphospecies (Figure 3). *P. corethrurus* is in the Rhinodrilidae family and belongs to the genus *Pontoscolex* where already 20 species are known, with three distinct subgenera: *Pontoscolex*, *Meroscolex* and *Mesoscolex*. *Pontoscolex* genus has presumably originated from the Guayana shield (Righi, 1984) where high species diversity within this genus has been observed. This morphospecies is known to be present in a wide range of habitats from poor soils of pastures to rich soils of primary forests. In 2014, two cryptic lineages in *P. corethrurus* complex was found on São Miguel Island of Azores archipelago (Cunha et al., 2014). One lineage was found in a pineapple plantation and the other one in a hostile environment on a volcano caldera (Furnas). As an invasive species, no synthesis of its impacts on the environment and the type of environments it invades exist. This information could be useful, especially after the detection of the cryptic lineages, to find possible differences among studies due to working on several species instead of one. To date, despite high number of studies on ecological aspects, less biological studies have been done on this morphospecies thus, information on ploidy degree, reproduction strategy, and phylogenetic placement of *P. corethrurus* are lacking. This thesis alongside the publication of *P. corethrurus* mitochondrial genome by Conrado et al. (2017) (Appendix 1) are contributing to fill out the gaps of knowledge on this morphospecies.

Box 3: Characteristics of *Pontoscolex corethrurus*, A) A juvenile specimen of *P. corethrurus*, B) Spherical cocoon of *P. corethrurus.*

- Species name : *Pontoscolex corethrurus*
- Familiy : Rhinodrilidae
- Taxonomic position: uncertain
- Presumed origin: Guyana shield
- Distribution: pan-tropical (peregrine)
- Habitat: from pastures to primary forests
- Ecological category: endogeic
- Reproduction type: probably parthenogenetic
- Ploidy degree: unknown



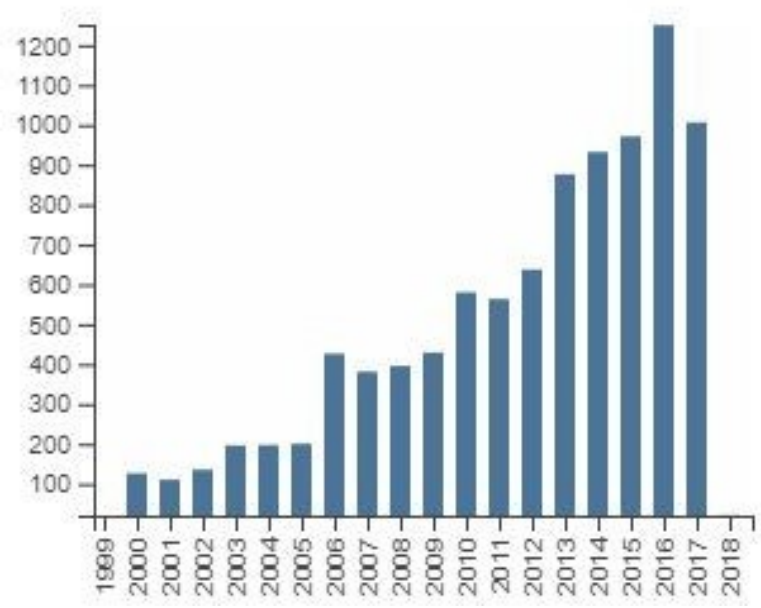


Figure 3: Sum of times, *P. corethrurus* was cited per year from 1999 to 2017, based on Web of Science information. In 2016 the highest number of citations have been recorded with more than 1200 times.

I.5. Thesis objectives and structure of the document

In my thesis, I was interested to understand the mechanisms and characteristics which have important roles in the invasion success of the pan-tropical invasive earthworm species; *Pontoscolex corethrurus*, based on all the studies done on this species. At the same time, as already two cryptic lineages were found within *P. corethrurus*, I wondered if this pan-tropical invasive earthworm could be a complex of several species, where different species under the same morphology are widespread in different parts of the world. Subsequently, I was interested to know the invasion roads and the genetic structure of this invasive earthworm species in different populations throughout the world. It is noteworthy to mention that these issues have been rarely investigated for tropical peregrine earthworm species. Finally, I wondered if for *P. corethrurus* morphospecies a complex of species with different ploidy degrees exist (polyploid complex). These objectives were studied through four chapters.

Chapter 1: What do 'positive' and 'negative' impacts of invasive species depend on?

Context: *Pontoscolex corethrurus*, which is one of the most widespread earthworm species in the tropical zone is one of the most studied in different disciplines of soil science. This species is specially known for its negative impacts on soil structure for strong soil compactions similar to bulldozer impacts but in some cases no compaction has been reported. A synthesis of all the studies done on this morphospecies is lacking.

Questions:

-What are the key traits in colonization success of *P. corethrurus*?

-What are the impacts of this species when introduced in a new area?

-Can we find the proof of cryptic species existence in literature based on its impacts and responses to environment?

Approach: Bibliographic survey of 265 papers on all the present aspects of knowledge on *P. corethrurus* from 1857 till 2017.

-This chapter was the subject of an article that has been published in the journal *Soil Biology and Biochemistry* in January 2018.

Chapter 2: How many invasive lineages within the complex of species *P. corethrurus*?

Context: Two cryptic lineages within *P. corethrurus* morphospecies were found by Cunha et al. (2014) in a hostile environment i.e., a volcano caldera and in a pineapple plantation, on São Miguel Island of Azores archipelago. We wondered if in a world-wide scale we could find more cryptic species within this complex.

Questions:

-What is the genetic structure and diversity within the *P. corethrurus* specimens?

-What is the phylogenetic placement of *P. corethrurus* type material, described for the first time by Fritz Müller (Müller, 1856) in 1856?

-Do we find quincunx formation of the setae at the last quarter of *P. corethrurus* body, for the other species in the genus *Pontoscolex*?

Approach: Phylogenetic and phylogenomic assessment using two mitochondrial (COI and 16S rDNA) and two nuclear (internal transcribed spacers 2 and 28S rDNA) markers and the approach of Anchored Hybrid Enrichment (AHE), accompanied with the examination of internal and external morphological characters.

- This article has been accepted for publication subject to minor revisions in *Molecular Phylogenetics and Evolution* journal on 11th of December 2017.

Chapter 3: Phylogeography and population genetics of an invasive peregrine earthworm species

Context: This chapter aims to investigate the phylogeography and population genetics of the most widespread lineage belonging to the *P. corethrurus* complex in a world-wide scale.

Questions:

-Have the cryptic species in *P. corethrurus* complex reached reproductive isolation?

-Has a 'super-clone' of the most invasive species in *P. corethrurus* complex invaded the world?

-What is the genetic diversity between the populations in the origin and invaded regions of of the most invasive species in *P. corethrurus* complex?

-What is the reproduction strategy of of the most invasive species in *P. corethrurus* complex?

Approach: Genotyping the specimens' genome by AFLP method and re-used COI sequences from chapter two, to construct haplotypes network for of the most invasive species in *P. corethrurus* complex.

-This chapter is presented in the form of an article that is in preparation for submission to the journal *Diversity and Distributions*.

Chapter 4: Ploidy degree of *Pontoscolex corethrurus* complex specimens

Context: Ploidy degree of *P. corethrurus* is still unknown despite being highly studied as a biological model in different disciplines. This information will help us understand the reproductive isolation between cryptic species within *P. corethrurus* complex. Meanwhile, it will help us to better define the type of molecular analysis (dominant or co-dominant markers), for future studies.

Questions:

-What are the ploidy degrees of *P. corethrurus* complex species?

-Does spermatogenesis exist for the specimens in this complex?

Approach: This chapter was done based on cytogenetics classical method.

-Although structured as a scientific article, this chapter will not be submitted for publication.

II. ***Chapter 1****: What do 'positive' and 'negative' impacts of invasive species depend on?*

II.1. Chapter's foreword

Synthesis of all the information available on an invasive earthworm species has never been done. This synthesis can give us valuable evidence on type of ecosystems a particular invasive species occupies, its impacts and responses to the environment, and the important characteristics which have made it a successful colonizer. Invasive earthworm species could alter soil structure and in some cases, have negative impacts which could be detrimental for agricultural crops. Thus, knowing their impacts in different types of ecosystems, could help us make appropriate decisions for control management policies of these species.

Pontoscolex corethrurus is one of the most studied invasive earthworm species in the tropical zone. This species is found in a wide range of different types of ecosystems; pasture, forest, urban area, and plantations. In this chapter, we intended to gather all the information available in the literature on *P. corethrurus*. We were interested to understand the mechanisms of invasion success of this species. At the same time, we wanted to synthetize the impacts of this species on the ecosystems it invades, to understand if it has "negative" impacts as is mostly considered for invasive species. Finally, we wanted to search for possible differences among studies, that could be explained by the use of specimens belonging to different cryptic lineages for the experiments. This chapter has been published as a scientific review article in *'Soil Biology and Biochemistry'* journal.

Bibliographic corpus on *P. corethrurus* was gathered from ISI Web of Science. Studies on this species were grouped into 6 major categories (Figure A 1): impact on the environment (33% of the studies), responses to environmental conditions (32%), earthworm community assessment (22%), biological features (3%), taxonomy (4%) and other subjects (i.e., genetics, biochemistry, geographical distribution and digestion) (6%). This chapter was realized by studying 265 articles. This study has been done in collaboration with Celine Pélosi, researcher of National Institute of Agronomic Research in France (INRA).

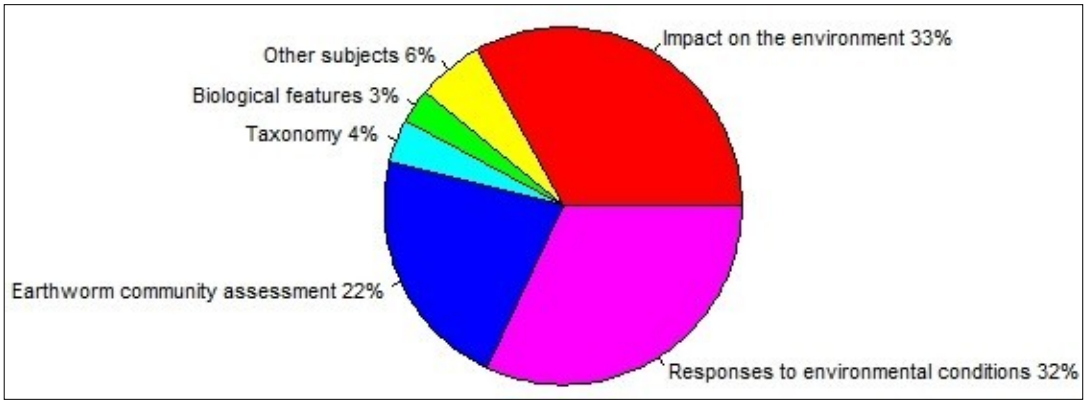


Figure A 1: Pie chart for the major study topics on *Pontoscolex corethrurus* based on Web of Science. "Impact on the environment" and "Responses to environmental conditions", are the most studied topics on this species with 33% and 32%, respectively.

II.2. Article

This article has been published in *Soil Biology and Biochemistry* journal in January 2018, situated in volume 116, pages 277-289. The title of this publication is: Harmful or useful? A case study of the exotic peregrine earthworm morphospecies *Pontoscolex corethrurus.*

Soil Biology and Biochemistry 116 (2018) 277–289

Contents lists available at ScienceDirect

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Review Paper

Harmful or useful? A case study of the exotic peregrine earthworm morphospecies *Pontoscolex corethrurus*

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ARTICLE INFO

Keywords:
Ecosystem engineers
Environmental impacts
Introduced earthworms
Invasiveness
Soil
Tropical ecosystems

ABSTRACT

Exotic peregrine earthworms are often considered to cause environmental harm and to have a negative impact on native species, but, as ecosystem engineers, they enhance soil physical properties. *Pontoscolex corethrurus* is by far the most studied morphospecies and is also the most widespread in tropical areas. The term of morphospecies is used in this review because *P. corethrurus* may in fact constitute a complex of cryptic species. This earthworm is found in a wide range of habitats, from apparently pristine to any kind of human-disturbed environment. This review synthesizes 265 studies describing the distribution, morphology, biological and ecological traits of this morphospecies, as well as its impacts on soil conditions and communities. We then discuss the characteristics necessary for this specific morphospecies to become a successful colonizer throughout the world and the positive and negative effects it can have on the ecosystems that it has invaded. We emphasize the lack of knowledge of *P. corethrurus* reproductive mode and ploidy level, of its population genetics, and of the potential existence of cryptic species. To finish, we highlight the fact that data on *P. corethrurus* interactions with non-earthworm soil macrofauna are scarce.

1. Introduction

Earthworms are generally described as ecosystem engineers that greatly impact the physical, chemical and biological properties of soil (Blouin et al., 2013). Of the 3000–3500 earthworm species that have been described (Csuzdi, 2012), about 150 species are considered to be peregrine (i.e., widely ranging, often owing to human activity; Blakemore, 2012). Most of these peregrine earthworm species are well adapted to human transport and can colonize disturbed habitats (Hendrix et al., 2008). Climate may act as a barrier to their dispersal while their abundance may be limited by soil fertility and plant cover quality (Ortiz-gamino et al., 2016). It is also recognized that introduced species may cause changes to the ecosystem to which it has been introduced. For instance, European *Lumbricidae* such as *Lumbricus rubellus* or *L. terrestris* that have invaded previously glaciated regions in Canada and the USA have dramatically affected nutrient cycling and the functioning of the native ecosystems (Eisenhauer et al., 2011; Suárez et al., 2006).

Most of the species that are deliberately or inadvertently introduced into a new region fail to survive, and the majority of those that do survive, do not become invasive pests (Williamson and Fitter, 1996). Introduced species pass through filters at four well-established spatio-temporal stages of invasion: introduction, establishment, landscape spread and integration (Vermeij, 1996). Species traits and environmental characteristics (Vermeij, 1996), as well as propagule pressure (propagule sizes, propagule numbers, and temporal and spatial patterns of propagule arrival) (Simberloff, 2009) may explain the success of these invasive species. Here, we discuss the case of *Pontoscolex corethrurus,* the quintessential peregrine earthworm in the tropics which has been successfully introduced worldwide. This endogeic earthworm tolerates a wide range of biotic and abiotic environmental conditions (Fragoso et al., 1999; Lavelle et al., 1987). It was first described in 1857 by Fritz Müller from Itajahy in the state of Santa Catarina in Brazil. Righi (1984) identified the Guyana shield as the original region of the *Pontoscolex* genus. Recently, Cunha et al. (2014), revealed the existence of two highly divergent genetic lineages within *P. corethrurus* in the island of Sao Miguel (Azores), suggesting the existence of cryptic species (i.e., different species which are not distinguishable morphologically). Thus, we choose to refer to the "morphospecies" *P. corethrurus* (i.e., a species distinguished from others only by its morphology) in this review. Cryptic species should be accounted for biological and ecological studies because different species may show differential

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http://dx.doi.org/10.1016/j.soilbio.2017.10.030
Received 13 July 2017; Received in revised form 24 October 2017; Accepted 26 October 2017

adaptations to diverse environments and respond differently to perturbations.

P. corethrurus is one of the most studied tropical earthworm morphospecies in soil science (Fragoso et al., 1997; Plisko, 2001). It is used in ecotoxicological studies (Römbke and García, 2000), and it has been recommended as a bioindicator for the assessment of soil quality and ecosystem disturbances (Brown et al., 2006). Some restoration strategies of degraded soils have included the introduction of the morphospecies. For instance, it was used for the biofertilisation of tropical agricultural lands (Senapati et al., 1999; Topoliantz et al., 2002), the remediation of polluted sites (Duarte et al., 2012; Ganihar, 2003; García and Fragoso, 2002; Liang et al., 2011), and the improvement of phytoextraction treatments (Jusselme et al., 2012). Additionally, its use has been proposed in vermicomposting (Chaudhuri and Bhattacharjee, 2011; Molina-Murguia et al., 2009; Nath and Chaudhuri, 2012; Sabrina et al., 2013) and as a source of protein in animal feed for poultry and pork, and in fisheries (Brown et al., 2006). Although *P. corethrurus* may have a positive impact on soil ecosystems in certain circumstances, it may also negatively affect soil physical properties by increasing soil compaction. It may also modify biogeochemical processes as well as communities of plants, microbes and native earthworms (Marichal et al., 2010).

Four hypotheses have been put forward for explaining the success of invasive species: (i) they have traits that favour each stage of the invasion process, (ii) they exploit empty niches, (iii) they are favoured by anthropogenic pressure on natives and (iv) they are no longer under predatory, parasitic or competitive pressure (Sakai et al., 2001). Here, our objectives were (i) to address each of these hypotheses in order to describe the colonization success of the earthworm *P. corethrurus* in its pan-tropical region; due to the scarcity of data on the pathogens, parasites and predators of this species, the fourth hypothesis was not developed; (ii) to discuss the effects of the morphospecies in the introduced areas and (iii) to identify evidence in the literature suggesting the use of different cryptic species in experimental studies. We also identified knowledge gaps and provided promising perspectives for future research.

2. Literature search

The literature search was carried out using the keyword "*Pontoscolex corethrurus*" in Topics of the Web of Science databases using the 'All Databases' option. This option contained 'Web of Science TM Core Collection', 'Current Contents Connect', 'KCI-Korean Journal Database', 'MEDLINE', 'SciELO Citation Index' and 'CABI' research engines. We found 302 references published between 1900 and 2017. We also searched for synonymous species described by Blakemore (2006): *Lumbricus corethrurus*, *Urochaeta corethrura*, *Pontoscolex arenicola*, *Urochaeta hystrix*, *Urochaeta dubia*, *Urochaeta, sp.*, *Urochaeta australiensis*, *Pontoscolex hawaiensis, Pontoscolex guangdongensis and Pontoscolex corethrurus mexicana*.

The articles written in English which were relevant for the review were sorted using the abstracts and the full texts. Moreover, the articles written in other languages and from which we could extract the information from the figures and tables, were also used. Finally, articles that did not focus specifically on *P. corethrurus* (e.g., when *P. corethrurus* was used for comparing results) were excluded. To complete the bibliographic corpus, some essential articles, which were not in the Web of Science, were collected from soil science specialists. The final corpus was composed of 265 references (Fig. 1). Most of the papers studied specimens collected outside the Guyana shield which is the putative native area of *P. corethrurus*. The greatest number of studies were conducted in India and Brazil, with 46 references each, followed by Mexico, with 39 references (Fig. 2).

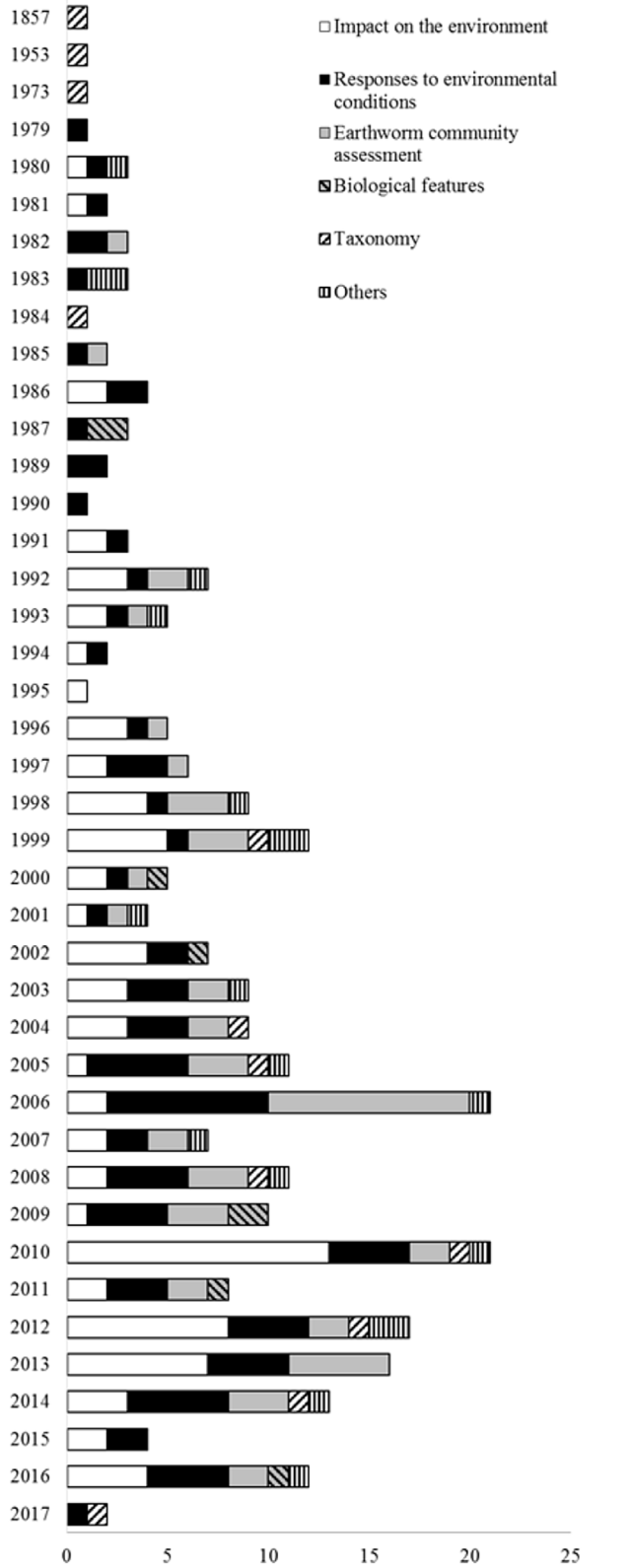

Fig. 1. Bibliographic corpus on *P. corethrurus* gathered in this review (source: ISI Web of Science). Papers were grouped into 6 different categories, corresponding to the most studied subjects on *P. corethrurus*: impact on the environment (33% of the studies), responses to environmental conditions (32%), earthworm community assessment (22%), biological features (3%), taxonomy (4%) and other subjects (i.e., genetics, biochemistry, geographical distribution and digestion) (6%).

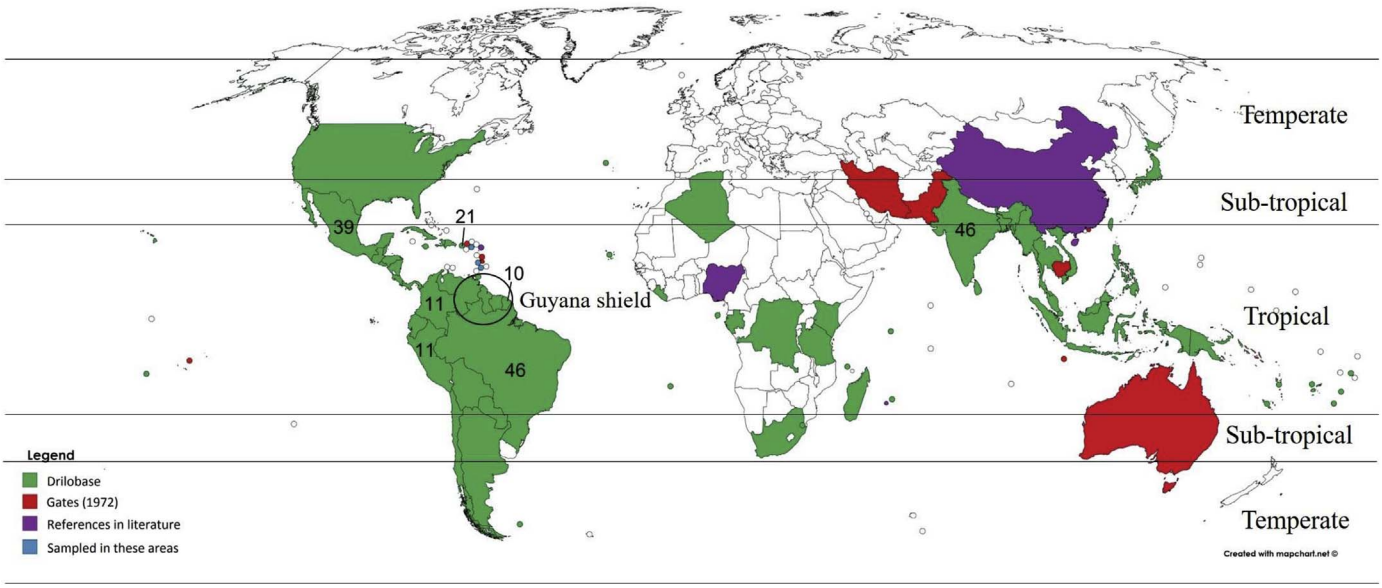

Fig. 2. Distribution map indicating the countries were *P. corethrurus* was found. Numbers show the countries where more than 10 studies were carried out on *P. corethrurus*. Four types of sources were used for this map and are presented in different colors: (i) in green, the locality specified in the Drilobase web site (http://taxo.drilobase.org/), (ii) in red, localities found in Gates (1972) but not mentioned in the Drilobase, (iii) in purple, four localities (China, Reunion and Guadeloupe islands, and Nigeria) found in the literature (China: (Hua et al., 2008; Huang et al., 2015; Paz-Ferreiro et al., 2015, 2014; Zhang et al., 2010), Reunion: (Boyer et al., 2013), Guadeloupe: (Lafont et al., 2007; Loranger-Merciris et al., 2012; Sierra et al., 2014), and Nigeria (Henrot and Brussaard, 1997; Mba, 1994): and (iv) in blue, three islands (St. Kitts, St. Lucia, St. Vincent) where *P. corethrurus* was sampled (S. James pers. com.) but this was not recorded in the literature. The map was done on https://mapchart.net/website. When *P. corethrurus* was recorded in a country, all the country is colored although the distribution of the species could be more restricted (for instance *P. corethrurus* is only present is the sub-tropical area of the United States). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. General results

3.1. Distribution and dispersal vectors

Pontoscolex corethrurus has a widespread distribution, shown in Fig. 2. The map shows that it is present not only in tropical regions but also in sub-tropical zones. For instance, Ortiz-gamino et al. (2016) recorded its presence in sub-tropical regions of Mexico, at an elevation of 1550–1619 m above sea level (m.a.s.l.), and an average temperature of 17° ± 2, where no other tropical species occur. This morphospecies was even recorded in the temperate zone, in the Azores archipelago (Cunha et al., 2014) and the Falkland islands (Reynolds and Jones, 2006). Gates (1972) mentioned that *P. corethrurus* was present in a greenhouse in the London suburb of Kew (United Kingdom), but there is no record of its expansion in this country.

Several vectors of passive dispersal may be involved in the spread of peregrine earthworms, e.g., transport of cocoons by streams and surface water, phoretic interactions with birds and mammals, transport by humans which can be accidental (with soil or potted plants) or commercial, for fishing bait and waste management industries (*P. corethrurus* is most probably extensively transported by humans). Dupont et al. (2012) proposed that the accidental transfer of this morphospecies from Cayenne to the Nouragues reserve in French Guiana, which was revealed using the Amplified Fragment Length Polymorphism (AFLP) method, could be due to deliberate soil transfer for scientific experiments and accidental soil transfer on tools and shoes. Moreover, Brown et al. (2006) indicated that *P. corethrurus* is commonly used as fish-bait in Brazil. While González et al. (2006) highlighted the fact that its dispersal to the Caribbean Islands can be explained by human migration prior to European colonization, Blakemore (2006) suggested oceanic drafting as another vector of dispersal.

3.2. Morphology

Our purpose was not to describe in detail *P. corethrurus* morphological diagnosis but instead to highlight the main external and internal morphological traits mentioned in the literature. When reviewing 13 studies on *P. corethrurus* taxonomy (see Table 1 for references), we noticed some heterogeneity in the descriptions. For instance, some differences in the positions of the clitellum and tubercula pubertatis, which are key traits in earthworm taxonomy, were observed among papers. The beginning of the clitellum position has been identified in either XIV or XV segments while the end of it has been found in three different segments: XXI, XXII, and XXIII. The beginning of the tubercula pubertatis has been found in XV, XVIII and XIX segments and its ends in XXI, XXII and XXIII segments. Moreover, female, male and spermathecal pores have not always been observed (Table 1). Some traits were homogenous among references, such as the position of spermathecae and calciferous glands. The quincunx formation of setae on the last quarter of the body was mentioned in several studies. It is a character commonly used for the diagnosis of this species. However, Moreno (2004) highlighted the possibility of mistaking this species with others of the *Pontoscolex* genus by considering only this characteristic. Another diagnostic characteristic of the morphospecies is a special caudal zone described by Eisen (1896) and Gates (1973) of 4–7 segments (Table 1). The typhlosole which begins approximately at segment XXI ends with this caudal zone (Gates, 1973).

3.3. Morphospecies traits and environmental characteristics

3.3.1. Reproductive strategy and fecundity

Earthworms are usually hermaphrodites, meaning that both male and female organ systems, such as testes and ovaries, occur within a single individual. In *P. corethrurus*, male reproductive organs are often absent or atrophied (Gates, 1973; Tsai et al., 2000) and cocoons are viable without mating, thus suggesting a parthenogenetic reproduction (Chaudhuri and Bhattacharjee, 2011). In rare cases, Gates (1973) observed some iridescence in *P. corethrurus* spermathecae that raised the possibility of biparental reproduction. Sexual reproduction has also been suggested to occur by Dupont et al. (2012), based on a population genetics study.

This morphospecies is a continuous breeder with a high fecundity

Table 1
Morphological characteristics of *P. corethrurus* described in 13 papers focusing on its taxonomy. External and internal morphological traits mentioned in more than 3 references were recorded. The positions are based on segments.

Morphological traits	Descriptions	
External characteristics		
Body length (mm)	Minimum	60 (2,4,8), 92 (11, 13), 95 (3),
	Maximum	75 (8), 120 (4,2), 100 (10), 111 (3), 128 (13), 148 (11), 155 (12)
Segment numbers	Minimum	129 (12), 145 (3), 160 (10), 166 (2), 167 (5, 11, 13), 193 (8), 200 (6), 212 (4)
	Maximum	165 (12), 200 (2, 10), 210 (8), 212 (3), 220 (13), 222 (5), 232 (11), 250 (6)
Pigmentation	Absence	(2, 4, 6, 10, 11)
	Presence	Pale brown (12), white to pink (13)
Clitellum	Position	XV-XXIII (2, 3, 4, 5, 10, 12) XIV-XXII (11, 13), XV-XXI (8), XV-XXII (1)
	Shape	Saddle shaped (2, 4, 5, 7, 8, 11, 12, 13)
Tubercula pubertatis	Position	½ XIX- ½ XXIII (3), XIX-XXI (2, 8), XIX/n-XXI, XXII/n (4), XIX/2, XIX/n, XX to XXI, XXII/n (5), XIX- ½ XXII (10), XVIII-XXI (11), XIX-XXII (12), XV-XXI (13)
	Shape	Longitudinal bands of irregular shape (4, 11, 12)
Dorsal pore	Absent	(2, 11, 12, 13)
Female pore	Absence	(8, 11, 13)
	Presence	(2, 5, 6, 10, 12)
	Number	One pair (10)
	Position	XIV-XV (10)
Male pore	Absence	(5, 8, 11, 12)
	Presence	(2, 4, 7, 13)
	Number	One pair (10)
	Position	XIX/XX (2), intersegmental in XX-XXI (10), XVII (13)
Spermathecal pores	Absence	(11, 13)
	Presence	(2, 3, 5, 7, 10, 12)
	Number	Three pairs (7, 10, 12)
	Position	On intersegmental furrows of VI/VII-VII/VIII-VIII/IX (7, 10, 12), VI/VII - VIII/IX (2, 5)
Tail's quincunx formation of setae	Presence	(2, 4, 5, 7, 8, 10, 11, 12, 13)
Caudal zone	Position	CVII-CXVIII (3), CXIII-CXXXIIII (5), CXX-CXXX (9),
	Number	4-7 (5), 5–7 (9) segments long
Internal characteristics		
Typhlosole	Presence	(2, 4, 5, 7, 10, 11, 12, 13)
	Caeca absence	(7, 11, 12, 13)
	Caeca presence	(2)
	Typhlosole position (start)	XXI-XXV (4), XXIII-XXV (10), XV (11, 13), XXII (12)
	Typhlosole position (end)	CVIII-CXXXVIII (5), CXIII-CXXV (10)
Spermathecae	Number	Two pairs (1), three pairs (2, 3, 5, 7, 8, 10, 11, 12, 13)
	Position	VII-IX (2, 3, 5, 7, 8, 10, 11, 12, 13), VIII-IX (1)
	Shape	Club-shaped (8), tubular (11)
Seminal vesicles	Absence	(5)
	Presence	(2, 4, 5, 7, 10, 11, 12, 13)
	Number	One pair (2, 4, 10, 11, 12, 13), 8 to 10 segments long (4, 5)
	Position	XII (2, 12), XV-XVII (11), XIII (13)
	Shape	Saccular (2, 12), follicular and flattened (11), taped shape (10)
Calciferous glands	Number	Three pairs (1, 2, 3, 5, 7, 8, 10, 11, 13)
	Position	VII-IX (1, 2, 3, 5, 7, 8, 10, 11, 12, 13)
	Shape	Lingular and flattened (2), oval shaped (11, 13), tubular (10, 12),

(1) (Beddard, 1893), (2) (Blakemore, 2006), (3) (Eisen, 1896), (4) (Gates, 1972), (5) (Gates, 1973), (6) (Müller, 1857), (7) (Narayanan et al., 2016), (8) (Nxele, 2012), (9) (Righi and Bittencourt, 1972), (10) (Righi, 1990) (11) (Shen and Yeo, 2005), (12) (Tripathi and Bhardwaj, 2005), (13) (Tsai et al., 2000).

rate (Gates, 1972; Vannucci, 1953). Under laboratory conditions, an adult of *P. corethrurus* can produce up to 145 cocoons per year (Arunachalam, 1987; Bhattacharjee and Chaudhuri, 2002; García and Fragoso, 2002). Cocoons have a short development time ranging from 21 ± 1 to 40 ± 9 days at 20–32 °C in laboratory (Arunachalam, 1987; Bhattacharjee and Chaudhuri, 2002; Chaudhuri and Bhattacharjee, 2011; Gates, 1972; Nair et al., 2009; Ortiz-ceballos et al., 2009). The hatching rate of cocoons is high, ranging from 78% to 97%, depending on the temperature, in moist soil conditions (Lavelle et al., 1987). Generally, one hatchling is present per cocoon (Bhattacharjee and Chaudhuri, 2002; Vannucci, 1953; but see Nair et al., 2009). Before depositing their cocoons, *P. corethrurus* individuals build spherical nest chambers (mean diameter of 5.97 ± 1.24 mm) with their buccal appendix and coat the chambers with a fine layer of soil and mucus (Ortiz-ceballos et al., 2009). Cocoons are then laid individually in these chambers (Hamoui, 1991; Vannucci, 1953). The nest chamber are surrounded by a 'feeding-chamber' where casts are deposited and where juvenile earthworms can come to feed once hatched (Ortiz-ceballos et al., 2009).

Research has revealed that *P. corethrurus* present a relative reproductive plasticity. For instance, this morphospecies has been shown to increase its rate of cocoon production and incubation period with increased temperature (Bhattacharjee and Chaudhuri, 2002). Moreover, García and Fragoso (2003) showed that individuals from the same population of *P. corethrurus* raised on different substrates displayed a possible physiological trade-off between cocoon number and cocoon weight, related to nitrogen (N) limitations. In their experiment, *P. corethrurus* produced fewer but bigger cocoons (> 50 mg) in soils with high N-availability and more but smaller cocoons (< 50 mg) when N was less available (García and Fragoso, 2003).

3.3.2. Feeding habits, soil nutrient content and plant composition of the ecosystem

The *P. corethrurus* diet is geophagous (i.e., feeding on soil) and can be classified between the polyhumic (i.e., ingesting soil with high organic matter content) and mesohumic (i.e., feeding indiscriminately on both mineral and organic particles) endogeic categories (Barois et al., 1999; Lavelle et al., 1987). Research has suggested that *P. corethrurus* may derive much of its tissue carbon (C) from rhizospheric sources (Spain et al., 1990) and fungal biomass may be its main source of N (Lachnicht et al., 2002).

Its abundance is positively affected by organic matter availability (García and Fragoso, 2002; Marichal et al., 2010; Ortiz-Gamino et al., 2016), N availability (Li et al., 2010; Marichal et al., 2010) and P availability (Marichal et al., 2011) in the soil. Therefore, this morphospecies prefers rich soils in terms of organic matter and leaf litter (Liu and Zou, 2002; Ganihar, 2003; García and Fragoso, 2003). It is,

however, worth noting that Ayala and Barois (2016) showed in a laboratory experiments that *P. corethrurus* was unable to grow in an extremely rich substrate of 75–100% organic matter, the mortality rate being between 56 and 100%.

P. corethrurus is also able to feed in environments where litter resources are low (Lavelle et al., 1987; Marichal et al., 2010; Ponge et al., 2006; Shilenkova and Tiunov, 2015). For instance, *P. corethrurus* reached a remarkably high density (200 ind.m^{-2}) in a soil extremely poor in organic matter, the alluvial sandy soil of a gallery forest along the Dong Nai River in the Cat Tien National Park, southern Vietnam. The results of a microcosm experiment suggested that the high abundance of these earthworms in poor sandy soils might be due to assimilation of labile carbon released to the soil from plant roots (Shilenkova and Tiunov, 2015).

Plant species differ in the quantity and quality of litter produced, and these differences may significantly affect earthworm populations (Zou, 1993): the density of *P. corethrurus* was higher in Hawaiian plantations considered to have high litter quality (*Albizia falcataria* plantations) compared with plantations with lower litter quality (*Eucalyptus saligna*). Plants may affect earthworm populations in other ways; for instance León and Zou (2004) showed that the shift from grass vegetation (*Axonopus compressus*) to woody plants (*Miconia prasina*) in secondary forests of Puerto Rico decreased the abundance and biomass of *P. corethrurus* through reducing fine root biomass.

3.3.3. Habitat

3.3.3.1. Vegetative cover. *P. corethrurus* seems to proliferate in disturbed habitats (Marichal et al., 2010) and is often found to be dominant in croplands, pastures, urban areas and gardens (Table 2). However, *P. corethrurus* has also been found in forests (Table 2). In particular, it was found to be dominant in the primary forests of the Manzillo Wildlife Refuge and of the Tortuguero National Park in Costa-Rica (Lapied and Lavelle, 2003), in the tropical rainforests of Puerto-Rico (Zou and González, 1997) and in the cloud forest at the top of Luquillo Mountains (Liu and Zou, 2002), a result highlighting that this species also lives in undisturbed ecosystems.

3.3.3.2. Soil moisture and temperature. *P. corethrurus* populations are generally found in areas where the annual mean temperature is above 20 °C. Reproduction being restricted to the 23–27 °C range, *P. corethrurus* growth to the adult stage is only possible between 20° and 30 °C (Lavelle et al., 1987). Contrary to other tropical species (e.g., *Meroscolex marcusi* and *Andiorrhinus caudatus*), *P. corethrurus* is resistant to dehydration (Ayres and Guerra, 1981). However, if the soil moisture is too low (depending on the soil type), *P. corethrurus* may go into diapause (i.e., temporary suspension in development) (Chuang et al., 2004; Guerra, 1994). The use of soil moisture treatments as a gradient of "optimal-stress" environmental conditions by Fragoso and Lozano (1992), showed that juveniles and adults of *P. corethrurus* use different strategies for tissue regeneration. In juvenile worms, caudal amputation resulted in the initiation of diapause and, consequently, in the activation of the process of regeneration, independently of environmental conditions. Adults were only capable of regenerating tissue during diapause, which mainly occurred under conditions of environmental stress (soil dryness in the experiment). In a laboratory experiment, Zhang et al. (2008) showed that soil dryness was the primary factor limiting the reproduction of *P. corethrurus*.

3.3.3.3. Soil physico-chemical characteristics. Although *P. corethrurus* is found in a wide range of soil types (e.g., Entisol-Oxisol, Vertisol, Ferrasol, Ultisol, Fluvisol, and Andosol soils), Huerta et al. (2007) showed in Tabasco, Mexico that it prefers sites with high silt content (Fluvisol). It tolerates a wide range of soil pH (García and Fragoso, 2002; Lavelle et al., 1987) and, although it is often found in relatively acidic soil i.e., from 4.5 to 6.8 (Table 2, Teng et al., 2013), it favors soils with higher pH. Studying a wide range of deforested soils of Eastern Amazonia, Marichal et al. (2010) revealed that *P. corethrurus* densities covaried with pH and also with silt. Although it seems to favour soils with high pH, Marichal et al. (2012) found a positive relationship between mortality and pH in soil sampled in Eastern Brazilian Amazonian soils with high pH values (7.41 and 7.96). Juveniles seem more sensitive to pH than adults; Topoliantz et al. (2005) found that treatments increasing pH, such as charcoal addition, promoted juvenile activity (i.e., casting).

3.3.3.4. Soil contaminants. Zavala-Cruz et al. (2012) proposed that the ability of *P. corethrurus* to colonize contaminated soils could be favoured by a genetic plasticity that confers a certain tolerance to pollutants or a specific genetic resistance to pollutants. *P. corethrurus* has indeed a broad tolerance towards soil contaminants and has been found in different polluted sites. For instance, *P. corethrurus* was the most abundant morphospecies (75% of the total abundance of the community) in a site contaminated with hydrocarbons after an oil spill about 20 years previously in Tabasco, Mexico (Hernández-Castellanos et al., 2013), suggesting a high tolerance to benzo(*a*)pyrene (BaP).

A similar tolerance to trace elements has been reported by Duarte et al. (2014) in a lead (Pb) mining site in Southern Brazil. They showed that *P. corethrurus* biomass, cast production and survival rates were reduced only at high Pb soil concentrations (9.716 μg g^{-1}), compared to low and intermediate Pb concentrations (maximum 4.278 μg g^{-1}). Similarly, *P. corethrurus* mortality, growth and cocoon production were affected only at high mercury (Hg) concentrations (50 and 100 μg g^{-1} soil) after 56 days in a laboratory experiment with soils from forested sites in French Guiana (Da Silva et al., 2016). Buch et al. (2017) worked on soils of two Brazilian forest conservation units that had been polluted by Hg due to atmospheric deposition. They found cocoon production and earthworm growth to be affected at much lower concentrations of Hg i.e., 8 μg g^{-1} than that reported by Da Silva et al. (2016). At this concentration, *P. corethrurus* individuals were not found to avoid the contaminated soil. As with other contaminants and earthworm species (Pelosi et al., 2014), the bioavailability of chemicals in soils is highly dependent on soil properties (Van Gestel and Weeks, 2004). Therefore, the effects of trace elements on *P. corethrurus* are likely to depend on soil type, moisture, temperature and many other soil characteristics.

Only a few studies have investigated *P. corethrurus* sensitivity to pesticides. Kale and Krishnamoorthy (1979) assessed the effects of the insecticide Sevin (i.e., 1-naphthyl-*n*-methylcarbamate) which was mixed with a clay loam in the laboratory. They found the lower concentrations (i.e., 37.5–75 ppm) to have a stimulatory effect on earthworm growth and survival rather than an inhibitory effect. However, the highest concentrations (i.e., > 150 ppm) resulted in growth delays and reduced rates of survival. Forster et al. (2006) showed that *P. corethrurus* was very sensitive to the fungicide carbendazim. This fungicide, forbidden in Europe since 2009, caused a decrease in *P. corethrurus* abundance during a three-month experiment under laboratory conditions using intact soil-core terrestrial model ecosystems (TMEs). This result was confirmed by Buch et al. (2013) who revealed that carbendazim at 3.16 mg a.i.kg^{-1} and the insecticide carbofuran at 5 mg a.i.kg^{-1} applied in boxes filled with artificial tropical soil (TAS, a substrate used in ecotoxicological tests (OECD, 1984)) had lethal effects on this morphospecies.

Finally, the response of *P. corethrurus* to herbicides is variable, as it is for the other contaminants mentioned above. Even at the highest concentrations of a glyphosate-based herbicide (GBH) (47 mg a.i.kg^{-1}), Buch et al. (2013) did not find any significant effect on mortality. Conversely, García-Pérez et al. (2014) showed that GBH could have lethal impacts on *P. corethrurus*, as the application of GBH to coffee plantations thrice a year caused significant reduction in *P. corethrurus* density (167 and 353 ind.m^{-2} with and without herbicide, respectively) and biomass (23 and 45 g m^{-2} with and without herbicide, respectively). However, in another study under laboratory conditions

Table 2
Relative abundance and dominance status of *P. corethrurus* based on papers published since 2000. The relative abundance of other species (sp.) is also presented. When the information was available, it is indicated when the species was considered dominant (D).

Habitat		Country	Soil pH	Relative abundance (%)				Ref[a]
				P. corethrurus	other exotic sp	native sp	undefined origin sp	
Forest	Secondary forest	Brazil	_	25.0	75.0	_	_	1
	Disturbed native forest	Brazil	5	_	100	_	_	2
	Forest of the Cahuita National Park	Costa Rica	_	68.2 (D)	31.8	_	_	3
	Peripheric primary forest of Tortuguero National Park	Costa Rica	_	100 (D)	_	_	_	3
	Primary forest of the Manzanillo Wildlife Refuge	Costa Rica	_	91.4 (D)	8.6	_	_	3
	Remote primary forest of Tortuguero National Park	Costa Rica	_	_	_	100	_	3
	Forest	Cuba	5.62	65.8 (D)	21.4	12.8	_	4
	Mixed forest	India	4.62	28 (D)	22.3	49.7	_	5
	Elfin woodland	Puerto Rico	_	14.7	16.5	68.8	_	6
	Flooded *Pterocarpus*	Puerto Rico	_	_	_	100	_	6
	Lowland moist forest	Puerto Rico	_	51.5	34	14.5	_	6
	Lowland dry forest	Puerto Rico	_	_	100	_	_	6
	Palo colorado forest	Puerto Rico	_	51.7	_	48.3	_	6
	Sierra palm forest	Puerto Rico	_	85.7	2.4	11.9	_	6
	Tabonuco forest	Puerto Rico	_	80.2	2.4	17.4	_	6
	Forest with coffee (*Coffea arabica*)	Puerto Rico	6.7–6.8	100 (D)	_	_	_	7
	Forest with fern (*Dicranoteris flexusosa*)	Puerto Rico	5.8–6.8	100 (D)	_	_	_	7
	Forest with *Selaginella* spp.	Puerto Rico	5.3–5.9	79.0 (D)	_	21.0	_	7
	Maricao State Forest	Puerto Rico	4.5–5.0	98.0 (D)	_	2.0	_	8
	Wet forest (well-drained areas)	Puerto Rico	4.7	97.0 (D)		1.0	2.0	9
	Wet forest (Tabonuco forest)	Puerto Rico	5.9	95.0 (D)			5.0	9
	Fresh water swamp forest	Singapore	_	86.0	_	_	14.0	10
	Bukit Timah nature reserve (foret)	Singapore	_	12.5	_	_	87.5	10
	Upper seletar resevoir park (foret)	Singapore	_	91.6	_	_	8.4	10
	Dry evergreen forest	Thailand	_	58.8	_	_	41.2	11
	Dry dipterocarp forest fired	Thailand	_	_	_	_	100	11
	Dry dipterocarp forest-non fired	Thailand	_	_	_	_	100	11
Wetland	Sungei buloh wetland reserve (covered with mangroves)	Singapore	_	87.2	_	_	12.8	10
Pasture and grassland	Cultivated pasture	Brazil	5.3	50.0	50.0	_	_	2
	Perennial pasture	Brazil	4.6	_	100	_	_	2
	Old pastures 1	Brazil	_	43.0	39.0	18.0	_	1
	Old pastures 2	Brazil	_	96.0 (D)	2.1	1.9	_	1
	Pasture	Cuba	5.6	11.3	74.8 (D)	13.9	_	4
	Pasture (extensive cattle farming) 1	Mexico	6.6	19.6	_	80.4	_	12
	Pasture (extensive cattle farming) 2	Mexico	6.6	37.0	63.0	_	_	12
	Pasture (semi-intensive cattle farming) 1	Mexico	5.5	46.0	54.0	_	_	12
	Pasture (semi-intensive cattle farming) 2	Mexico	5.6	_	100	_	_	12
	Rifle range (grass field)	Singapore	_	54.1	_	_	45.9	10
	Grassland	Thailand	_	55.6	_	_	44.4	11
Culture and plantation	Grain crop (converted from an old pasture)	Brazil	_	87.0 (D)	8.3	4.7	_	1
	Grain crop field 1	Brazil	_	0	66.5	33.5	_	1
	Grain crop field 2	Brazil	_	0	30.0	70.0 (D)	_	1
	Sugarcane (*Saccharum* sp.) 2	Brazil	_	10.0	60.0	30.0	_	1
	Sugarcane (*Saccharum* sp.) 1	Brazil	_	41.0	50.0	9.0	_	1
	Manduirana plantation	Brazil	5.6	_	100	_	_	2
	Banana plantation	Costa Rica	_	100 (D)	_	_	_	3
	Mixed fruit plantation	India	_	20.0	8.3	71.7 (D)	_	13
	Pineapple plantation	India	_	10.4	1.9	87.7 (D)	_	13
	Rubber plantation	India	4.7	71.8 (D)	5.4	22.8	_	14
	Rubber plantation	India	4.5	76.5(D)	3.3	20.2	_	5
	Agricultural ecosystem	Malaysia	6.1	7.8	_	_	92.2 (D)	15
	Rice paddy	Thailand	_	27.3	_	_	72.7	11
	Cassava plantation	Thailand	_	55.8	_	_	44.2	11
	Forest plantation	Thailand	_	93.3	_	_	6.7	11
	Mango plantation	Thailand	_	71.4	_	_	28.6	11
	Sugarcane plantation	Thailand	_	_	_	_	100	11
Urban area and gardens	Lawn	Brazil	4.6	25.0	75.0	_	_	2
	Bribri village	Costa Rica	_	100 (D)	_	_	_	3
	Cahuita village	Costa Rica	_	91.8 (D)	8.2	_	_	3
	Puerto Viejo village	Costa Rica	_	89.7 (D)	10.3	_	_	3
	Campus of National University of Singapore	Singapore	_	16.7	_	_	83.3	10
	Kranji wireless station	Singapore	_	80.8	_	_	19.2	10
	Singapore botanic gardens	Singapore	_	91.7	_	_	8.3	10
	Household area	Thailand	_	27.2	_	_	72.8	11
	Office building area 1	Thailand	_	52.0	_	_	48.0	11
	Office building area 2	Thailand	_	51.0	_	_	49.0	11

[a] References: (1) (Nunes et al., 2006), (2) (Ressetti, 2006), (3) (Lapied and Lavelle, 2003), (4) (Martinez and Sanchez, 2000), (5) (Chaudhuri and Nath, 2011), (6) (González et al., 2007), (7) (Borges et al., 2006), (8) (Hubers et al., 2003), (9) (González et al., 1999), (10) (Shen and Yeo, 2005), (11) (Somniyam and Suwanwaree, 2009), (12) (Ortiz-gamino et al.,

2016), (13) (Dey and Chaudhuri, 2014), (14) (Chaudhuri et al., 2008), (15) (Teng et al., 2013).

by the same authors, earthworms exposed to a *Coffea* litter polluted by GBH produced the same number of cocoons as *P. corethrurus* fed with the unpolluted litter (García-pérez et al., 2016).

3.4. Impact of P. corethrurus on its environment

3.4.1. Physical impacts on soil structure

P. corethrurus is known to compact soil. As a consequence of its feeding activity, small aggregates are progressively transformed into larger aggregates which tend to accumulate in the absence of other agents that break down these larger aggregates; the soil is thus progressively compacted (Alegre et al., 1996; Blanchart et al., 1997). The accumulation of casts by *P. corethrurus* at the soil surface under moist soil conditions may result in the formation of a continuous muddy layer of earthworm casts if "decompacting" activities by other invertebrate populations are too weak. The growth of plants is then prevented and when droughts occur, this layer turns into a compact thick crust. As a consequence, large patches of bare soil impermeable to water and air are generated (Chauvel et al., 1999). Alegre et al. (1996) observed a significant increase in bulk density from 1.12 to 1.23 g cm^{-3} and a decrease in porosity from 58% to 53% in the presence of *P. corethrurus* in a loamy soil in Peru. Similar changes were found in a reciprocal transplant study by Barros et al. (2001) where blocks of forest soil with 48% porosity (in an experimental station in Central Amazonia in Brazil) were transferred to a pasture with 16% porosity where *P. corethrurus* was very abundant (400 ind.m^{-2}) and vice versa. After 1 year, the transplanted blocks of forest soil presented a porosity of 26%, whilst the transplanted blocks of pasture soil presented a porosity of 34%.

By contrast, in certain circumstances, *P. corethrurus* contributed to soil bioturbation processes and decreased soil compaction. Zund et al. (1997) demonstrated that the presence of *P. corethrurus* decreased bulk density and increased aeration of a compacted Oxisol from Australia. Moreover, Hallaire et al. (2000) showed, in a sandy loam soil in Yurimaguas, Peru, that *P. corethrurus* activity induced a compaction of the surface soil, through a coalescence of casts, in plots without organic inputs whereas they created a crumb structure in plots with high soil organic matter contents. Although it is generally accepted that soil compaction by macroaggregation occurs when soil organic matter (SOM) is missing, Sparovek et al. (1999) showed that *P. corethrurus* inoculation, with or without organic matter amendment, resulted in soil compaction in an acidic Oxisol of Brazil.

3.4.2. Chemical impacts

3.4.2.1. Organic matter mineralization and nutrient cycling. Earthworm activity *via* the production of casts is recognized as an important factor affecting C, N and phosphorus (P) cycles in the soil and CO_2 and N_2O fluxes from the soil to the atmosphere (Chapuis-Lardy et al., 2010; Jiménez et al., 2003; Lavelle et al., 1998). The activity of such endogeic geophagous earthworms is often considered to increase C mineralization in the short term and favour C storage through the stabilization of SOM in stable micro-aggregates in the long term (Lavelle et al., 1997; Lavelle and Martin, 1992; Lavelle and Spain, 2001). The influence of earthworms on SOM and nutrient dynamics may depend on a number of factors including the time frame in question but also inherent soil properties, the form of management in place (Fonte et al., 2010) and the interaction with plants (Fonte et al., 2012). Using microcosm experiments, Fonte et al. (2010) showed that, in the first 15 cm layer of the soil, *P. corethrurus* decreased the total soil C by 3% under the Quesungual slash-and-mulch agroforestry system of western Honduras (QSMAS). The QSMAS is an agricultural system with short fallow periods and promoted by extension agents with the intention of reducing the slash-and-burn agricultural strategy (Pauli et al., 2005). By comparing treatments with and without earthworms in maize crops under no tillage in the Peruvian Amazonia, Desjardins et al. (2003) showed that the total carbon content of the 0–10 cm depth was dramatically reduced by 28% in the earthworm – inoculated plots. The results of a 5 months incubation of *P. corethrurus* with added rice and soybean residues suggested that incorporation of organic C by earthworm was higher with smaller rice residues than with larger and woodier soybean residues (Coq et al., 2007).

Earthworm activity is recognized to be an important factor in regulating CO_2 fluxes from the soil to the atmosphere (Speratti and Whalen, 2008). We found only one laboratory study investigating this issue with *P. corethrurus*. The study showed that its presence in Ferralsol of Madagascar induced a significant increase in CO_2 emissions (Chapuis-Lardy et al., 2010).

In addition to its effects on C dynamics, *P. corethrurus* is known to enhance N mineralization and availability (Araujo et al., 2004; González and Zou, 1999; Lafont et al., 2007; Lavelle et al., 1992; Tapia-Coral et al., 2006). For instance, Lavelle et al. (1992) showed that mineral N concentrations ranged from 133.1 to 167.8 μg N per gram dry soil in fresh casts of this morphospecies fed on an Amazonian Ultisol. This was approximately five times higher than the concentration in the non-ingested soil. Similarly, Pashanasi et al. (1992) and Araujo et al. (2004) found that the introduction of *P. corethrurus* significantly increased the soil microbial biomass-N (bio-N) and mineral-N availability in various experiments. However, in a mesocosm experiment, Fonte and Six (2010) found no significant effect of *P. corethrurus* on potentially mineralizable N using a method that measured relatively labile sources of organic N.

Finally, it is generally accepted that earthworms increase P availability across a wide range of agroecosystems (Jiménez et al., 2003; Lopez-Hernandez et al., 1993). A significant increase of exchangeable phosphate was observed in the casts of *P. corethrurus* in several experiments carried out in mesocosms using topsoil dug to a maximum of 25 cm depth (Chapuis-Lardy and Brossard, 1995; Chapuis-Lardy et al., 1998, 2009; Lopez-Hernandez et al., 1993; Sabrina et al., 2013), thus confirming its important contribution to phosphate cycling in tropical soil surface layer. Surprisingly, Fonte and Six (2010) observed a decrease in P availability in presence of *P. corethrurus* in the surface 15 cm of mesocosms that were incubated under field conditions within the QSMAS in western Honduras. They proposed that increased P enrichment and availability in casts comes at the expense of lower P content and availability in non-ingested soil.

3.4.2.2. Metal mobility and availability in soils. In general, earthworms increase the availability and mobility of essential (e.g., Zn, Cu, Mn, Fe) and non-essential (e.g., Cd, Pb, Hg) metals in both contaminated and uncontaminated soils (Sizmur and Hodson, 2009). Using a sequential extraction procedure, Duarte et al. (2012) revealed that *P. corethrurus* significantly reduced the amount of Pb in the soluble and exchangeable forms in the soil, and increased the Pb bound in Fe and Mn oxides in the casts. This can be beneficial for soil bioremediation. In addition, Jusselme et al. (2015) found that *P. corethrurus* has an indirect impact on the Pb phytoextraction ability of *Lantana camara*. The presence of earthworms enhanced *L. camara* biomass by about 1.5–2 times, thereby increasing the uptake of Pb two to threefold (Jusselme et al., 2015).

3.4.3. Impact on biotic factors

3.4.3.1. Effect on other earthworm species. Pontoscolex corethrurus may reach high densities in some areas. Marichal et al. (2010) surveyed 270 sites in Brazil and Colombia and showed that where *P. corethrurus* occurred, its average density was 90.2 ind.m^{-2}, ranging from 5.3 to 567 ind.m^{-2}. The density of *P. corethrurus* populations is often inversely correlated with the density of other earthworm species (Chaudhuri and Nath, 2011; González et al., 1996; Lapied and Lavelle, 2003; Römbke et al., 2009).

Some studies have suggested that an increase in density of *P.*

corethrurus could cause the loss of native species populations (Fragoso et al., 1995; Lapied and Lavelle, 2003). However, the coexistence of this morphospecies with native species has been observed in several sites of different forests in Cuba, India, Puerto Rico, in pastures in Brazil, Cuba and Mexico and in cultures and plantations in Brazil and India (Table 2). The coexistence or replacement of native earthworms by exotic ones may depend on the disturbance history and the state of naturalness of the landscape (González et al., 2006). Different relationships among *P. corethrurus* and native earthworms may depend on the specific context of the study area. For instance, in a mid-altitude Tabonuco forest (400 m above sea level) in Puerto Rico, Hendrix et al. (1999) showed that the niches of *P. corethrurus* and the native earthworm *Estherella* sp. Overlapped completely in sites rich in N resources. On the other hand, in a tropical forest in Puerto Rico, Lachnicht et al. (2002) found the activity of *Estherella* sp. and *P. corethrurus* to be spatially separated, and it appeared that they excluded each other from bottom and surface layers. Marichal et al. (2010) suggested that the replacement of native species by *P. corethrurus* is a result of changes in the environment, such as deforestation in tropical rainforest areas, that affect both groups of species differently, rather than the result of competition between invasive and native species. They proposed that while native species tend to disappear because of the destruction of their habitats and reduction of their food sources, *P. corethrurus* can occupy the soil with increased pH, C and nutrient contents created by the deforestation and burning.

In some cases, the dominance of native species over *P. corethrurus* was found, such as in pineapple plantations of West Tripura in India where the endogeic native species *Drawida assamensis* was dominant although *P. corethrurus* was present (Table 2, Dey and Chaudhuri, 2014).

Lastly, *P. corethrurus* may coexist with other exotic earthworm species (Table 2) such as *Dichogaster* spp in Brazil and Costa Rica(Lapied and Lavelle, 2003; Nunes et al., 2006), *Ocnerodrilus occidentalis* and *Drawida barwelli* in Puerto Rico and Cuba (González et al., 2007; Martinez and Sanchez, 2000) *Amynthas gracilis* and *Octolasion tyrtaeum* in Mexico (Ortiz-gamino et al., 2016) and *Metaphire houlleti*, *M. posthuma*, *Perionyx excavatus* and *Amynthus alexandri* in India (Dey and Chaudhuri, 2014).

3.4.3.2. Effect on nematodes. Earthworms can have either a direct (e.g., by ingestion) or an indirect (i.e., by physical and chemical changes of soil properties) impact on plant-feeding nematodes (Blouin et al., 2005; Lafont et al., 2007; Senapati, 1992; Wurst, 2010). Under laboratory conditions, Boyer et al. (2013) revealed a decrease in *Heterodera sacchari* and *Pratylenchus zeae* populations, two plant parasitic nematodes, in the presence of *P. corethrurus*. They highlighted a transit effect on nematode populations during the passage through the earthworm gut. However, Lafont et al. (2007) and Loranger-Merciris et al. (2012) found that the density of the banana feeding nematodes *Radopholus similis, Pratylenchus coffeae* and *Helicotylenchus multicinctus* did not significantly decrease in presence of *P. corethrurus* in microcosm experiments, although they observed significantly less root damage induced by nematodes. Finally, *P. corethrurus* may have a positive effect on total nematode densities, as shown by Villenave et al. (2010) in a 5-month field mesocosm experiment conducted in Madagascar. The authors revealed that *P. corethrurus* stimulated the microbial community, which increased the density of the dominant microbiovorous bacterial-feeding and fungal-feeding nematodes.

3.4.3.3. Effect on soil microorganisms. Endogeic earthworms have developed complex interactions with soil microorganisms. In particular, the digestion process in the earthworm gut is known to enhance microbial activity (Barois and Lavelle, 1986; Drake and Horn, 2007). The feeding activity of *P. corethrurus* has been shown to result in increased microbial biomass and activity in casts after soil was passed through the gut and excreted (Barois, 1992; Barois and Lavelle, 1986; Bernard et al., 2012). Thus, *P. corethrurus* has provided a basis for the 'sleeping beauty' hypothesis where water and soluble-C in the form of intestinal mucus produced by the earthworm awakens dormant microbial communities in the gut, thereby increasing mineralization of the stable forms of SOM ingested (Lavelle et al., 1983). While dormant microorganisms may be activated during their transit through the gut, others remain unaffected, and yet others digested in the intestinal tract (Drake and Horn, 2007). Barois (1992) pointed out dissimilarities of gut microbial activity among *P. corethrurus* populations suggesting that different populations might show differences in physiological genetics and/or in the intensity of the mutualism with the soil microbial communities. This latter study also demonstrated that temperature has a direct effect in triggering microbial activity within the gut of *P. corethrurus*.

3.4.3.4. Effect on plants. Earthworms generally have positive effects on plant growth in the tropics (Brown et al., 1999) by affecting soil maccroaggregation and availability of nutrients. We previously highlighted (section 3.4.1) that soil macroaggregation by *P. corethrurus* often resulted in increased soil bulk density, and decreased total soil porosity and water infiltration, along with some changes in the soil moisture patterns. Such changes in a sandy loamy soil might be beneficial to some crops such as shown by Alegre et al. (1996) for rice cowpea and maize. Pashanasi et al. (1996) showed that the inoculation of *P. corethrurus* at a density of 90 ind.m^{-2} had a positive effect on soil properties and plant production in low-input cropping systems at Yurimaguas (Peru), although this positive effect varied depending on rainfall, plants and organic inputs. In particular, maize seemed to respond better than rice to earthworm effects, while cowpea did not respond at all.

A major factor affecting plant growth, in relation to the presence of earthworms, is the availability of resources. If the casts, which are enriched in nutrients necessary for plant growth such as N, P, and potassium (K) (Chaudhuri et al., 2012; Lopez-Hernandez et al., 1993) are deposited close to plant roots, they can have significant positive impacts on plant growth (Lavelle et al., 1992). For instance, Loranger-Merciris et al. (2012) showed that *P. corethrurus* enhanced dessert banana growth through increased P availability in its casts. In another study, the presence of *P. corethrurus* increased aboveground biomass of *Brachiaria decumbens* by 30%, *via* increased availability of soil nutrients (Fonte et al., 2012).

The activity of *P. corethrurus* may also promote plant health. Teng et al. (2016) demonstrated that the severity of banana blood disease (i.e., a destructive bacterial infection caused by *Ralstonia solanacearum*) decreased after the inoculation of *P. corethrurus*. This process was explained by a higher plant biomass in comparison to controls, as roots were exposed to high densities of beneficial microorganisms through burrowing and casting activities of the earthworms (Teng et al., 2016).

4. Discussion

4.1. Components of P. corethrurus invasion success

Identifying traits correlated with invasiveness is a central goal in invasion ecology. It is generally agreed that distinct characteristics are important during different stages of the invasion process (e.g., Ribeiro et al., 2008). In particular life history traits (i.e., traits involved in reproduction, growth and survival) may help to differentiate potentially successful and unsuccessful invaders (Sol et al., 2012). During the first stage of the invasion, which is the arrival of a species in a new habitat, one or more propagules of a species must first be carried and survive the dispersal. Most long-distance introductions of *P. corethrurus* to new areas are the direct or indirect result of human activities. This earthworm was thus transported throughout the world (Fig. 2) and these events that were probably recurrent are extremely difficult to date.

Once propagules are introduced, a successful invader must establish

a reproducing population. *P. corethrurus* is a continuous breeder with a high fecundity rate, a high hatching success and a short development time (Lavelle, 1981). Organisms with such characteristics are often classified as r-selected and recognized to be frequently colonizing species (Bufford and Daehler, 2011). The fact that *P. corethrurus* can reproduce by parthenogenesis predisposes this species to invasiveness. Indeed, the ability of a single individual to establish a population is an important characteristic of many invasive species (e.g. Dybdahl and Drown, 2011). Yet, parthenogenetic species lack the capacity to generate novel genetic variation necessary for evolvability (i.e., the ability of a population to adapt in response to environmentally induced stress, Waddington, 1965) due to the absence of bi-parental reproduction and genetic recombination. It has been suggested however, that *P. corethrurus* is capable of bi-parental reproduction (Dupont et al., 2012; Gates, 1973). Such a mixed-mating system, allowing reproduction through inbreeding and outbreeding according to mating possibilities, is a trait that may favour the rapid establishment of an exotic species in new areas (Dupont et al., 2007). The possibility of sexual reproduction should be investigated by genotyping parents and offspring from cross experiments. Moreover, knowledge of the ploidy level of *P. corethrurus* could help to better understand its reproductive mode. Indeed, parthenogenesis is closely linked to polyploidy in earthworms and odd number of chromosomes are often incompatible with sexual reproduction (Shen et al., 2011).

Competitive ability is another trait that may confer an advantage for invasive species during establishment. Many studies have documented invaders that show a superior ability to exploit local resources when compared with native residents (Sakai et al., 2001). Plasticity, i.e., the ability of an organism to cope with a wide variety of habitats and conditions, is thus an important factor in the success of the establishment step (Bufford and Daehler, 2011). *P. corethrurus* is described as euryecic (i.e., of wide ecological plasticity). For instance, *P. corethrurus* has a broad tolerance towards soil contaminants. Its fitness (i.e., individual reproductive success to participate in next generation pool gene) in different polluted sites is affected only at high pollutant concentrations. *P. corethrurus* also presents a reproductive plasticity; an adjustment of cocoon production (number and weight) and incubation period have been observed in different situations (Bhattacharjee and Chaudhuri, 2002; García and Fragoso, 2003).

Another important sign of plasticity of this species is its flexible diet. Although *P. corethrurus* prefers rich soils in terms of organic matter and leaf litters, it is able to proliferate in extremely poor soils (Shilenkova and Tiunov, 2015). Marichal et al. (2010) proposed that *P. corethrurus* can occupy soils where other earthworm species are not present or have disappeared due to soil use and management.

In addition to the invasiveness of the species, another component of the invasion success is the invasibility of the recipient ecosystem (Mitchell et al., 2006). The hypothesis of ecological opportunity proposes that extinction of native species, and in consequence the creation of 'empty' niches, promote the establishment of exotic species (Elton, 1958). Since human-caused environmental changes may alter native species survival, they may favour a few introduced species that would competitively displace many other species from a region (Tilman and Lehman, 2001). Land use history plays thus a major role in determining the abundance and community structure of earthworms and the establishment of exotic earthworms in areas previously inhabited by native worms. For example, in the tropics, the conversion of forest to pastures has been associated with significant decreases in soil macroinvertebrate diversity (Lavelle and Pashanasi, 1989) and an increased dominance of a few exotic earthworm species that can persist along gradients of plant succession after disturbance (León et al., 2003; Zou and González, 1997). Although invasion by *P. corethrurus* has been also observed in undisturbed habitats (González et al., 2006; Hendrix et al., 1999), it seems that land use conversion is a main reason for *P. corethrurus* dominance in different parts of the world (Marichal et al., 2010; Zou et al., 2006).

Once initial colonization and establishment have occurred, invasive species may spread from long- and short-distance dispersal. The rate of range expansion will obviously be influenced by propagule pressure and dispersal capacity but also by the ability of individuals to survive and reproduce in the new range where the invasive species may encounter novel selective regimes (Sakai et al., 2001). The evolution of such local adaptation requires genetic variation. Little is known about the genetic composition of *P. corethrurus* populations. Studies of population genetics might provide valuable information about the process of invasion, for instance by comparing the genetic composition of recently established populations with populations in the native range.

The last phase of the invasion process is the integration of the species in the ecosystem and its impact on the environment. The impact of *P. corethrurus* on soil physical structure may be either detrimental or beneficial. Depending on the SOM content, its activity may either promote soil compaction, especially when populations of other "decompacting" species are not present, or contribute to soil bioturbation (Hallaire et al., 2000). Moreover, *P. corethrurus* is known to accelerate biogeochemical fluxes (González et al., 2006). In particular, its casting activity may increase the N and P availability in agroecosystems. *P. corethrurus* presence may thus be beneficial for plant growth. Teng et al. (2016) also demonstrated its positive effect on plant health.

The impact of *P. corethrurus* on other earthworm species and in particular on native species is still an open question. Some studies have stated that an increase in densities of *P. corethrurus* might directly cause the disappearance of native species and that once established in areas inhabited by native species, its effects on soil properties prevent the recolonization by native species populations (Fragoso et al., 1995; Lapied and Lavelle, 2003). However, *P. corethrurus* has been observed in coexistence with native species in some disturbed sites (Table 2). These observations suggest the absence of competitive exclusion as proposed by Marichal et al. (2010).

The literature about biotic interactions with *P. corethrurus* is almost exclusively about earthworm/earthworm and plant/earthworm interactions except for a few studies on nematodes. Information about the interaction between *P. corethrurus* and other soil macrofauna species is lacking. For instance, *P. corethrurus* has been observed in termite galleries (Gates, 1972) but, to our knowledge, no studies on their interactions have been carried out. Moreover, almost no data exist on parasites, pathogens and predators of this species, though such information could improve our understanding of *P. corethrurus* invasiveness.

4.2. Evidence of cryptic diversity?

Moreno (2004) mentioned the possibility that studies interested in *P. corethrurus* could have mistakenly studied other species in the same genus. Although several complexes of cryptic species have been recently described in earthworms, highlighting the difficulties of morphological diagnosis in this taxon (King et al., 2008; Novo et al., 2010; Pérez-Losada et al., 2009; Shekhovtsov et al., 2016), there is not much data available in the literature on the genetic diversity within the *P. corethrurus* morphospecies (Cunha et al., 2014; Dupont et al., 2012). In three populations of *P. corethrurus* in the Azores archipelago, Cunha et al. (2014) revealed the existence of two genetically divergent lineages which were morphologically indistinguishable. They showed that one of the lineages was able to cope with the extreme conditions found in the caldera of a volcano where it tolerates a mixture of non-anthropogenic chemical and physical stressors. This lineage could correspond to a new species and, in this case, the adaptation to the caldera environmental conditions cannot be interpreted as the sign of plasticity of *P. corethrurus* but instead as the result of a speciation process. This example illustrates that cryptic species may be different in their biological and ecological features and preferences (Birky et al., 2010). Thus, it is conceivable that the variable impacts of *P. corethrurus* on the environment, such as its compacting and de-compacting effects,

are evidence of two different species. In this particular case, it seems however that opposite effects are most likely due to soil characteristics. Diaz Cosin et al. (2011) highlighted that comparing published data dealing with species belonging to a complex of cryptic species is dangerous, as the authors could have incorrectly identified the species. They recommended that authors deposit the individuals used in the experiments into a collection in order to eliminate this uncertainty.

By comparing *P. corethrurus* description in different published papers (Table 1), some morphological variability was observed. Further studies are now needed in order to determine if this variability could be explained by the existence of several cryptic species (e.g., James et al., 2010). To test this hypothesis, the concordance between morphological and phylogenetic identification of *P. corethrurus* should be tested in samples coming from its whole distribution range. Moreover, investigations of the reproduction mode and ploidy level of the different lineages would help to test two alternative hypotheses concerning the observation of sexual characters in some *P. corethrurus* specimens: (i) some populations may be mixture of sexual and asexual lineages and (ii) a unique lineage may have a mixed reproductive strategy allowing shifts from sexual to parthenogenetic reproduction according to the environmental conditions.

5. Conclusion

P. corethrurus is the most common and most studied tropical earthworm morphospecies; this review integrated both the most recent and earliest information on its biology and what makes it a successful invader. Its impacts on the environment and other soil organisms were found to be strongly influenced by soil characteristics as well as land use and management. Most of the studies that have been reviewed here have sampled the specimens in the introduced range of the species. Although in some parts of the world this morphospecies has probably reached the integration stage of the invasion process and has established strong biological interaction within ecosystems, in its more recently introduced ranges, *P. corethrurus* populations may not have reached this stage yet. Thus, throughout its distribution area and given the stage of the invasion process that has been reached, population dynamics may be different and studies of ecological processes may not be comparable.

Acknowledgements

The authors are grateful to the service of “prêt inter-bibliothèque” of the University Paris-Est Créteil and to George Brown and Raphael Marichal who helped to provide numerous full-texts of articles. The authors are also grateful to Patrick Lavelle for its stimulating comments and extensive reading of the manuscript and to Naoise Nunan for English language editing.

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III. *Chapter 2: How many invasive lineages within the complex of species* **Pontoscolex corethrurus*?***

III.1. Chapter's foreword

Few studies on speciation process and cryptic species discovery have been done on peregrine earthworm species. These kinds of studies are even rarer for species in tropical zone. Knowledge on cryptic species existence is especially important for the taxa which are used as biological models e.g., soil bioindicator, bioremediation agent, and ecotoxicological model, because working on different species could give us biased results. One of the known peregrine earthworm species in the tropical zone is *Pontoscolex corethrurus*. This morphospecies is also one of the most studied in soil science. In 2014, Cunha et al. (2014) found two cryptic lineages within *P. corethrurus* on São Miguel Island of Azores archipelago. One lineage was found in a pineapple plantation and the other one in a volcano caldera (Furnas). During this chapter, we were interested to investigate if more cryptic lineages within *P. corethrurus* complex could be found in samples coming from the tropical and sub-tropical zones. We were also interested to assign the type locality samples (i.e., samples from where *P. corethrurus* was described for the first time in 1856 in Santa Catarina state, southern Brazil), of this morphospecies to one of the lineages revealed by the phylogenetic/phylogenomic trees. Meanwhile, as one of the main characteristics to diagnose this morphospecies is the quincunx formation of the setae in the last quarter of its body, we intended to evaluate the taxonomic value of this morphological character. The possibility of mistaking this morphospecies with other species in the genus *Pontoscolex* by considering this character has been already highlighted.

This study was done on samples from 116 sites through 25 countries, which came from a vast collaboration with scientists from all over the world (Figure B 1). These collaborations were from; Taiwan, Norway, France, United Kingdom, Brazil, Martinique, Mexico, Peru and United States.

This article has been accepted for publication subject to minor revisions in *Molecular Phylogenetics and Evolution* journal on 11th of December 2017.

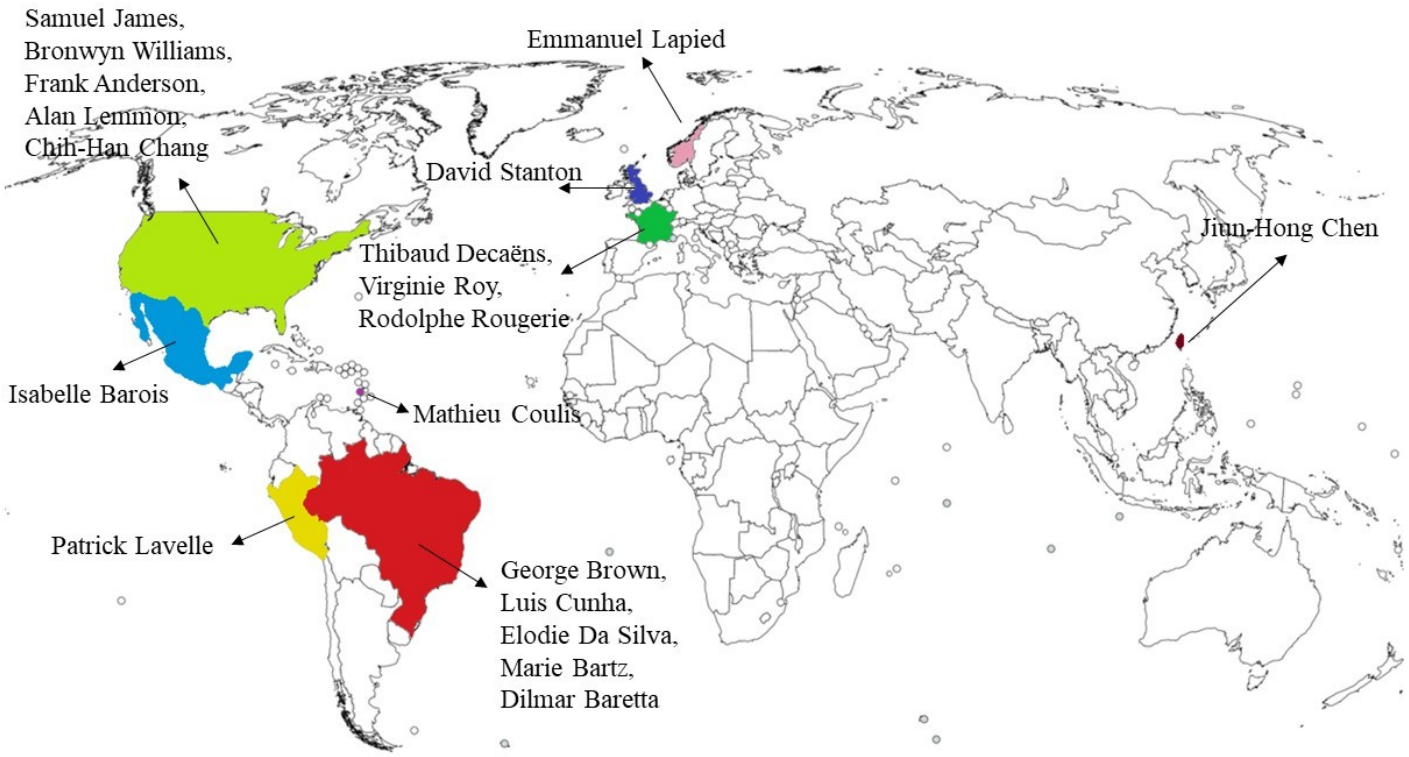

Figure B 1: Worldwide collaboration established during chapter 2 of my thesis.

Molecular Phylogenetics and Evolution 124 (2018) 60–70

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Complex taxonomy of the 'brush tail' peregrine earthworm *Pontoscolex corethrurus*

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A B S T R A C T

Pontoscolex corethrurus is the most widespread earthworm species in tropical and sub-tropical zones and one of the most studied in soil science. Although, ecological interactions of *P. corethrurus* with its environment are well documented, the taxonomic status of the species remains unclear. In this study, we investigated phylogenetic relationships within the genus *Pontoscolex*, in particular focusing on morphologically indistinguishable (i.e., cryptic) lineages. A total of 792 specimens collected from 25 different countries and islands all over the world were analyzed using two mitochondrial (COI and 16S rDNA) and two nuclear (internal transcribed spacers 2 and 28S rDNA) markers, and a total of 11 morphological characters both internal and external were investigated in all genetically characterized lineages. A large-scale multilocus sequence data matrix was also obtained for *Pontoscolex* spp. specimens using the Anchored Hybrid Enrichment (AHE) method. Multilocus phylogenetic and phylogenomic analyses, combined with species delimitation methods; including single locus (mPTP, ABGD) and multilocus (BPP) approaches, revealed congruent results. Four cryptic species were supported within the *P. corethrurus* species complex, and four potentially new species within the genus *Pontoscolex*. One widespread lineage (L1), within *P. corethrurus* complex was observed in the current population of Fritz Müller's garden where *P. corethrurus* was first described in 1856. Cryptic lineages were observed in sympatry at several localities. This, in combination with observed heteroplasmy in COI gene in one population raises an important question of reproductive isolation between these species.

1. Introduction

Speciation not accompanied by morphological changes results in formation of cryptic species (Bickford et al., 2007). Although this phenomenon has been recognized for centuries (Winker, 2005), application of molecular approaches over the last few decades has provided a

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https://doi.org/10.1016/j.ympev.2018.02.021
Received 6 November 2017; Received in revised form 20 February 2018; Accepted 20 February 2018
Available online 28 February 2018
1055-7903/

rapid and increasingly inexpensive means for unraveling these taxonomic challenges. Bickford et al. (2007) emphasized that strong environmental constraints may drive morphological stasis through stabilizing selection or adaptation to specific hosts (i.e., strong selection on behavioral or physiological characters). An alternative hypothesis is that cryptic species might be common in environments that hamper transmission of visual signals. In soil for instance, chemical signaling may play a more crucial role than morphological traits in sexual recognition (Novo et al., 2013).

Over the past decade, several genetically distinct, yet morphologically indistinguishable lineages have been identified within several earthworm morphospecies, including; *Hormogaster elisae* Alvarez, 1977 (Marchán et al., 2017; Novo et al., 2010, 2009), *Metaphire paiwanna* Tsai et al., 2000 (Chang et al., 2008), *Allolobophora chlorotica* Savigny, 1826 (Dupont et al., 2011; King et al., 2008), *Aporrectodea caliginosa* Savigny, 1826 (Pérez-Losada et al., 2009), *Lumbricus terrestris* Linnaeus, 1758 (James et al., 2010), *Lumbricus rubellus* Hoffmeister, 1843 (Donnelly et al., 2013), *Pontoscolex corethrurus* Müller, 1856 (Cunha et al., 2014), *Eisenia nordenskioldi* Eisen, 1879 (Shekhovtsov et al., 2016a, 2013), and *Eisenia nordenskioldi pallida* Malevick, 1956 (Shekhovtsov et al., 2016b). These cryptic species were rarely observed in sympatry or occupying the same ecological niche within the same habitat (King et al., 2008). Competition theory predicts that morphologically similar species should not coexist (Chesson, 1991), even though coexistence could be facilitated by differences in their biology (Zhang et al., 2004) and ecology. For instance, some of *A. chlorotica* lineages overlap in geographic distribution (i.e., sympatry) but are allotopic (i.e., do not occupy the same macrohabitat; Dupont et al., 2016) and have contrasting ecological preferences (Lowe and Butt, 2007).

Even in the rare cases where cryptic earthworm species have been found in sympatry, hybridization is uncommon. Limited hybridization has been, however, documented between *Lumbricus terrestris* and *L. herculeus* (Martinsson and Erséus, 2017), which were considered as one for a long time (James et al., 2010). Martinsson and Erséus (2017) have also detected limited hybridization among *L. rubellus* cryptic lineages using one nuclear and one mitochondrial gene. Similarly, Dupont et al. (2016) demonstrated that hybridization events were rare between lineages within the *A. chlorotica* species complex, but that historical hybridization can be inferred from patterns of mitochondrial introgression.

These complexes of cryptic earthworm species are mostly known from temperate and subtropical regions whereas a few cases of cryptic diversity were reported in tropical zone (Chang et al., 2008, 2007; Chang and Chen, 2005). Here, we investigated the genetic diversity within specimens that are commonly attributed to *Pontoscolex corethrurus* (Müller, 1856), a pantropical earthworm that is tolerant to a wide range of biotic and abiotic environmental conditions, traits that make it a successful colonizer (Fragoso et al., 1999; Lavelle et al., 1987). It is believed that *P. corethrurus* reproduces by parthenogenesis, however sexual reproduction is also possible (review in Taheri et al., 2018). *P. corethrurus* belongs to the family Rhinodrilidae (James, 2012), and it is believed to have originated from the Guayana shield area (Righi, 1984). A total of 20 nominal species have been formally described in the genus *Pontoscolex* on the basis of morphological diagnoses (Feijoo and Celis, 2012; Moreno, 2004). They were clustered in three distinct subgenera: *Pontoscolex*, *Meroscolex* and *Mesoscolex* (Borges, 1992). *P. (Pontoscolex) corethrurus* is the most widespread earthworm in the tropics (Plisko, 2001; Römbke et al., 2009) and subtropics (Taheri et al., 2018). It was described for the first time in 1856 by Fritz Müller from individuals collected in Itajaí (currently Blumenau), in Santa Catarina state, southern Brazil (Müller, 1856). The quincunx formation of setae on the last quarter of the body is the most commonly used character for the diagnosis of this morphospecies (Fig. 1A).

Pontoscolex corethrurus has been extensively used as a biological model in soil science (Duarte et al., 2012; Fragoso et al., 1997; Ganihar, 2003; García and Fragoso, 2002; Henrot and Brussaard, 1997; Jusselme et al., 2012; Liang et al., 2011; Moreno, 2004; Nath and Chaudhuri, 2012; Plisko, 2001; Senapati et al., 1999; Topoliantz et al., 2002). Because of their major roles in many aspects of soil fertility, food web ecology and ecosystem functioning, earthworms are common subjects of ecological and toxicological researches (Edwards, 2004; King et al., 2008). A comprehensive knowledge of boundaries between species is then crucial when using earthworms as biological models, as different species may have different impacts on their environments and respond differently to environmental stresses (Domínguez et al., 2005; Donnelly et al., 2013; Kille et al., 2013; Römbke et al., 2016; Voua Otomo et al., 2013). Despite the widespread interest by scientists in *P. corethrurus*, only three studies investigated the genetic characteristics of this morphospecies (Conrado et al., 2017; Cunha et al., 2014; Dupont et al., 2012). Interestingly, Cunha et al. (2014) found two genetically divergent lineages within *P. corethrurus* samples from the island of São Miguel (Azores) suggesting for the first time the existence of cryptic species in this morphospecies. They showed that the lineage found in the Furnas volcano could cope with extreme conditions found in the caldera, where it tolerates a mixture of non-anthropogenic chemical and physical stresses.

The objectives of the present study were to characterize the genetic structure and diversity within the *P. corethrurus* complex at a global scale, and to use these data to infer phylogenetic placement of the type material of *P. corethrurus* described by Fritz Müller. For this purpose, we reconstructed phylogenetic relationships among lineages belonging to the genus *Pontoscolex* using two mitochondrial and two nuclear genes and checked our results using a large-scale multilocus sequence data matrix obtained for a restricted number of samples. Moreover, we examined morphological traits generally used to identify *P. corethrurus* to assess their taxonomic value.

2. Material and methods

2.1. Phylogenetic approach

2.1.1. Samples

A total of 792 earthworms morphologically close to *P. corethrurus* were collected by soil scientists and biologists from 25 countries and islands in tropical, subtropical and temperate zones (Table 1). These countries and islands included Fiji, Gabon, Madagascar, Brazil, Cuba, Dominican, French Guiana, Jamaica, Mexico, Peru, St. Croix, St. Kitts, St. Lucia, St. Vincent, Trinidad and Tobago, Martinique, Hawaii, India, Malaysia, Philippines, Taiwan, Thailand, and the Azores, which in total represent 6 of the biogeographic realms used in Hendrix et al. (2008). Specimens from the two localities of Azores studied by Cunha et al. (2014) were added to our samples. Between 1 and 22 sites were sampled in each country or island (Fig. 2 and Table A.1 of Supplementary data). One of the sampling sites was Fritz Müller's garden, the probable type locality from where this species was first described in 1856 (Müller, 1856). Individuals from this site were considered as topotypes (i.e., a specimen originating from the type locality of the species or subspecies to which it is thought to belong) for *P. corethrurus*. GenBank sequences for mitochondrial and nuclear markers corresponding to *P. corethrurus* were also added to our data, including cytochrome oxidase I (COI) (JN036370, JQ279700, AB543229, JN887898, JN185607, JQ279698, JQ279699, JN887896, JN887897), 16S rDNA gene (JN793524, AB543235, JN887906, JN887905), and the 28S rDNA gene (AY101571, KJ912259). These sequences were from Australia, Brazil, French Guiana, India and Japan. Two specimens of *P. (Pontoscolex) spiralis* (only known from a few locations in the Caribbean; Borges and Moreno, 1990; James and Gamiette, 2016) from Guadeloupe and Puerto Rico were added. This species has a distinct morphology making it easy to recognize and was used as a reference point in the genus *Pontoscolex*. After collection, specimens were washed in distilled water, and preserved in 100% ethanol.

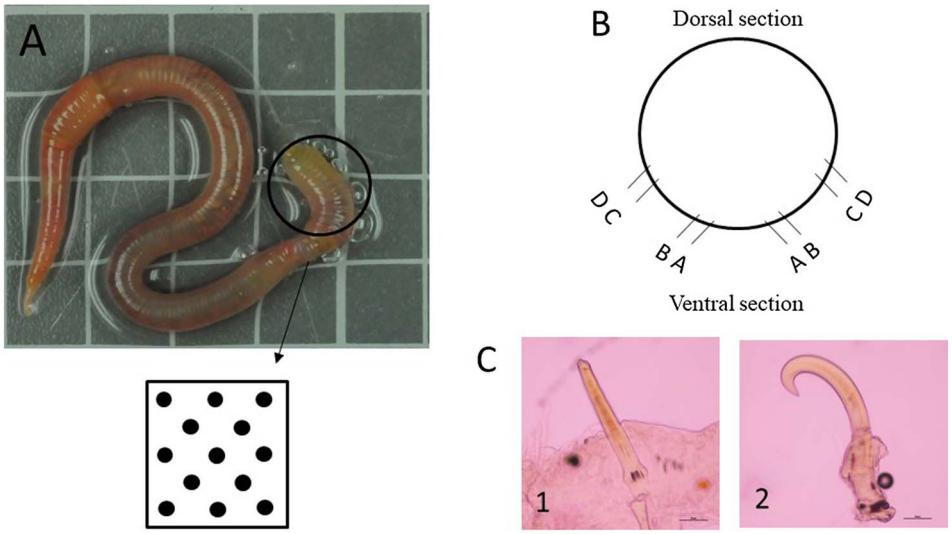

Fig. 1. Morphological characteristics of *Pontoscolex* spp.: (A) Quincunx formation of setae observed on the last quarter of some species within *Pontoscolex* genus. A characteristic used to diagnose. *P. corethrurus*; (B) A, B, C, and D setae positions, on top is the dorsal and on down the ventral part of an earthworm and (C) Hooked-setae in comparison with non-hooked-setae. Both pictures represent lateral views. (1) setae of an individual from the Philippines (Tawi Tawi island, L1). (2) setae of an individual from French Guiana (Pararé C, *P.* sp. 1).

2.1.2. DNA extraction, amplification, and sequencing

A 20-mg piece of dorsal tegument was cut between the tail and the clitellum. DNA extraction was performed using the Nucleospin tissue kit (Macherey Nagel, France). Two mitochondrial regions; fragments of the cytochrome oxidase I (COI) and the 16S-rDNA genes, and two nuclear sequences; a fragment of the 28S-rDNA gene and internal transcribed spacer 2 (ITS2), were amplified. The COI fragment corresponds to the standard DNA barcode for animals (Hebert et al., 2003) and was amplified using primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). The PCR reaction mixture contained 10 ng of DNA template, 0.2 µM of each primer, 0.5 mM of dNTPs, 1 mg/ml of BSA, 2.5 mM of $MgCl_2$, 1U of Flexi*Taq* polymerase (Promega, France) and 1X of *Taq* buffer, in a total volume of 25 µl. PCR conditions were: an initial denaturation step at 94 °C for 3 min and 10 amplification cycles at 94 °C for 30 s, 44 °C for 45 s, 72 °C for 60 s; another 30 cycles of amplification at 94 °C for 30 s, 50 °C for 45 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. For specimens showing poor PCR success, two alternate COI primer pairs were used; the first one was LepF (5′-ATTCAACCAATCATAAAGATATTGG-3′) and LepR (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) (Hajibabaei et al., 2006), and the second one, designed from this study for the genus *Pontoscolex* using Primer3 (http://www.simgene.com/Primer3), was *Ponto*F (5′-CTAGGAGTGTGGGCTGGAAT-3′) and *Ponto*R (5′-AGCAGGATCAAAGAAGGAGGT-3′). The 16S rDNA gene fragment was amplified using primers LR-J-12887 (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al., 2002) and LR-N-13398 (5′-CGCCTGTTTAACAAAAACAT-3′) (Simon et al., 1994). The ITS2 fragment was amplified using primers 5.8Smuss-F (5′-CGCAGCCAGCTGCGTGAATTAATGT-3′) (Kallersjo et al., 2014) and ETTS1-R (5′-GCTTAAGTTCAGCGGGT-3′) (Vilgalys and Hester, 1990) and the 28S rDNA fragment was amplified using primers F1 (5′-GAGTACGTGAAACCGTCTAG-3′) and R1 (5′-CGTTTCGTCCCCAAGGCCTC-3′) (Pérez-Losada et al., 2009). For all primer pairs, PCR conditions were: an initial denaturation step at 94 °C for 3 min and 40 amplification cycles with 94 °C for 30 s, annealing temperature differed between primers; i.e., COI Lep at 49 °C, 16S and ITS2 at 50 °C, 28S at 54 °C and COI *Ponto* at 55 °C for 45 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were purified, and forward and reverse strands sequenced by Eurofins (https://www.eurofinsgenomics.eu/). Sequences were aligned and edited manually using BioEdit v.7.1.9 (Hall, 1999) and Seaview v.4.5.4 (Gouy et al., 2010). For the protein-coding gene COI, alignment was translated to amino acids using MEGA 6 (Tamura et al., 2013) to detect frame-shift mutations or stop codons; which may indicate the presence of pseudogenes. In cases of ambiguous sequencing results (e.g., ambiguous base calls, unexpected position of a sequence in the reconstructed tree), DNA was re-extracted and re-sequenced. Heterozygous positions were either treated as missing data (N) for phylogenetic reconstructions or considered as informative polymorphism (used for phasing, see below) for haplotype network reconstructions and heteroplasmy analyses.

2.1.3. Analyses of molecular data

2.1.3.1. Phasing and haplotype networks. Heterozygous sites were observed for some sequences of nuclear markers. In order to derive information from these sites, phasing was performed on these sequences. Haplotypes were inferred using the PHASE program implemented in the software DnaSP v.5, based on alignments containing available unphased sequences from the present study. A haplotype network was constructed based on derived haplotypes after phasing, in Network v. 5.0.0.1 with median joining calculations (Bandelt et al., 1999).

2.1.3.2. Phylogenetic trees. The 16S rDNA, COI, 28S rDNA and ITS2 alignments were concatenated using Seaview v.4.5.4 and haplotypes were generated using DnaSP v.5 (Librado and Rozas, 2009). Topological and branch-length congruence of the mitochondrial (COI and 16S rDNA) and nuclear (ITS2 and 28S rDNA) datasets were tested using Concaterpillar 1.8a (Leigh et al., 2008). Concaterpillar uses the likelihood ratio test for congruence developed by Huelsenbeck et al. (1996) in a hierarchical framework to identify congruent subsets of genes. DNA sequences were analyzed under the GTR model proposed by the software. The mitochondrial and nuclear datasets were found to be topologically congruent (raw P-value = 0.17, Weibull-smoothed P-value = 0.16). They were also congruent with each other according to the branch length congruence test (P = 0.16). Absence of significant incongruence between partitions (p = 0.72) was also showed by the incongruence length difference (ILD) test of Farris et al. (1995), also known as the partition homogeneity test, in PAUP v. 4 (Swofford, 2002). Heuristic searches were performed on 500 pseudoreplicates with ten random additions of taxa, holding one tree per iteration to a maximum of 100 trees. Phylogenetic analyses were thus performed on the concatenated sequences.

The best fitting evolutionary model was selected with MrModelTest (Nylander, 2016) using the Akaike Information Criterion for each individual partition and for the total concatenated dataset. For Bayesian Inference, appropriate models of substitution per gene was: GTR + G, HKY + I + G, GTR + I and GTR + G for the 16S rDNA, COI, 28S rDNA and ITS2 datasets, respectively. The Bayesian search was carried out with MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) using four simultaneous Markov chains, 2 million generations and sampling every

Table 1
Samples origins, number of sample sites, cryptic lineages found and the number of mitochondrial (COI and 16S) and nuclear (ITS2 and 28S) sequences used for Pontoscolex specimens collected in each country. Missing data where the amplification was not performed are shown with a dash symbol (–).

Biogeographic realm	Country or Island	Nb of Sites	COI	16 s	ITS2	28 s	Cryptic lineage	Other species
Australian	Fiji	1	–	1	–	–	1	
Ethiopian	Gabon	1	20	13	15	1	1	
Ethiopian	Madagascar	2	15	14	1	1	1	
Neotropical	Brazil	14	139	109	70	11	1, 3, 4	*P.* sp. 4
Neotropical	Cuba	1	3	3	1	1	3	
Neotropical	Dominican	1	–	2	–	–	1	
Neotropical	French Guiana	22	237	169	61	26	1, 2	*P.* sp. 1 and *P.* sp. 2
Neotropical	Jamaica	1	–	2	–	–	1, 3	
Neotropical	Mexico	3	38	39	38	5	1, 3	*P.* sp. 3
Neotropical	Peru	16	19	15	5	5	1	
Neotropical	St. Croix	1	–	1	–	–	1	
Neotropical	St. Kitts	1	–	1	–	–	1	
Neotropical	St. Lucia	1	1	2	1	1	1	
Neotropical	St. Vincent	1	1	2	1	1	1	
Neotropical	Trinidad & Tobago	1	1	1	1	–	1	
Neotropical	Martinique	3	15	4	4	4	1	
Neotropical	Guadeloupe	1	1	1	1	–	–	*P. spiralis*
Neotropical	Peurto Rico	1	1	1	1	1	–	*P. spiralis*
Oceanic	Hawai	1	1	7	1	1	1	
Oriental	India	2	15	4	4	2	4	
Oriental	Malaysia	1	1	3	–	1	1	
Oriental	Philippeans	10	10	57	2	2	1	
Oriental	Taiwan	21	61	15	5	5	1, 3, 4	
Oriental	Thailand	7	64	65	1	1	1	
Palearctic	Azores	2	8	6	4	4	1, 4	
Total	25	116	653	537	217	73		
Genbank	Australia, Brazil, Fr. Guiana, India, Japan	–	JQ279700-L1-Fr.GUI* JQ279698-L1-Fr.GUI JQ279699-L1-Fr.GUI AB543229-L1-JPN* JN887898-L1-IND* JN036370-L1-IND JN185607-L1-IND JN887896-*P.*sp.3-IND JN887897-*P.*sp.3-IND	JN793524-L1-IND AB543235-L1-JPN JN887905-*P.*sp.3-IND JN887906-*P.*sp.3-IND	–	AY101571-L1-AUS* KJ912259-L1-BRA*	L1	*P.* sp. 3, IND

Fr. GUI*: French Guiana.
JPN*: Japan.
IND*: India.
AUS*: Australia.
BRA*: Brazil.

100 generations, with a burn-in of 2000. Resulting p-files were examined in Tracer v.1.6 (Rambaut et al., 2014) to evaluate convergence and to ensure sufficient burn-in for the trees. Maximum likelihood (ML) analysis was performed with PhyMl online analysis (http://www.atgc-montpellier.fr/phyml/, Guindon et al., 2010) for the concatenated dataset using the global GTR + I + G model of substitution using default parameters. Clade support was assessed using bootstrap with 1000 pseudoreplicates. Trees were visualized and edited using FigTree v. 1.4.2. (Rambaut, 2014).

2.1.3.3. Molecular species delimitation analyses. For species delimitation, we followed a two-step procedure: we first used single locus methods applied to the complete COI haplotype dataset (79 haplotypes) in order to infer species hypotheses, which were tested in a second step using multilocus analyses applied to the concatenated dataset from the 4 genes (63 sequences out of 1474; i.e., those specimens for which we had the 4 genes sequences) in order to confirm putative species.

For single locus analysis, the Automatic Barcode Gap Discovery (ABGD) method developed by Puillandre et al. (2012) was performed. 79 COI haplotypes were used in PAUP v. 4 software to have the distance matrix under the appropriate model for this gene i.e., HKY + I + G as an input on the ABGD webpage http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. Initial partitions were selected as they are typically more stable on a wide range of prior values and are generally closer to the number of groups described by taxonomists than recursive partitions (i.e., primary partitions assume a single gap for the entire data set, while the recursive partition splits the primary partitions into secondary partitions, and so on until no further splittings occur) (Puillandre et al., 2012).

Another single locus analysis was the multi-rate Poisson Tree Process (mPTP) analysis which is a model that accommodates different rates of coalescence within clades (Kapli et al., 2017). We performed the mPTP analysis on the COI ML tree reconstructed by PhyMl, using the online mPTP implementation http://mptp.h-its.org/#/tree.

We then tested the species hypotheses inferred from ABGD and mPTP using the multilocus Bayesian Phylogenetics and Phylogeography (BPP) v.3.1 analysis applied to a dataset combining all four genes

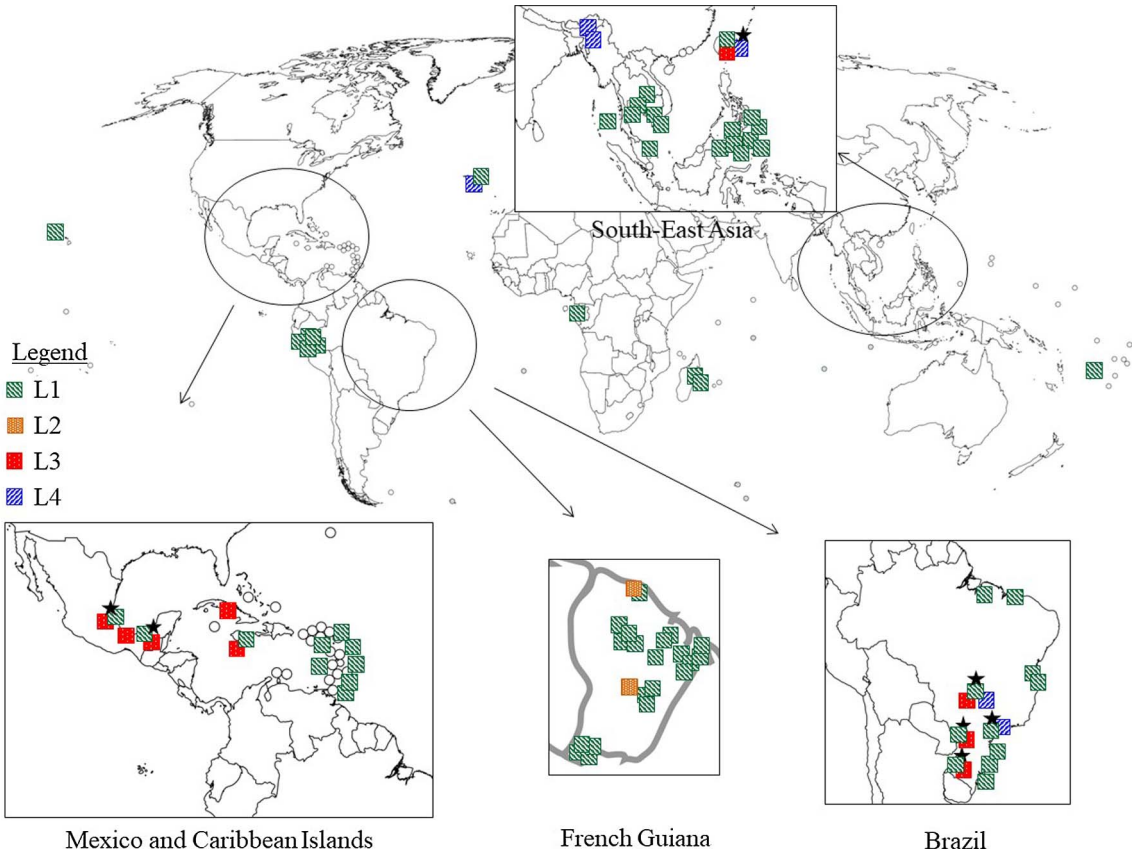

Fig. 2. Cryptic lineages in *Pontoscolex corethrurus*. Mexico and Caribbean islands, French Guiana, Brazil and South-East Asia are magnified in the squares. Cryptic lineages are shown by different colors: L1 (green lineage), L2 (orange), L3 (red) and L4 (blue). L1 is the most widespread lineage. Sites with sympatry of cryptic lineages are marked by a star. Refer to Appendix for more information on the sampled sites within each country. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sequenced: 16S rDNA, COI, 28S rDNA and ITS2. BPP uses the multi-species coalescent to analyze DNA sequence data, and can accommodate incomplete lineage sorting and uncertainty in the topology of the species tree (Rannala and Yang, 2003; Yang, 2002). We used the joint species delimitation and species-tree inference (unguided species delimitation), for which the topology from the concatenated Bayesian reconstruction was used as a guide tree, and species numbers were used based on the ABGD and mPTP approaches. As different parameters in this approach could influence the results, we used the parameters proposed by Martinsson and Erséus (2017). Two analyses, A and B, were run to ensure robust results within analyses, with different parameters as follows: in analysis A the population size parameter (θs) was assigned the gamma prior G (2, 100), with mean of 2/100 = 0.02 and the divergence time at the root of the species tree (τ0) was assigned the gamma prior G (2, 20); for analysis B, the population size parameter (θs) was assigned the gamma prior G (2, 400), with mean of 2/400 = 0.005, and (τ0) was assigned the gamma prior G (2, 40). The analyses were run for 200,000 generations with a burn-in of 10,000 generations and a sample frequency of 5. A and B each were run three times to confirm consistency between runs. Lineages delimited with a posterior probability of > 0.95 in all analyses are considered as well supported.

At the end, we calculated the mean genetic differentiation between putative species based on ABGD, mPTP and BPP species delimitations. This analysis was done based on 79 haplotypes of COI using Kimura's 2-parameter (Kimura, 1980) model of sequence evolution by MEGA v.6. (Tamura et al., 2013). This model was chosen for facilitating comparisons with other studies focusing on genetic divergence between earthworm species (Chang and James, 2011; Porco et al., 2013; Römbke et al., 2016; Voua Otomo et al., 2013).

2.2. Phylogenomic approach

We also produced a genome wide multilocus sequence dataset for eight of the *Pontoscolex* species sampled for the phylogenetic analysis described above. Using the results of the latter, one specimen per lineage was selected for phylogenomic study, plus the Rhinodrilidae outgroup taxa *Andiorrhinus* sp. and *Urobenus* sp. For this analysis, Cunha et al. (2014) lineage samples were not available.

We used Anchored Hybrid Enrichment (AHE) following Lemmon et al. (2012) and Lemmon and Lemmon (2013), and the workflow as described here: http://anchoredphylogeny.com/workflow/ (accessed 22 September 2017). Data were collected in the Center for Anchored Phylogenomics at Florida State University. A custom probe kit was prepared following Hamilton et al. (2016) by analyzing 2 Clitellate genomes (*Helobdella robusta* and *Lumbricus rubellus*) and one Polychaete genome (*Capitella teleta*), and 130 transcriptomes derived from broad taxon sampling of the Annelida. These transcriptomes were obtained for and used in Anderson et al. (2017) and by the Halanych Lab at Auburn University (unpublished data). In total, 594 anchor loci were targeted in the probe kit. After merging the overlapping read pairs (Rokyta et al., 2012), reads were mapped to the target regions using *Helobdella*, *Dendrobaena*, and *Mesenchytraeus* as references (for details of the assembly, and orthology methods, see Hamilton et al., 2016). In order to avoid contamination caused by indexing error, consensus sequences resulting from the assembly were screened to remove those originating from fewer than 650 loci. Orthology was assessed on the resulting consensus sequences using a neighbor-joining approach based on alignment-free pairwise sequence distances. Finally, alignments were performed in MAFFT (v7.023b, with –genafpair and –maxiterate 1000 flags, Katoh and Standley, 2013) and trimmed to remove poorly aligned regions.

After read assembly, quality control, alignment and alignment

trimming, sequence data were used to derive phylogenetic trees using ASTRAL (Mirarab et al., 2014; Mirarab and Warnow, 2015), which calculates individual gene trees and assembles them into a supertree; and PhyMl online (http://www.atgc-montpellier.fr/phyml/, Guindon et al., 2010) analysis in which concatenated alignments were used as input data for ML estimation and run under the GTR + G + I model selected by SMS (Lefort et al., 2017) using the AIC criterion. Clade support was assessed using bootstrap with 1000 pseudoreplicates.

2.3. Morphological analyses

Based on phylogenetic, phylogenomic and delimitation results, at least three specimens per lineage were chosen for morphological and anatomical study. Some key external characteristics of *Pontoscolex*, such as the positions of the clitellum and tubercula pubertatis, the shape and (ir)regularity of setae (A, B, C, and D) on the body (Fig. 1B), and presence or absence of genital markings, were carefully examined. Quincunx formation of setae in the last quarter of the body was also verified for each lineage. Some key internal characteristics of the genus were also observed, including presence or absence of seminal vesicles, shape and positions of calciferous glands, gizzard and hearts.

3. Results

3.1. Phylogenetic relationships and species delimitation within the genus Pontoscolex

A total of 1474 sequences were acquired in this study and 15 sequences were obtained in GenBank. The lengths of each marker after alignment were: 492 bp for 16S rDNA (32 haplotypes/ 540 sequences), 634 bp for COI (79 haplotypes/659 sequences), 712 bp for 28S rDNA (27 haplotypes/73 sequences), 409 bp for ITS2 (48 haplotypes/217 sequences). The DNA sequences were deposited in GenBank; the Accession Numbers are shown in Supplementary data (Table A.2). Our concatenated 4-genes dataset (47 haplotypes/63 specimens; see Table 1) had a total length of 2247 bp. The trees obtained from the analyses of the concatenated dataset using Bayesian inference (BI) and Maximum Likelihood (ML) methods revealed congruent topologies (Fig. 3 for BI and Fig. A.1 of Supplementary data for ML). The estimated genealogies for the four genes based on BI showed good convergence and high ESS (estimated sample size) values (i.e., > 200).

The number of candidate species (9 species) recovered by ABGD (initial partitions) was very stable across all prior intraspecific divergences (P = 0.1 to P = 0.0017), except for the extreme value of P = 0.0010 (for which 40 species were recovered). The same results were observed for mPTP procedure of species delimitation (Fig. 3).

All three runs of the multilocus species delimitation analyses A and B in BPP produced congruent results with the single locus approach. Rannala and Yang (2003) suggested that putative species could be considered distinct if their posterior probability (PP) exceeded a threshold of 95–99%. In our results, except for L2 and L3 in analysis B (which had a PP of 99%), all the other lineages for both analyses A and B had a PP of 100%, suggesting that these lineages correspond to nine distinct species (Fig. 3).

Results of COI K2P genetic distances between the 9 putative species are shown in Table A.3 of Supplementary data. The lowest genetic divergence found in *P. corethrurus* complex is 14.1% between L3 and L4 and, the highest value is 20.7% between L1 and L2. For other *Pontoscolex* species, the lowest value was between *P.* sp. 2 and *P.* sp. 3 (17.3%) and the highest, between *P.* sp. 1 and *P.* sp. 4 (25.4%).

3.2. Phylogenomic data

For the 8 in-group *Pontoscolex* samples plus two Rhinodrilidae outgroup samples, an average of 577 loci > 125 bp, 575 loci > 250 bp, 554 loci > 500 bp and 115 loci > 1000 were captured. The final data set consisted of 609 loci, comprising 238,549 sites, of which 63,124 were variable and 27,483 were informative (genomic data are available at: http://purl.org/phylo/treebase/phylows/study/TB2:S22250?x-access-code=3cfa49fbd797d19078a0fe3adbd8525d&format=html. Missing data made up 25.12% of the matrix. ML tree topology was exactly the same to those obtained from four genes except that lineage L4 was absent in phylogenomic analyses (no samples provided). All nodal support values are 1000 except for the node connecting *P. spiralis* to the tree which is 888. The phylogenomic trees (Fig. A.2 of the Supplementary data for ML tree) were highly similar to the concatenated 4 gene trees.

3.3. Morphological traits of the recovered lineages

The results of the comparative analysis of key morphological traits from at least three specimens per putative species are shown in Table 2. All the individuals were consistent with respect to the morphological characters defining the genus *Pontoscolex*, which are the position of gizzard on the 6th segment, calciferous glands in 7th, 8th and 9th segments, and hearts in the 10th and 11th segments. Among the nine lineages recovered by ABGD/mPTP and confirmed by BPP approaches, five could be consistently distinguished morphologically: one matches the formally named species *P. spiralis,* and four other unnamed lineages are designated as *P.* sp. 1, *P.* sp. 2, *P.* sp. 3 and *P.* sp. 4 according to their position in the phylogenetic tree. The four remaining lineages could not be distinguished morphologically and were named L1, L2, L3 and L4. They constituted a monophyletic group in the phylogenetic tree (Fig. 3) and were separated from *P.* sp. 1, *P.* sp. 2, *P.* sp. 3 and *P.* sp. 4 by *P. spiralis*. Moreover, the topotypes clustered with lineage L1 and the samples from the Azores were grouped with both L1 and L4. Consequently, this clade was representative of the *P. corethrurus* complex. Sequences from GenBank were all grouped with L1, but four sequences from India (COI sequences JN887896 and JN887897, and 16S sequences JN887906 and JN887905) fell within *P.* sp. 3 lineage. All the lineages had quincunx formation on the tails, except *P. spiralis* and *P.* sp. 4 that had regular A, B, C, and D setae all over the body. The position of the clitellum was different between the *P. corethrurus* complex and *P. spiralis*, *P.* sp. 3, and *P.* sp. 4. This character could not be recorded for *P.* sp. 1 and *P.* sp 2 because all specimens were juveniles. *P.* sp. 1 was the only lineage where hooked setae were observed (Fig. 1C), with regular A setae row (Fig. 1B). Additionally, *P.* sp. 2 had regular A and C setae all over the body. Almost all lineages had seminal vesicles, but in the *P. corethrurus* complex it was not observed in all specimens. Genital markings were observed for *P.* sp. 3, *P.* sp. 4, and *P. spiralis* individuals at two positions.

3.4. Geographic distribution and relationship between cryptic lineages in the P. corethrurus complex

The geographic distributions of the *P. corethrurus* lineages are shown in Fig. 2. L1 was the most widespread lineage. In seven sites, different cryptic lineages were observed in sympatry: L1/L3 (2 sites) in Mexico, L1/L3/L4 in Taiwan, and in Brazil L1/L3 (2 sites), L1/L3/L4, and L1/L4. Relationships among these lineages were further investigated using a haplotype network obtained with phased nuclear sequences. Because of very few polymorphisms and low resolution of the 28S rDNA marker (9 polymorphic sites on a total of 712 bp), we present only the results for ITS2 (Fig. 4). The ITS2 alignment comprised a total of 371 sites: 300 sites were invariable, 27 were polymorphic, 2 contained gaps and 42 contained missing data. The length of the alignment was shorter than for the ITS2 alignment mentioned earlier (371 vs. 409 bp), because only *P. corethrurus* complex sequences were used for this analysis and these contained no indels or gaps in the alignment. Heterozygous ITS2 sites were observed for 192 sequences from the *P. corethrurus* complex. The analysis was done on the 38 haplotypes used in the tree resulting from the analysis of the concatenated dataset. Each lineage of the *P.*

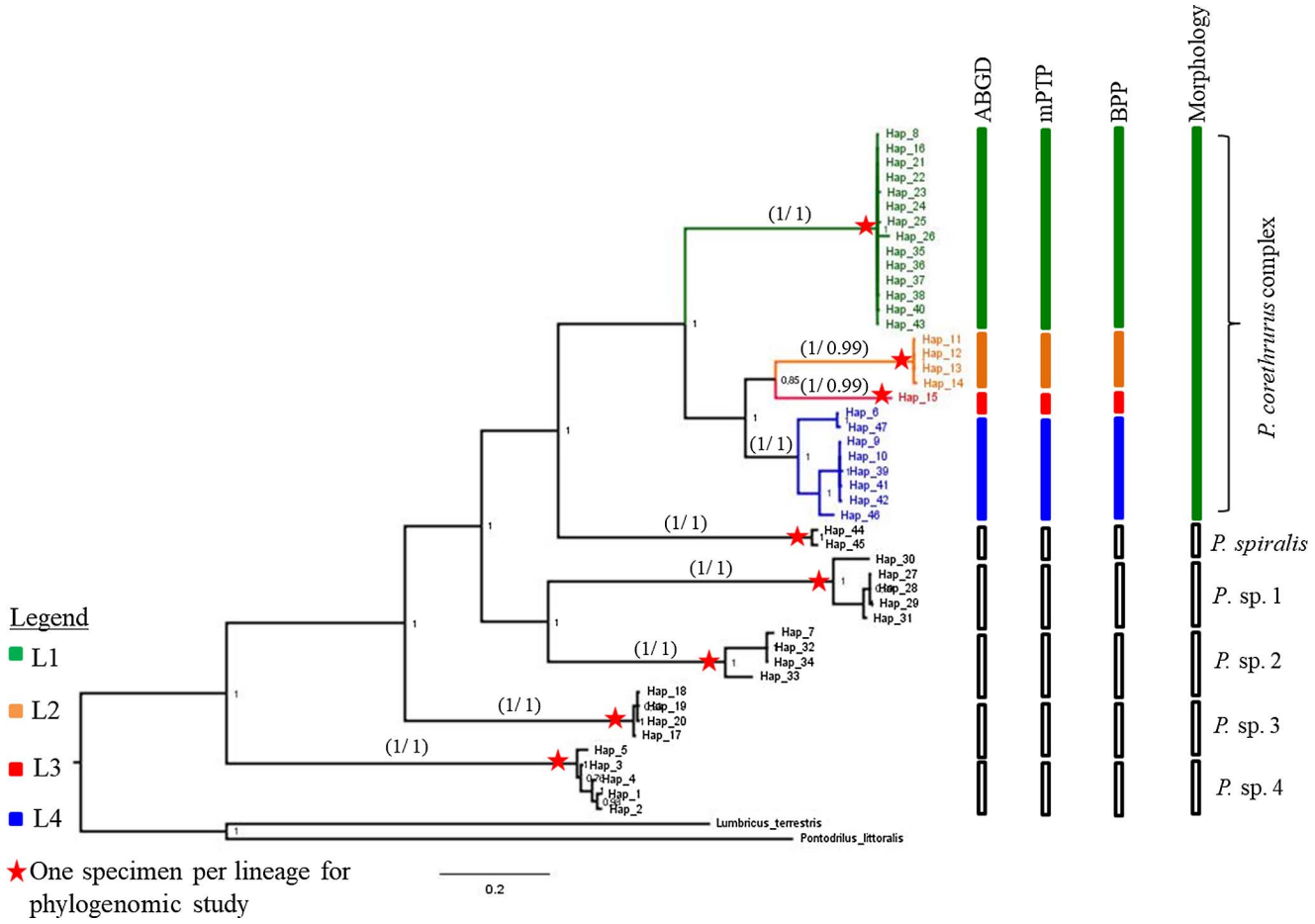

Fig. 3. Bayesian haplotype tree of the four genes (16S rDNA + COI + 28S rDNA + ITS2). Bayesian posterior probability values > 0.85 are disposed at the node of each cluster. Species delimitation analyses by ABGD, mPTP, BPP and morphological approaches are shown by bars. Posterior probability values based on BPP analysis for species delimitation are shown on each branch (Analysis A/Analysis B). Generally species with both PP > 0.95 are considered as well supported species. Outgroup species and species other than *P. corethrurus* (*P. spiralis*, *P.* sp. 1, *P.* sp. 2, *P.* sp. 3, and *P.* sp. 4) are shown in black text, without coloration. Color codes for lineages are the same as in Fig. 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

corethrurus complex was separated in the haplotype network except for haplotype 5 which was shared between L3 and L4 and was observed in the following locations; Furnas (Azores), Rabung and Hualtu (India), National Taiwan University (Taiwan) in L4, Mancha, Tlalcotlen and Hayas (Mexico), and Palmar de la Jarreta (Cuba) in L3.

Interestingly, three specimens from one site in Taiwan (National Taiwan University) showed heteroplasmy for COI mitochondrial gene. The DNA of these samples was extracted 2 times and sequenced 3 times in order to exclude the contamination hypothesis. We observed that for each heterozygous site, one possible case corresponded to the L3 lineage and the other to L4.

4. Discussion

4.1. New species within the genus Pontoscolex

Molecular analyses of 792 specimens belonging to the genus *Pontoscolex* representing a global sampling, from the tropics and subtropics, revealed the existence of nine genetically divergent lineages. One of these lineages corresponded to the species *P. spiralis* which is taxonomically well-known (Borges, 1996). Four lineages showed distinct morphological traits that allow us to differentiate them from the morphospecies *P. corethrurus* and from *P. spiralis*. These species were named *P.* sp. 1, *P.* sp. 2, *P.* sp. 3 and *P.* sp. 4. We were unable to assign these four species to previously established names and some of them could be new to science.

P. sp. 1 presented hooked setae and *Pontoscolex (Pontoscolex) kuneguara* is the only species within the genus reported to have hooked-setae on the body excluding the tail. Yet, hooked setae were observed over the entire body of *P.* sp. 1, including the tail, thereby differentiating these specimens from *P. kuneguara*. *Pontoscolex* sp. 2 was characterized by regular A and C setae, a characteristic also reported for *P. hingstoni* (Stephenson, 1931) and *P. cuasi* (Christoffersen, 2008; Righi, 1984). In the former, seminal vesicles extend up to the 31st segment while for the latter to the 25th. However, we were unable to determine the seminal vesicles position in our specimens of *P.* sp. 2 because they were juveniles. The presence of irregular setae observed on specimens representing the *P.* sp. 3 lineage indicate that this lineage could be assigned to either the *Pontoscolex* or *Mesocolex* subgenus. The subgenus *Pontoscolex* contains 15 species with and without regular setae; in *Mesoscolex* only one species is currently known, and it has irregular setae; and all four described species in the subgenus *Meroscolex,* have regular setae (Moreno, 2004). As *P.* sp. 3 was only represented by four juveniles from a single population in Mexico (i.e., Mancha), we were unable to compare the clitellum and tubercula pubertatis positions with those of described species of *Pontoscolex*. Despite small body size (in comparison with other specimens) and juvenile state, big seminal vesicles were remarkable during dissection of this lineage. It is noteworthy that two sequences from GenBank for COI (JN887896 and JN887897) and 16S (JN887906 and JN887905) genes, labeled as *P. corethrurus* from India, clustered with this lineage. This result highlights the risk of mistaking *P.* sp. 3 with *P. corethrurus* and

Table 2
Morphological (internal and external) characters of Pontscolex specimens found in each lineage and species. Missing data or information are shown with a dash symbol (–).

Lineage	Clitellum position (segments)	Tubercula Pubertatis position (segments)	Genital markings (segments)	Setae shape	Regular setae	Quincunx formation on tail	Seminal Vesicles	Gizzard (segment)	Calciferous glands (segments)	Calciferous glands shape	Hearts (segments)
Pontoscolex corethrurus complex (L1, L2, L3, L4)	15–23 or 1/2 15–1/2 23	19–21	No	Non hooked	None	Yes	Yes/no	6	7, 8, 9	Tubular	10, 11
Pontoscolex spiralis	16–24	20–22	Yes, 8–12 and 19–23	Non hooked	A, B, C, D	No	Yes	6	7, 8, 9	No data	10. 11
P. sp. 1	16–24	20–22	Yes, 8, 9 and 19–22	Hooked	A	Yes	Yes	6	7, 8, 9	No data	10, 11
P. sp. 2	Juveniles	20–22 (intersegmental)	No	Non hooked	A, C	Yes	Yes	6	7, 8, 9	No data	10, 11
P. sp. 3	Juveniles	No data	No	Non hooked	None	Yes	Yes	6	7, 8, 9	Tubular	10, 11
P. sp. 4	13–23	19–1/2 22	Yes, 5, 6, 9 and 18-22AB	Non hooked	A, B, C, D	No	Yes	6	7, 8, 9	Oval parallel tubular duct from side	10, 11

reveals that *P.* sp. 3 is also present in India. Moreno (2004) emphasized that a source of misidentification could be the use of the quincunx formation of setae as diagnostic character for *P. corethrurus.* The quincunx formation indeed exist in *P.* sp. 1, *P.* sp. 2 and *P.* sp. 3 as well. Apart from *P. spiralis*, regular setae all over the body was also observed for *P.* sp. 4 which was found in Manaus (Brazil). We assume that *P.* sp. 4 belongs to either *Meroscolex* or *Pontoscolex* subgenus.

Additional investigations (molecularly and morphologically) are necessary in the future to disentangle the diversity within the genus *Pontoscolex*. Ideally, these questions should be addressed through genetic comparison with the actual type specimens of already named species, a very challenging task since in most cases the type specimens are lost, such as for *P. corethrurus,* or difficult to gain access to, and the molecular analysis of tissues is also almost impossible because of DNA preservation issues.

4.2. Single and multilocus methods revealed a complex of cryptic species in *P. corethrurus*

In the phylogenetic trees, the *P. corethrurus* complex of cryptic species was represented by a monophyletic clade composed of four morphologically indistinguishable lineages (L1, L2, L3 and L4) separated from the other species by *P. spiralis*. The haplotype network for ITS2 which is a fast-evolving nuclear sequence where signal of hybridization or mating with nonspecific relatives are often shown (Vollmer and Palumbi, 2004), confirmed the differentiation of the four lineages within the *P. corethrurus* complex (Fig. 4).

Single (ABGD/mPTP) and multilocus (BPP) delimitation methods produced congruent results. Single locus delimitation methods were often criticized, because when applied among closely related species, these approaches may result in an over- or under-estimation of species diversity (Meyer and Paulay, 2005; Roe et al., 2010; Roe and Sperling, 2007; Will and Rubinoff, 2004). Alternatively, multilocus analysis constitutes a valuable source of information for species delimitation because it lowers the risk of incorrect results based on single locus datasets (Dupuis et al., 2012; Roe et al., 2010). Our multilocus analysis with 4 genes was run with different values of the priors for the population size (θs) and the divergence time at the root (τ0) (Rannala, 2015) as it has been shown that different parameters (particularly θ priors) give different results i.e., smaller values give higher probabilities for species splits (Rannala, 2015). Our study confirms this prediction; as θ prior was smaller, higher probability was observed for L2 and L3 (PP = 1 compared to PP = 0.99). Other PP values for all the other lineages in both analyses were the same (PP = 1).

We recognize that low levels of locus sampling (i.e., 4 locus) invite criticisms that the data may not be adequate to address phylogenetic questions. Therefore, we included the AHE method to evaluate the validity of a result from lower sampling intensity. Here, we did not go into details of lineage sorting or conflicts among gene trees, yet the outcome of the AHE analysis was quite clear. Four genes results were obtained by conventional sequencing, and at a lower cost per sample than the AHE, but in spite of having about 1% as many sequence positions, produced similar results. We expected that recombination among lineages would have led to incongruent multilocus topologies. Our results suggested a lack or limited recombination among lineages and that low effective population sizes and long evolutionary periods of time, led to complete lineage sorting of multiple independent loci. Overall, our results from both single locus and multilocus analyses support the hypothesis that four cryptic species exist in this morphospecies.

4.3. Phylogenetic placement of the topotypes and cryptic species distribution

The lineage L1 matched the topotype samples from Fritz Müller's garden. Therefore, we suggest that this lineage corresponds to *Pontoscolex corethrurus* sensu stricto. Additionally, L1 is the most

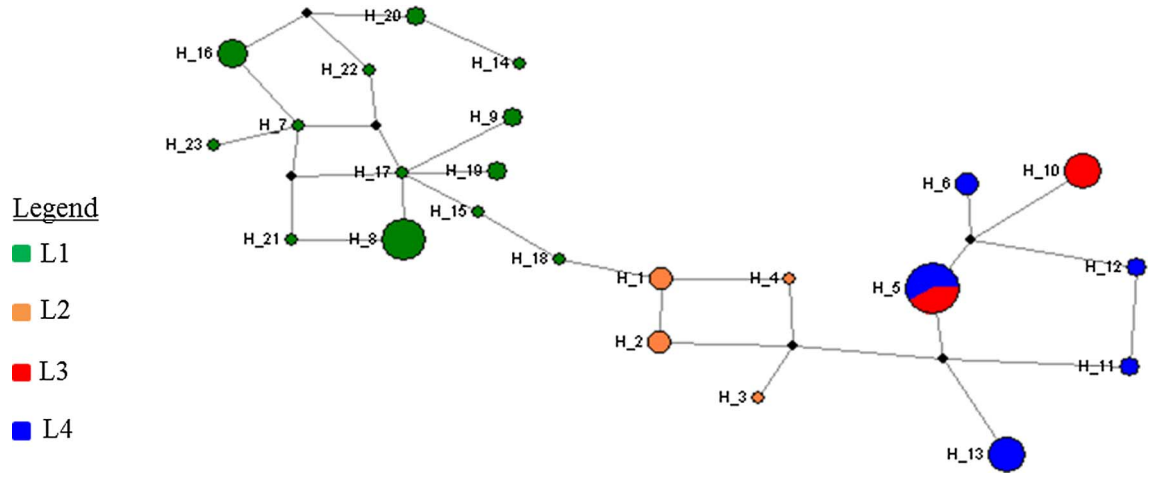

Fig. 4. ITS2 marker haplotype network of the four main *P. corethrurus* lineages after phasing Color codes for lineages are the same as in Fig. 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

widespread lineage, being recorded in 100 out of a total of 116 sampled sites. This suggests that most of the studies done on *P. corethrurus* were most probably on this lineage and not on other species in the complex.

L4 corresponds to the cryptic species already found by Cunha et al. (2014) for the Azores. We found this lineage in Taiwan, India and Brazil as well. Cunha et al. (2014) revealed that this species has a remarkable tolerance to hostile conditions (i.e., high soil temperature, high carbon dioxide and low levels of oxygen and elevated metal bioavailability) as it was found on Furnas volcano of the Azores. It is noteworthy that in the other countries (Brazil, Taiwan and India), L4 was found in non-hostile environments i.e., in gardens of National Taiwan University and Embrapa in Brazil, and in grass area of village center/grass lawn in front of a forest reserve in India. Further investigations to understand the geographical distribution of L4 are necessary.

Sympatry of lineages was observed in seven of 116 localities. It occurred in two sites in Mexico between lineages L1/L3, in four sites in Brazil between L1/L3/L4, L1/L4, and two sites L1/L3, and in one site in Taiwan between L1/L3/L4. Interestingly, in the latter population (National Taiwan University, Taipei, Taiwan), COI heteroplasmy was observed for three specimens, with all the heterozygous sites corresponding either to the L3 haplotype or to the L4 haplotype. The ITS2 network also showed that haplotype 5 is shared between L3 and L4 specimens (i.e., in the sites of Mexico, India, Azores, Taiwan and Cuba) (Fig. 4). This result suggests sexual reproduction for L3 and L4 and that reproductive isolation is not yet complete between these lineages. The observed heteroplasmy could be explained by paternal leakage (i.e., low-level paternal contribution of mtDNA to progeny) (Birky, 2001; Kmiec et al., 2006). Sequencing lacks the sensitivity to show mtDNA mutations that are present at low percentages (i.e., 0–20%) (Wong and Boles, 2005) which could explain why heteroplasmy was not detected with the 16S rDNA gene. The observed heteroplasmy cannot be due to contamination, as we sequenced the specimens from re-extracted DNA three times, even if true we would observe the heteroplasmy for 16s rDNA as well. Further population genetics analyses are necessary to investigate the reproductive isolation between L3 and L4 in the complex and to test whether retention of genetic polymorphisms due to past hybridizations could explain this phenomenon. Reproductive isolation among these species, could also be tested by laboratory cross-breeding experiments. These types of experimentations have already found the evidence of reproductive isolation between *Eisenia fetida* and *Eisenia andrei* (Domínguez et al., 2005), and between cryptic lineages of the *Hormogaster elisae* (Marchán et al., 2017).

5. Conclusion

We showed that the *Pontoscolex corethrurus* complex consists of at least four cryptic species. Further, based on modern sampling from the type locality for *P. corethrurus*, we suggest that L1 represents *P. corethrurus* sensu stricto. Morphologically cryptic diversity in *P. corethrurus* could be problematic given its presumed wide distribution and common use in soil studies, as different species may have different impacts on and respond differently to the environment. It is possible that published studies in the past may have dealt with species that were not necessarily *P. corethrurus* sensu stricto (L1) but the risk is relatively low since L1 is the most widespread species in the complex. We echo the advice of Martinsson and Erséus (2017): it is important to define species boundaries as objectively as possible, especially for taxa used as biological models. One key to objectivity, especially with the continuous progress of our ability to delimit biological units, is to make results reproducible by preserving voucher specimens, and by publically releasing sequences (Wen et al., 2017).

Acknowledgements

S. Taheri acknowledges the University Paris-Est and French government by Campus France for her PhD grant. The authors thank the Fritz Müller Museum of Blumenau for allowing collecting on property grounds and the funding agencies for support of this study: G. Brown, E. da Silva, M. Bartz and S. James thank CNPq for fellowship support; L. Cunha, D. Stanton and G. Brown acknowledge support of NERC, EU, Fundação Araucária and the Newton Fund; D. Baretta and M. Bartz acknowledge FAPESC support; C.-H. Chang thanks support from National Science Foundation, USA (EF-1550795). We would like to thank Patrick Lavelle for sampling specimens from Peru. We are grateful to Michelle Kortyna, Sarah Banker, and Alyssa Bigelow for laboratory assistance and to Jean-Emmanuel Longueville, Sean Holland, Kirby Birch, and Ameer Jalal for bioinformatics support.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.02.021.

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IV. Chapter 3: Phylogeography and population genetics of an invasive peregrine earthworm species

IV.1. Chapter's foreword

Population genetics studies help us in determining the genetic variation within and among populations, in native and invaded areas, while phylogeography allows determining the invasion roads of a taxa. These issues were rarely addressed in earthworms. To date, phylogeography studies were carried out on only two peregrine invasive earthworm species: *Aporrectodea rosea* and *Aporrectodea trapezoids.* One of the most well-known peregrine earthworms, is *Pontoscolex corethrurus.* Phylogeography of this species has never been studied but population genetics of this species was investigated for the first time by Dupont et al. (2012). In Dupont et al. (2012) study, samples from French Guiana i.e., in the original zone of genus *Pontoscolex*, were studied using AFLP markers. Due to easy transportation and propagation of this species from different places by humans, high genetic diversity in populations in close relations with urban areas were detected. In the second chapter of my thesis, we showed that this morphospecies corresponds to a complex of four cryptic species. Among them, we found that *P. corethrurus* sensu stricto (*P. corethrurus* L1) was widely spread, showing high invasion capabilities. In the present chapter, we were first interested to investigate whether the cryptic species in *P. corethrurus* complex have reached reproductive isolation. Then, we focused on *P. corethrurus* L1 to investigate if a 'super-clone' has invaded the world, and to compare the genetic variation between populations in native (Guayana shield) and invaded regions of this species. Finally, we used population genetic analyses (e.g., gametic disequilibrium) to study the reproduction strategy of *P. corethrurus* L1. This chapter is written in the form of a scientific article and will be submitted to the journal *Diversity and Distributions*. This study was conducted through international collaborations from France (Thibaud Decaëns), Brazil (George Brown and Luis Cunha), and Taiwan (Jiun-Hong Chen), on a total of 299 AFLP profiles and 662 COI sequences obtained from specimens sampled in: Brazil, Peru, French Guiana, Trinidad and Tobago, Mexico, St. Lucia, St. Vincent, Martinique, Azores, Gabon, Madagascar, Thailand, Malaysia, Taiwan, Philippines, and Hawaii.

Global phylogeography and population genetics of the peregrine earthworm *Pontoscolex corethrurus*

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IV.2. Abstract

Aim: *Pontoscolex corethrurus* is the most invasive earthworm in (sub)tropical zone. This parthenogenetic earthworm probably originated from the Guayana Shield. A recent phylogenetic study revealed four cryptic species in *P. corethrurus* complex (L1, L2, L3 and L4) and L1 was particularly widespread. Moreover, sympatry was observed in several populations. Our aims were to study the genetic admixture between cryptic species in sympatry and to achieve a large scale phylogeographical study for L1. Our tested hypothesis were that (i) a "super-clone" has invaded the world, (ii) the clonal diversity is lower in the introduced range than in the native zone, and (iii) L1 has a mixed reproductive strategy.

Location: Brazil, Peru, French Guiana, Trinidad and Tobago, Mexico, St. Lucia, St. Vincent, Martinique, Azores, Gabon, Madagascar, Thailand, Malaysia, Taiwan, Philippines, Hawaii

Methods: In three populations where L1, L3 and L4 were detected, genetic admixture was analyzed using 57 AFLP profiles. Analyses of genetic variation of L1 populations were done using both nuclear (226 AFLP profiles) and mitochondrial (479 COI sequences) genetic information.

Results: No evidence of hybridization between species was obtained. A weak COI genetic diversity was revealed for L1 with one major haplotype (H1) which was shared by 72% of the specimens. Asexual reproduction was confirmed by the presence of clones in several populations revealed by AFLPs. However, no clones were shared among populations. Absence of clones, high gene diversities and weak values of gametic disequilibrium were observed in some populations.

Main conclusions: Long invasion history and multiple re-introductions of specimens from different parts of the world may explain the similar level of genetic diversity in introduced and native regions. The relatively high clonal diversity allowed to reject the 'super-clone' invasion hypothesis. Since recombination was suggested by gametic equilibrium in some populations, mixed reproduction strategy of *P. corethrurus* L1 is likely.

IV.3. Introduction

Many species have been reported as having extremely widespread distributions. Among these cosmopolitan species, earthworms are poorly represented with the exception of a few taxa (Hendrix et al., 2008). Because of the limited dispersion ability of earthworms it is even striking that out of 3000-3500 described species (Csuzdi, 2012), about 150 species are considered peregrine (i.e., widely ranging, often owing to human actions; Blakemore, 2012). The proportion of peregrines on total described species in temperate region is much higher than in tropical region (i.e., tropical: 51 of an estimated several thousand species; temperate: 45 out of 500–600 estimated species) (Hendrix et al., 2008). In addition to some advantageous traits such as high fecundity, small size, resistant cocoons, wide and rapid dispersal, and feeding tolerance, some of these peregrine species are known to have a parthenogenetic reproduction often accompanied by polyploidy (reviewed in Fernández et al., 2011). Two main features allow parthenogenetic species to invade and occupy open habitats faster than sexually reproducing species ; (i) a double intrinsic rate of natural increase of a population and (ii) the ability of one individual to establish a new colony (Cuellar, 1977). Moreover, this mode of reproduction is generally impervious to inbreeding depression and fit genotypes are not broken up by sexual recombination.

The elucidation of genetic structure patterns and variation within and among populations in distant locations has the potential to provide insights into the geographic origin, migration pathways and colonization history (e.g. single *vs* multiple introductions) of these species (Sakai et al., 2001). For instance, molecular markers have proved to be powerful tools for inferring the evolutionary history of the peregrine earthworm species *Aporrectodea trapezoides* (Fernández et al., 2011, 2016) which has Palearctic origin but a current worldwide distribution. Analyzing two mitochondrial and two nuclear markers, Fernández et al. (2011) revealed a relatively high clonal diversity in this parthenogenetic species whereas one clone was shared by almost one third of the sampled individuals and was widely distributed. Such "super-clones" are thought to be "general-purpose genotypes" (Lynch, 1984) which show a broad ecological tolerance resulting from interclonal selection in temporally variable environments (Vorburger et al., 2003).

One of the most well-known and widespread tropical peregrine earthworms is *Pontoscolex corethrurus* (Taheri et al., 2018). This endogeic earthworm originates from Guayana Shield in South America (Righi, 1984), and is tolerant to a wide range of biotic and abiotic environmental conditions (Fragoso et al., 1999; Lavelle et al., 1987). It was described as parthenogenetic although the possibility of sexual reproduction could not be ruled out (Gates 1973, Dupont et al. 2012). Although, this morphospecies has been frequently used as biological model in various experiments (reviewed in Taheri et al. 2018), only two studies were interested in its population genetics (Dupont et al., 2012; Cunha et al., 2014). Dupont et al. (2012) investigated the genetic variability of *P. corethrurus* in its native range using AFLPs (Amplified Fragment Length Polymorphism). Among the six populations from French Guiana, a higher level of genetic diversity was found in

populations originating from the most disturbed sites while a higher number of clones was observed in the other populations. In Cunha et al. (2014), three populations from the São Miguel Island in the Azores archipelago where *P. corethrurus* was introduced were studied using two mitochondrial markers and AFLPs. Besides the discovery of two highly divergent genetic lineages, they found a higher intra-lineage genetic diversity in the population of earthworms living in pineapple greenhouses, most probably because of repeated introductions. A recent study by Taheri et al. (*in revision*) using both phylogenetics and phylogenomics approaches confirmed the high complexity of the genus *Pontoscolex*. This study showed that the commonly used species, *P. corethrurus,* corresponds in reality to a complex of four distinct and cryptic species (defined as *P. corethrurus* L1, L2, L3 and L4). *P. corethrurus* L1 was the most common of the complex in different areas. Meanwhile this latter species was present in the location where this morphospecies was first described by Fritz Müller in 1857 (Müller 1857). It was thus suggested that L1 corresponds to *P. corethrurus* sensu stricto. Moreover, different species in the complex were found in sympatry in various locations and the question of hybridization between them was raised.

Here, our aim was to achieve a large scale phylogeographical study of this well-known peregrine earthworm, investigating samples coming from its native and introduced range. First of all, specimens were assigned to four cryptic species composing the complex using one mitochondrial marker. Then, we tested whether AFLPs allowed to distinguish 3 of the cryptic species that were found in sympatry, namely L1, L3 and L4. In a second time, the AFLPs were used to investigate the genetic admixture between these three cryptic species in different populations where they were found in sympatry in order to test the hybridization hypothesis. At last, we choose to focus on the most widespread species of the complex that is considered as *P. corethrurus* L1. The genetic variability and the genetic differentiation of the different L1 populations were studied in order to test the following hypothesis: (i) a "super-clone" has invaded most of the introduced areas, (ii) the clonal diversity is lower in the introduced range than in the native one because mutations did not have time to accumulate, and (iii) this species has a mixed reproductive strategy.

IV.4. Methods

IV.4.1. DNA material and COI sequences

A total of 299 specimens belonging to the *Pontoscolex corethrurus* complex were collected from six countries in tropical and subtropical zones (Table C 3 in Supplementary data). These countries included: French Guiana which is situated in the Guayana shield (i.e., the putative native zone of the morphospecies), Brazil, Mexico, Gabon, Taiwan, and Thailand. In each country, 1-8 sites were sampled. After collection, specimens were washed in distilled water, and conserved individually in 100% ethanol. A piece of 20 mg of dorsal tegument was cut between the tail and

the clitellum. DNA extraction was performed using the Nucleospin tissue kit (Macherey Nagel, France). A total of 662 sequences of a fragment of the cytochrome oxidase I gene (COI) were obtained from a previous study (Taheri et al., *in revision*).

IV.4.1.1. COI sequences analyses

A phylogenetic tree based on 662 COI sequences was built (Figure C 6 in Supplementary data) using the model HKY+G which was the best fitting evolutionary model selected with MrModelTest (Nylander, 2016) using the Akaike Information Criterion. Two specimens of *Pontoscolex spiralis* from Guadeloupe and Puerto Rico were chosen as outgroups (Genbank accession numbers XXX[15]). Maximum likelihood (ML) analysis was performed with PhyMl online analysis (http://www.atgc-montpellier.fr/phyml/, Guindon et al., 2010). Clade support was assessed using bootstrap with 1000 pseudoreplicates. Trees were then edited using FigTree v. 1.4.2. (Rambaut, 2014).

A total of 479 COI sequences from 49 locations were assigned to *P. corethrurus* L1. Among them, 269 sequences from 12 populations were used for a population genetics analysis. For these samples, number of polymorphic sites, number of haplotypes, haplotype (gene) diversity and nucleotide diversity (Pi; Nei, 1987) were calculated using DnaSP v. 5 software (Librado & Rozas, 2009). A haplotype network was constructed based on derived haplotypes in Network v. 5.0.0.1, using median joining calculations (Bandelt et al., 1999).

IV.4.2. AFLP procedure

AFLP analysis was carried out according to Vos et al. (1995) with few modifications: approximately 50 ng/µl of purified genomic DNA of each specimen was digested with two digestive enzymes. The first digestion was done in 10µl with Taq1 (20U, New England BioLabs, Ipswich, MA, USA (NEB)), Buffer Taq1 (10X, NEB), BSA (1mg.ml-1, NEB) and incubated for 1h30 at 65°C by thermal cycler (T100TM, BIO-RAD Laboratories Inc., Foster city, CA, USA). The second digestion of the DNA from the last step was done in 15µl with a solution of: EcoRI (20U, NEB), Buffer EcoRI (10X, NEB), BSA (1mg.ml-1, NEB) and incubated for 1h30 at 37°C. We verified DNA digestion quality by gel electrophoresis and negative controls were run at each time. Adapters were ligated using 50pmoles μl^{-1} of double- stranded Taq1 adapter (Taq top 5'-GACGATGAGTCCTGAC and Taq bottom 5'-CGGTCAGGACTCAT, Eurofins Genomics, Germany) and 5pmoles µl-1 of double-stranded EcoRI adapter (Eco top 5'-CTCGTAGACTGCGTACC-3' and Eco bottom 5'-AATTGGTACGCAGTCTAC-3', Eurofins

[15] Sequences are deposited on GenBank, currently waiting for accession numbers.

Genomics, Germany), T4 DNA ligase buffer (10X, Promega, Madison, WI, USA), ATP (10mM, NEB), BSA (1mg.ml-1, NEB), T4 DNA ligase (3U, Promega). Samples with a total volume of 50µl (prepared solution + digested DNA from previous step + adjusted water) was then incubated for 3h at 37°C. Digestion-ligation production of 20µl was diluted with 40µl of AE buffer (QIAGEN Sciences, Maryland, USA). Then each dilution was divided in 3 separate samples of 15µl each. Pre-selective PCRs contained 5pmoles of E01 primer (GACTGCGTACCAATTCA) and 5pmoles of T01 primer (GATGAGTCCTGACCGAA), MgCl2 (25mM, Promega), dNTPs (10mM, InvitrigenTM, Life technologies, Carlsbad, CA, USA), GoTaq buffer (5X, Promega), DNA Taq polymerase (5U, Promega), adjusted with water to have a total volume of 50µl in each sample (added to diluted ligation product). The PCR pre-selective was done by an initial denaturation step at 94°C for 2min, followed by annealing setup of 30 cycles containing; 94°C for 30s, 56°C for 1 min and 72°C for 60s, and finally elongation setup of 72°C for 10 minutes. After this setup, the three sub samples were reassembled in one, and then 1µl of each sample was diluted in 19µl of AE buffer (QIAGEN). Selective PCR reactions contained 5µl of pre-amplified DNA with 15µl of a solution containing MgCl2 (25mM, Promega), dNTPs (10mM, Invitrigen), GoTaq buffer (5X, Promega), 5pmoles.µl-1 T32 primer (5'-GATGAGTCCTGACCGAAAC-3') or 5pmoles.µl-1 T38 primer (5'-GATGAGTCCTGACCGAAACT-3'), DNA Taq polymerase (5U, Promega) and one primer combination E32-FAM was used (5'-GACTGCGTACCAATTCAA-3'). A touchdown thermal cycling (PTC-100) started with denaturing setup of 94°C for 2 min, following by 9 cycles; 94°C for 30s, 65°C for 30s with 1°C diminution per cycle, 72°C for 60s, following by 26 cycles containing 94°C for 30s, 56°C and 72°C during 1min and finally 72°C for 10 minutes. After each setup DNA solutions were centrifuged by ROTANTA 460R (Hettich Lab Technology, Tuttlingen, Germany). Amplified products were mixed with formamide (Hi_DiTM, Applied Biosystems, Foster city, CA, USA (AB)) and a Genescan^{TM}-500LIZ^{TM} (AB) size standard (9.5µl of formamide and 0.5 µl of GenescanTM-500LIZ^{TM} for 2µl of amplified product). Fragments were separated on a ABI PRISM^{TM} 3130 Genetic Analyzer (platform INSERM, Henri Mondor hospital, Créteil). Raw data were visualized and the fragments manually scored using Genemapper V5 (Applied Biosystem) software. Processed data were exported as presence/absence matrix.

IV.4.2.1. Reproducibility of AFLP profiles

Genetic markers, including AFLPs, can be prone to genotyping errors with various potential sources (Bonin et al., 2004). Therefore, we estimated the genotyping error rate of our dataset by re-genotyping and blind scoring of 54 randomly chosen individuals (25% of the samples) within *P. corethrurus* L1 from 15 locations (i.e., Bahia cacao, Bahia pasture, Belem, Caxiuana, Joinville, Orléans, Sao Paolo, Cayenne, Mancha, Tlalcotlen, Mitaraka, PararéA, PararéB, Taiwan National University, and Chachoengsao). The error rate was calculated by mismatch error rate based on the formula proposed by Bonin et al. (2004), multiplied by the total number of markers (i.e., 394).

IV.4.2.2. AFLP analyses of cryptic lineages

The Structure software (Pritchard et al., 2000) was used to assign the individuals for which COI sequences was not available to the L1, L3 and L4 cryptic species of the *P. corethrurus* complex. For that, a 'reference' data set was built, composed of 113 AFLP profiles from L1, L3 and L4 individuals of known COI sequences. We first checked if AFLP data allowed to distinguish the lineages within the complex using the Structure software by modelling cluster assignments for K = 1–5 clusters using non-admixture model. We made 5 independent runs for each K to confirm consistency across runs in all simulations. In a second time, we used the 'reference' data to assign 54 AFLP profiles from individuals for which COI sequences were not available, to the cryptic species of the *P. corethrurus* complex. The used model was non-admixture, and K was set between 1 to 5, with 5 independent runs per K.

In three populations (MNH, TLC and NTU) coexistence of cryptic species was found, Structure was run to find potential hybrids among them. The model selected was admixture, and K was set between 1 to 5 with 15 independent runs per K. For each analysis, we performed a burn-in period of 10000 iterations and 100000 Markov chain Monte Carlo iterations.

After each analysis, to determine the most likely value of K, we used the ΔK method of Evanno et al. (2005) implemented in Structure Harvester (Earl & vonHoldt, 2012). Results from different replicate runs were combined into one output using Clumpp software v. 1.1.2 (Jakobsson & Rosenberg, 2009). Results visualization were done by Distruct (Rosenberg, 2007).

We also analyzed possible hybridization occurrence within MNH, TLC and NTU populations with the program NewHybrids (Anderson & Thompson, 2002; Anderson, 2008) based on statistical model-based Bayesian methods. The software considers six genotype categories: pure species A, pure species B, F1 hybrid, F2 hybrid, and the F1 backcross to pure species A or pure species B, with the results of estimated posterior probability to assign each individual to one of the six genotypic classes. We assigned each individual to the most likely (probability>0.50) NewHybrids genotypic class. Given the large number of polymorphic loci (308) a burn-in period of 75,000 repetitions was defined, and 100,000 MCMC iterations, with no previous population information, and 'Jeffery's like priors' were considered.

IV.4.3. Population genetics of P. corethrurus *L1*

A total of 226 AFLP profiles corresponding to 12 populations from 5 countries (Brazil, French Guiana, Mexico, Gabon and Thailand) were used to investigate the genetic variation within and between populations of *P. corethrurus* L1. Genetic diversity statistics, including number of different haplotypes by considering the 'error rate' calculated from replicates, genotype diversity, gene diversity, proportion of variable markers and the frequency down-weighed marker value

(DW) (Schönswetter & Tribsch, 2005) were calculated using AFLPdat program in RStudio (RStudioTeam, 2015). Genotype and gene diversity were calculated based on Nei's formula (Nei, 1987). Similar haplotypes within locations were removed and further analyses on *P. corethrurus* L1 were carried out on a dataset without clones.

In order to evaluate the evidence for recombination, measures of multilocus gametic disequilibrium were calculated and tested for significance with 500 randomizations in Multilocus software (http://www.bio.ic.ac.uk/evolve/software/multilocus/), using 3 different methods. Since in a pair of binary character data, the presence of all four possible combinations of characters (0/0, 1/0, 0/1, 1/1) indicate incompatibility which is more parsimoniously explained by sexual recombination than by three mutation events, the proportion of compatible pairs of loci was computed to probe the predominant mating system in the populations. Moreover, the index of association (I_A) and an alternative measure of index of association that is less sensitive to the number of loci ($\bar{r}_d$, Agapow & Burt, 2001) were computed. We further tested for gametic disequilibrium based on the distribution of allelic mismatches between pairs of genotypes over all loci using an exact test implemented in Arlequin. To adjust for multiple comparisons, the SGoF method (Carvajal-Rodríguez et al., 2009) as implemented in the software Myriad (http://myriads.webs.uvigo.es/MyriadsReadme.htm) was applied.

We identified loci with greatly increased differentiation between populations using a genomic scan approach. These F_{st} outlier may be interpreted as signature of (i) divergent selection (ii) intrinsic (i.e. environment independent) pre- or post-zygotic genetic incompatibilities (Bierne et al., 2011) or (iii) differential introgression from a sister species (Gosset & Bierne, 2013). Tests for F_{st} outliers were carried out using Bayescan 2.01 (Foll et al., 2010), running with 100,000 iterations (sample size 5000 * thinning interval 10 + 50000 burn-in), after 20 pilot runs (5000 iterations each), and selecting outliers at a threshold of prior odds (PO) >10, as suggested by the manual for datasets with hundreds of loci. Further analyses of genetic structure were carried out on two datasets: the original dataset and the dataset without outlier loci.

We quantified the amount of genetic differentiation of population groups using a hierarchical Analysis of Molecular Variance (AMOVA) implemented in Arlequin V3.5 (Excoffier & Lischer, 2010). In this analysis, the AFLP data set was partitioned at three levels: groups of native *versus* introduced populations, among-populations within groups and among all populations. One thousand random permutations were used to infer the significance of the variance components. In addition, an unbiased estimate of differentiation among populations, $\theta^{(II)}$ was obtained using the Bayesian method proposed by Holsinger et al. (2002) and implemented in the software Hickory v1.1. The data were run with the default parameters using the f-free model.

To illustrate the relationships among populations, split networks were constructed using the software Splitstree version 4.1.4.6 (Huson & Bryant, 2006) on AFLP profiles. We used the distance-based Neighbor-Net (N-net) method for construction of networks. The N-net provides good visualization of the data when it presents complex evolutionary steps or reticulate

relationship among genotypes (Huson & Bryant, 2006). The networks were constructed based on Nei's distance (GD) matrix between populations calculated with a Bayesian method using AFLP-Surv version 1.0 (Vekemans et al., 2002) with non-uniform distribution by assuming deviation from Hardy-Weinberg equilibrium; F_{is} values were estimated by Hickory software using the full model (Holsinger et al., 2002). Analyses were done with 1000 permutations and 1000 bootstrap values.

IV.4.4. Principal stages of the analyses

A workflow of principal stages of the analyses is presented in Figure C 1: first, assignment of AFLP sequences in *P. corethrurus* complex to already known COI lineages (L1, L3 and L4); second, potential hybridizations of species in *P. corethrurus* complex (L1, L3, and L4) for three populations in sympatry based on AFLP profiles; finally, the phylogeography and population genetics study of *P. corethrurus* L1 populations based on 479 COI sequences and 226 AFLP profiles.

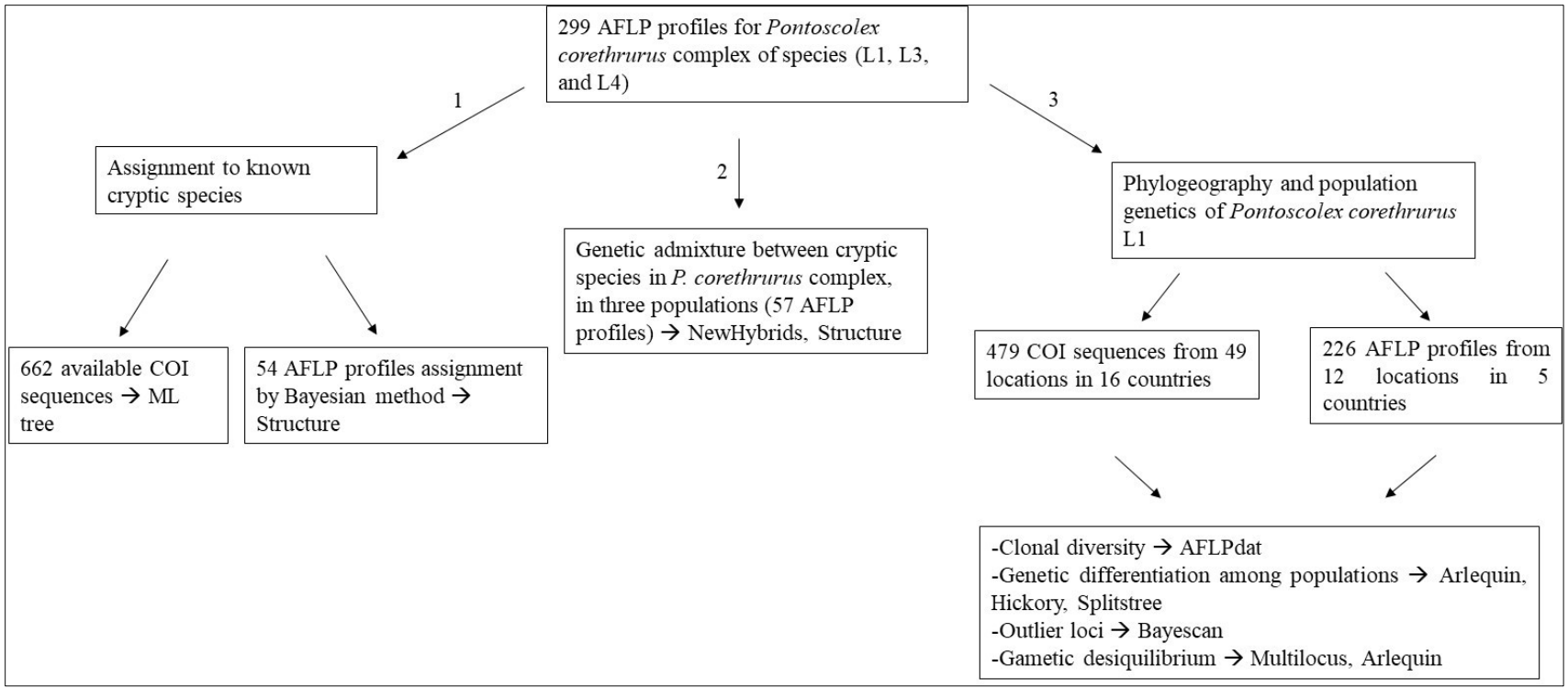

Figure C 1: Workflow diagram for the analyses in this study. Three principal stages are shown in this diagram. In each stage the name of the software used is indicated.

IV.5. Results

IV.5.1. Genetic relationships among cryptic lineages

Phylogenetic analysis using the COI gene revealed that individuals from the 12 studied populations corresponded to lineages L1, L3 and L4. Within the reference dataset, Structure analysis of AFLP data supported the presence of only two clusters (ΔK= 2.36). One cluster corresponded to all L3 samples (cluster red in Figure C 2) while the other cluster corresponded to all L1 samples and to L4 samples (cluster green in Figure C 2). Because of small number of individuals in L4 (i.e., 4 from NTU), this lineage was not recognized as a separate cluster. Further, we assigned the AFLP profiles for which we didn't have mitochondrial sequences, to these clusters: 53 out of 54 samples were assigned to the cluster corresponding to L1/L4 and 1 sample was assigned to the cluster corresponding to L3. Because L4 samples were extremely rare and only observed in one population (NTU), we assumed that all specimens assigned to the cluster in green in Figure C 2 belonged to the L1 lineage.

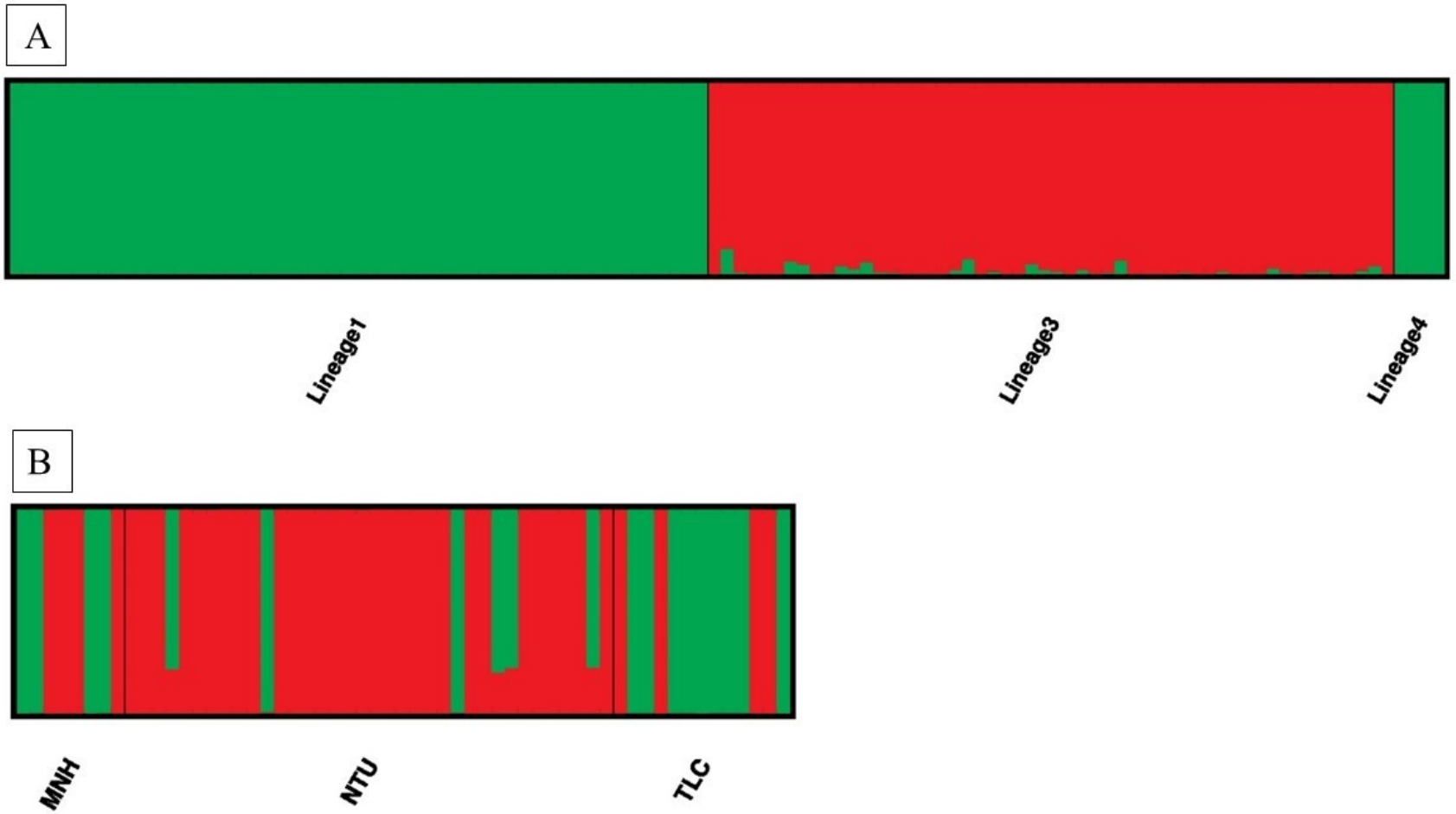

Figure C 2: Structure results on AFLP profiles: A) unknown individuals assignation based on 'reference' data in Taheri et. al. (in revision), colors are selected based on the previous study; Lineage 1 green and L3 red, B) mixture model results for three populations where sympatry of cryptic species were found; MNH, NTU and TLC.

IV.5.2. Reproductive isolation among cryptic species **P. corethrurus** *L1 and L3*

Sympatry of cryptic species was observed in three locations: two sites in Mexico, MNH with 4 individuals in L1 and 4 individuals in L3, TLC with 9 individuals in L1 and 4 individuals in L3, and one site in Taiwan NTU with 2 individuals in L1, 30 individuals in L3 and 4 individuals in L4. In these three populations, none of the individuals were diagnosed as hybrids between L1 and L3 species by Structure. The four admixed individuals that can be observed in Figure C 2 corresponded to L4 individuals. NewHybrids analysis confirmed the absence of first generation hybrids within these three populations. The four individuals of L4 were presented as backcrosses of F1 and L1 (results of this analysis are not shown).

IV.5.3. Phylogeography of **P. corethrurus** *L1 species based on COI sequences*

An extensive global dataset of COI sequences obtained in L1 specimens was used to assess the distribution of mitochondrial genetic diversity among populations. Thirteen COI haplotypes were inferred from 479 sequences within 49 locations. COI haplotype network is shown in Figure C 3. Haplotype one (H1) was shared by the majority of individuals i.e., 364 out of 479, followed by haplotype two (H2) with 64 individuals. Five haplotypes (i.e., H3, H7, H8, H9, H10) were exclusively found in the native range (i.e., French Guiana). Six other haplotypes (i.e., H4, H5, H6, H11, H12, H13) were found outside this zone and in a worldwide scale i.e., in Brazil, Peru, Thailand, and Martinique. The highest number of polymorphic sites (11 and 10 respectively) and the highest haplotype diversity (0.696 and 0.561 respectively) were observed in CAX and MIT. MIT and CAY had the highest nucleotide diversity (00673 and 0.00503, respectively). In 5 populations (PARA, PARB, PARC, ORL and LOP), no polymorphic sites were observed.

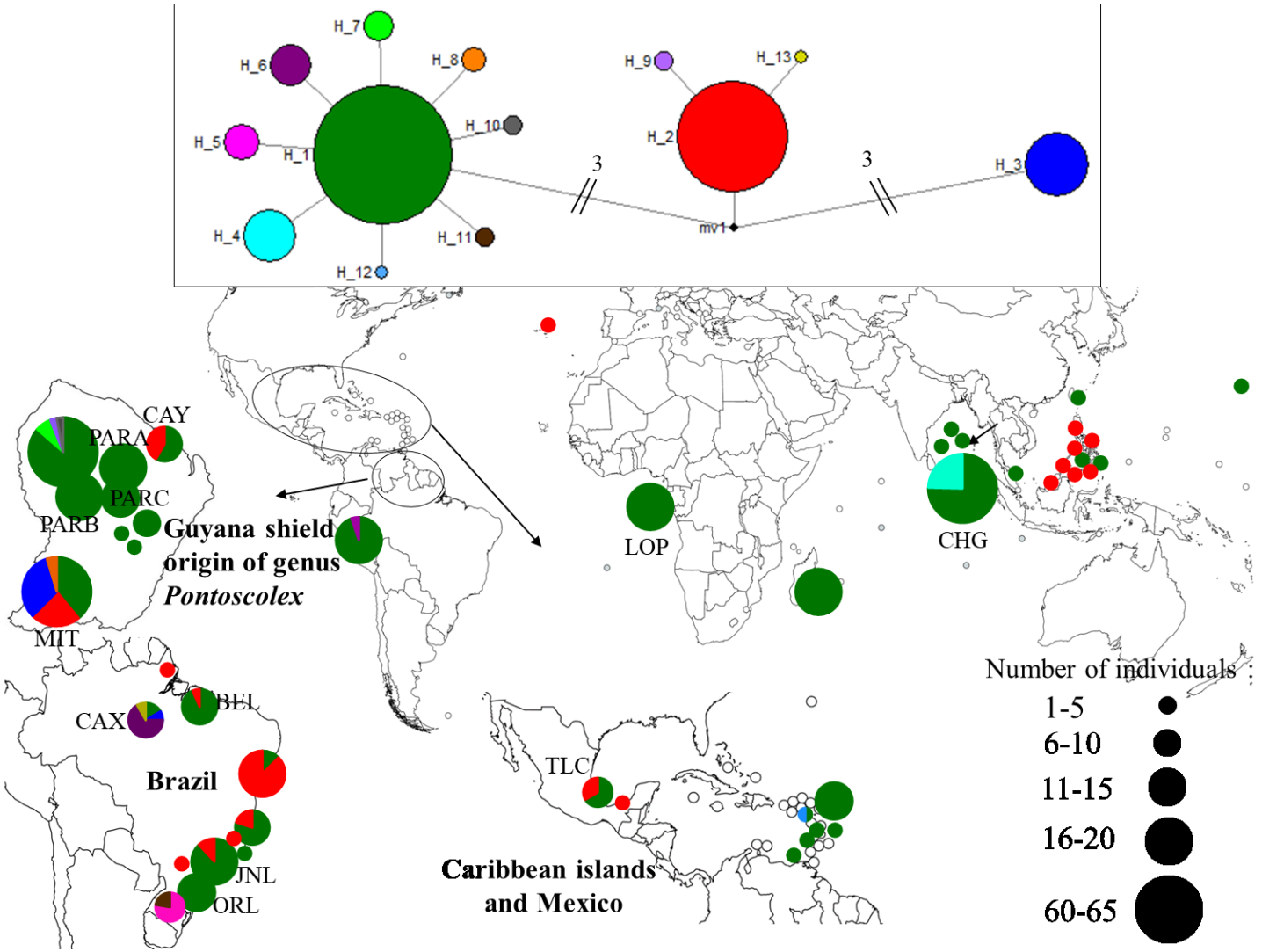

Figure C 3: *P. corethrurus* L1 COI haplotype network based on 479 sequences in 49 locations, throughout the tropical zone. 72% of specimens have the same haplotype (H1). Twelve populations for which population genetic analyses based on COI and AFLP profiles are done, are indicated with abbreviations under the related haplotypes. The intensity of each haplotype is related to the number of individuals within a location. Each line represents one mutation among haplotypes, unless they are indicated.

IV.5.4. Genetic variation of populations in **P. corethrurus** *L1 species based on AFLP profiles*

High number of clones were found for LOP location in Gabon with 17 similar haplotypes out of 20 individuals, followed by CHG location in Thailand with 10 similar haplotypes out of 35 sequences (Table C 1). In five populations, no clones were found i.e., CAY, PARC, BEL, JNL, and TLC. Despite low number of clones for ORL, PARA and PARB (3 out of 24, 3 out of 19 and 1 out of 8 sequences), gene diversity values were relatively low; 0.083, 0.052, 0.087, respectively. The highest gene diversity was found in JNL and CAY i.e., 0.168 and 0.165 respectively, where no clones were found. Frequency down-weighed marker value (DW) shows the rarity of each marker within the dataset. Therefore, CAY, MIT, CAX and LOP had the highest rates of rare alleles in comparison with other populations with 1804, 830.653, 708.9 and 692.7, respectively. LOP with the highest number of clones and the lowest gene diversity, had a high DW value, meaning that the AFLP haplotypes within this population are highly different to the other haplotypes of the total dataset. The smallest DW values were found in JNL and PARA with 354.068 and 362.161 values respectively.

Further analyses were done on data set without clones, reducing the dataset from 226 profiles to 185. Each population showed significant gametic disequilibrium and character compatibility except LOP where the sample size and diversity were extremely low (4 different haplotypes 2% of variable markers). All indices were congruent (Table C 1). The highest values of gametic disequilibrium were observed in TLC (Mexico) and the lowest in JNL, suggesting a higher rate of recombination in the later.

Table C 1: Overall genetic variability in 12 locations through 5 countries for *P. corethrurus* L1, based on AFLP markers and COI gene. Studied parameters situated above NoG (number of genotypes) are done based on datasets with clones i.e. for %VM (proportion of variable markers), polymorphic sites, and DW, while for parameters downward NoG, clones are removed from the dataset i.e. for genotype and gene diversities. For PCLP (Proportion of compatible locus pairs), I_A, and $\bar{r}_d$, significant values (P<0,002) are shown in bold.

Marker	Countries	French Guiana[16]					Brazil				Mexico	Gabon	Thailand
	Sites	CAY	MIT	PARA	PARB	PARC	BEL	CAX	JNL	ORL	TLC	LOP	CHG
AFLP (308 markers)	N	17	20	19	8	13	21	18	22	24	9	20	35
	VM%[17]	52.284	31.472	21.574	22.335	46.701	41.878	48.223	51.523	22.589	28.173	2.030	34.518
	DW (from means)[18]	1804	830.653	362.161	406.032	516.553	570.668	708.9	354.068	426.114	467.167	692.7	471.039
	NoG[19]	17	17	16	7	13	21	14	22	21	9	3	25
	Genotype diversity	1	0.979	0.965	0.964	1	1	0.935	1	0.978	1	0.416	0.973
	Gene diversity	0.165	0.112	0.052	0.087	0.163	0.132	0.138	0.168	0.083	0.113	0 .012	0 .110
	PCLP[20]	**0.91**	**0.97**	**0.99**	**1.00**	**0.94**	**0.93**	**0.96**	**0.88**	**0.98**	**0.99**	1.00	**0.96**
	I_A	**6.78**	**6.82**	**11.91**	**18.51**	**2.54**	**12.77**	**22.34**	**2.23**	**13.37**	**42.52**	1.29	**11.98**
	$\bar{r}_d$	**0.03**	**0.06**	**0.15**	**0.21**	**0.01**	**0.08**	**0.12**	**0.01**	**0.16**	**0.39**	0.21	**0.09**
	LD (%)	8.66	14.78	8.32	9.67	8.49	19.84	16.04	4.06	34.17	19.64	0.00	20.12
COI (633 bps)	N	12	63	16	16	15	15	12	17	13	9	20	61
	NPS[21]	6	10	0	0	0	6	11	5	0	6	0	1
	Number of haplotype	2	4	1	1	1	2	4	2	1	2	1	2
	Haplotype diversity	0.53	0.696	0	0	0	0.133	0.561	0.221	0	0.5	0	0.377
	P_i (per site)[22]	0.00503	0.00673	0	0	0	0.00126	0.00426	0.00174	0	0.00474	0	0.0006

[16] French Guiana is the only country in this study which is located in the Guayana shield; the origin of genus *Pontoscolex*
[17] Variable markers
[18] Frequency-down-weighted marker values
[19] Number of genotypes
[20] Proportion of compatible locus pairs
[21] Number of polymorphic sites
[22] Nucleotide diversity

The AFLP data set was used in a global analysis for outlier detection. The PO threshold set was 10 in Bayescan analysis which identified 33 markers as outliers, out of 308 polymorphic markers (Figure C 4). All analysis of population differentiation was carried out with and without outliers.

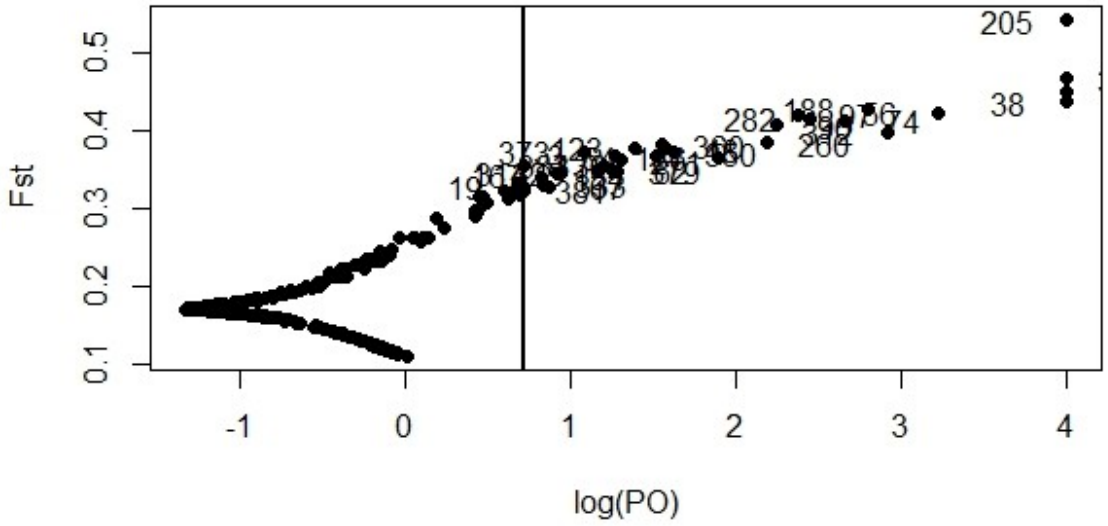

Figure C 4: Bayescan results done on AFLP profiles. F_{ST} is plotted against the log10 of the posterior odds (PO). The vertical line shows the critical PO used for identifying outlier markers. The 33 markers (out of 394) on the right side of the vertical line are candidates for being under selection for *P. corethrurus* L1 specimens.

The genetic differentiation Φ_{ST} measured by AMOVA analysis was 0.401 (0.352 without outliers); 60 % of the variation was partitioned within populations, 33 % was attributed to differences among populations within groups, and 7 % of the variation was due to differences between native and introduced (65%, 30%, 5% respectively without outliers). All three hierarchical levels were significant with $P<0.002$ and $P<0.001$, with and without outliers, respectively (Table C 2). A similar level of genetic differentiation was obtained using the f-free model with Hickory software: $\theta^{(II)} = 0.342$ and 0.295 for dataset with and without outliers respectively.

Table C 2: Analysis of molecular variance (AMOVA) of 308 AFLP loci for 5 natives and 7 introduced populations of *P. corethrurus* L1. Between parenthesis values for analysis without outliers.

	Variance components	**% of total variation**	**P-value**	**Φ statistics**
Among groups (native *versus* introduced)	2.84 (1.61)	7.16 (5.13)	0.000 (0.000)	Φ_{CT} = 0.072 (0.051)
Among populations within groups	13.06 (9.45)	32.91 (30.10)	0.000 (0.000)	Φ_{SC}= 0.354 (0.317)
Among all populations	23.78 (20.34)	59.93 (64.77)	0.001 (0.002)	Φ_{ST}= 0.401 (0.352)

Relationships between populations are illustrated by the Neighbor-Net results which are shown in Figure C 5. Figure C 5 (A) correspond to N-net without outliers, where less reticulates are shown than in Figure C 5 (B) which is with outliers. Based on both results, LOP (Africa) and CAY (French Guiana) are the most differentiated to each other and to the other locations while the sample from Asia (CHG) is genetically closed to the populations from South America.

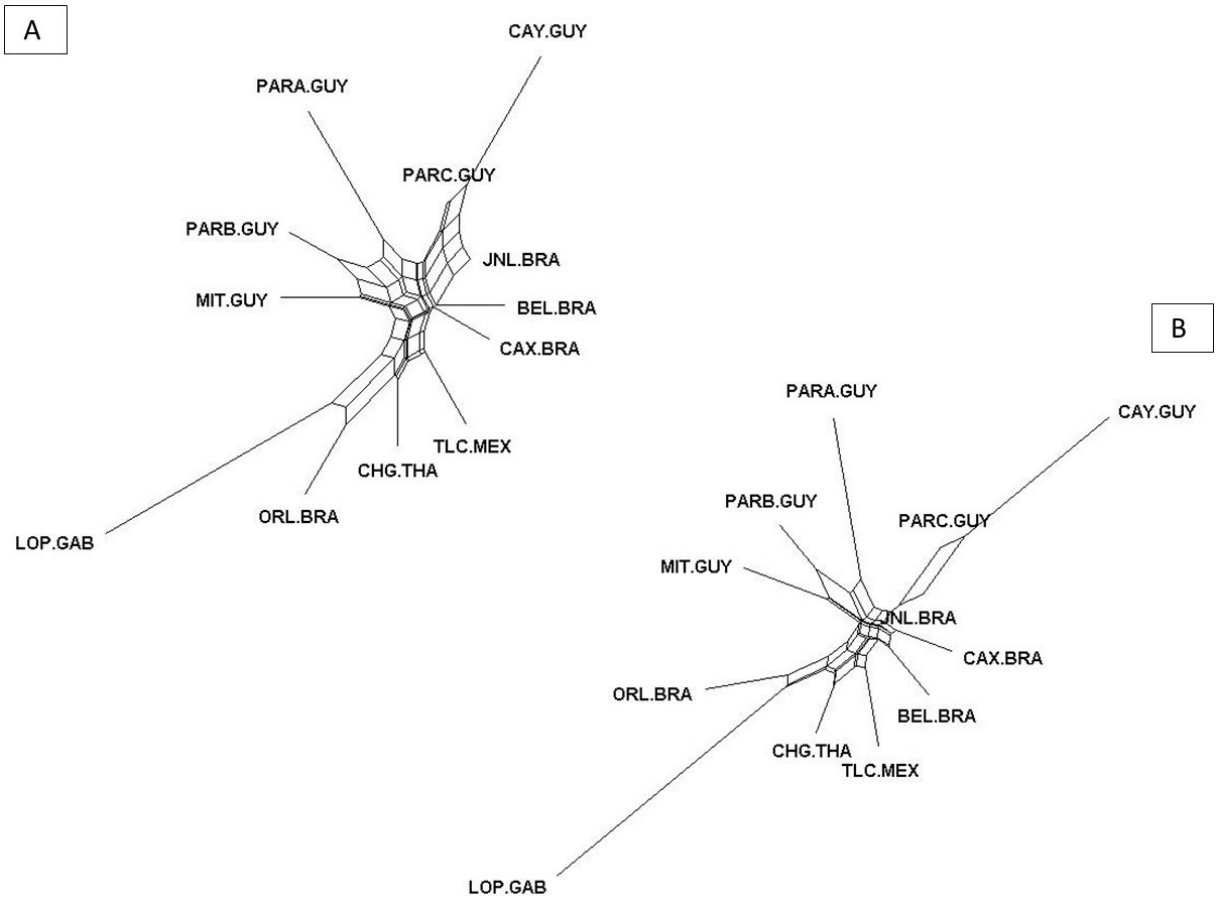

Figure C 5: Neighbor-Net networks done on AFLP profiles with Splitstree based on Nei's genetic distance matrix for 12 populations in *P. corethrurus* L1 : A) network done with 394 markers, B) network done without the 33 outliers found by Bayescan software.

IV.6. Discussion

The *P. corethrurus* complex of species is known to be composed of at least 4 cryptic species (Taheri et al. *in revision*) and 3 of them (L1, L3 and L4) were found in sympatry in 3 populations (MNH, TLC, and NTU) in the introduced range. Previous results of COI heteroplasmy suggested that reproductive isolation was not complete between L3 and L4 (Taheri et al. *in revision*). This could not be tested here because of a very low number of L4 specimens (4 individuals within the NTU population); hence distinction between L1 and L4 was not possible using AFLPs markers. Instead, we investigated whether L1 was reproductively isolated to L3. Based on Structure and NewHybrids results, reproductive isolation among *P. corethrurus* L1 and L3 species was established within the 3 populations where these species were found in sympatry. Moreover, we evaluated the possibility that differential introgression of alleles from sister-hybridizing species would results in strong differentiation among L1 populations at F_{st} outlier loci (Gosset & Bierne, 2013). Although Bayescan analysis found 33 F_{st} outliers out of 308 polymorphic AFLP markers, the different analyses of genetic structure gave similar results

when carried out with and without the F_{st} outliers; allowing to exclude the hypothesis of strong genetic differentiation due to introgression in *P. corethrurus* L1. Altogether, our results confirm that L1 and L3 correspond to independent biological species.

Interestingly, in *P.* corethrurus different lineages show different distribution patterns, which is not uncommon pattern in peregrine species, and that may be associated with different ability to respond to environmental constraints (Novo et al., 2015; Schult et al., 2016). The plasticity among lineages shows its extreme by the L4, for which a healthy population was found living inside a geothermal field (Cunha et al., 2014), under an extreme geogenic stress (high temperature and low oxygen levels). In contrast L2 has been solely found in French Guiana in a well-defined geographic area (Taheri et al., *in revision*).

In the rest of the study, we focused on *P. corethrurus* L1 which is the most widespread species within the *P. corethrurus* complex. Samples collected all around the world from a total of 49 localities were used for a phylogeographical study of COI haplotypes. Overall, an extremely low COI haplotype diversity was observed with a total of 13 haplotypes in the whole data set, in comparison with other earthworm species. For instance, for *Dendrobaena octaedra,* 24 COI haplotypes were found through three locations in Southern Finland (Knott & Haimi, 2010). Two haplotypes were extremely well represented all over the world: H1 (72 % of the sequences) and H2 (13% of the sequences; Figure C 3). None of these haplotypes were present in only one locality: the samples from RGS, with H5 and H11. Despite the very extensive geographical range of *P. corethrurus* in the tropical and sub-tropical zone, and the limited potential for natural dispersal of earthworms (Costa et al., 2013), our sampling of four continents did not reveal very divergent mitochondrial haplotypes. In this study, three major haplotypes were found; H1, H2 and H3. H1 was only separated from H2 by four mutations and six mutations from H3. Similar results were found for the parthenogenetic earthworm *Dendrobaena octaedra*: based on COI network haplotype, four main haplotypes existed for this species; H1, H3, H18 and H21, respectively (Knott & Haimi, 2010). H1 was separated from H18 by two mutations and one mutation from H3 and H21. Several factors may be suggested to explain the lack of haplotype diversity: a slow evolutionary rate of the cytochrome oxidase I gene, a recent origin of the species, a selective sweep, or genetic drift associated with historical events, such as a recent global population bottleneck (López-Legentil & Turon, 2007), in addition to the reproductive mode of the species.

Interestingly, the analysis of 308 polymorphic AFLP markers revealed a low genetic diversity in most of the populations. In particular, gene diversity was extremely low (< 0.100) in LOP, PARA, ORL and PARB populations (Table C 1). The congruence between AFLPs and mitochondrial data suggests that the low genetic diversity of this species is not due to a marker-specific effect but to either life-history traits or population and species history. Two main explanations for the low level genetic diversity *P. corethrurus* L1 would be the occurrence of recent population bottlenecks and asexual reproduction.

The low genetic diversity was linked to a significant number of similar genotypes (i.e., clones) in several populations. For instance, in LOP populations only 15% of the AFLP haplotypes were different (84% in PARA, 88% in ORL and PARB). The important number of clones highlight the prominence of the parthenogenesis reproduction in several populations.

This is also confirmed by the high levels of gametic disequilibrium (Table C 1) that were observed in several populations such as TLC (I_A = 42.52; $\bar{r}_d$ = 0.39), CAX (I_A = 22.34; $\bar{r}_d$ = 0.12), PARB (I_A = 18.51; $\bar{r}_d$ = 0.21) and ORL (I_A = 13.37; $\bar{r}_d$ = 0.16). It is however worth noting that no AFLP genotype was shared among populations, an observation allowing to reject the hypothesis that a "super-clone" was responsible of the success of *P. corethrurus* invasion.

Moreover, the levels of COI and AFLPs genetic diversity were variable among populations. In French Guiana, three populations showed only the H1 haplotype (PARA, PARB and PARC) while higher haplotype diversity was observed in the two other populations from the native range. In CAY, H1 and H2 were found and in MIT, 3 other haplotypes, in addition to H1 were detected. This is confirmed with the AFLPs. For instance, gene diversity was two times higher in CAY population per comparison with PARA and PARB. A similar situation was observed in the introduced range of the species: some populations showed high genetic diversity indices, such as CAX in Brazil whereas in ORL (another Brazilian population) and in LOP (Gabon), the COI gene was monomorphic (only H1). These results suggest that successive introduction from multiple sources has enhanced genetic diversity in certain introduced localities that could be occurring since humans arrived and dispersed in Amazonia (Cunha et al., 2016). An alternative explanation would be that relatively high genetic diversity has accumulated through mutation events in some populations that have been founded long time ago. At last, high genetic diversity could also be due to sexual reproduction.

In JNL and PARC populations, low values of gametic disequilibrium estimators (e.g. I_A = 2.23; $\bar{r}_d$ = 0.01 and I_A = 2.54; $\bar{r}_d$ = 0.01 respectively, Table C 1) and absence of clones suggested sexual reproduction within these populations. Altogether, these results suggest that where individuals have recently invaded an area, the reproduction strategy is parthenogenesis. This mode of reproduction is considered as particularly advantageous for an invading species. Once the species is established in the introduced area, sexual reproduction may then occur. Such a mixed-reproduction strategy helps the maintenance of genetic diversity within this species, and helps the foundation of new invasive populations in other parts of the world.

IV.7. Conclusion

Through this study we have highlighted the importance of using nuclear genome analysis alongside mitochondrial genes for phylogeography and population genetics studies. With COI gene, an invasion of a 'super-clone' could be concluded, while the absence of shared AFLP profiles among populations, rejected this hypothesis. Results confirmed that *P. corethrurus* L1 was reproductively isolated from *P. corethrurus* L3. Meanwhile, the plastic reproduction strategy, in *P. corethrurus*, shifting from sexual to asexual mode, explains the success of this species and the existence of exceptionally fit genomes supported by higher reproductive rates (with no clonal population senescence after many generations), elegantly defining a mode of escape to Muller's ratchet (Muller, 1932). Parthenogenesis of *P. corethrurus* L1 enables a quick replacement of losses and make the specimens efficient colonizers when compared with species with obligatory sexual reproduction strategies.

IV.8. References

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IV.9. Supplementary data

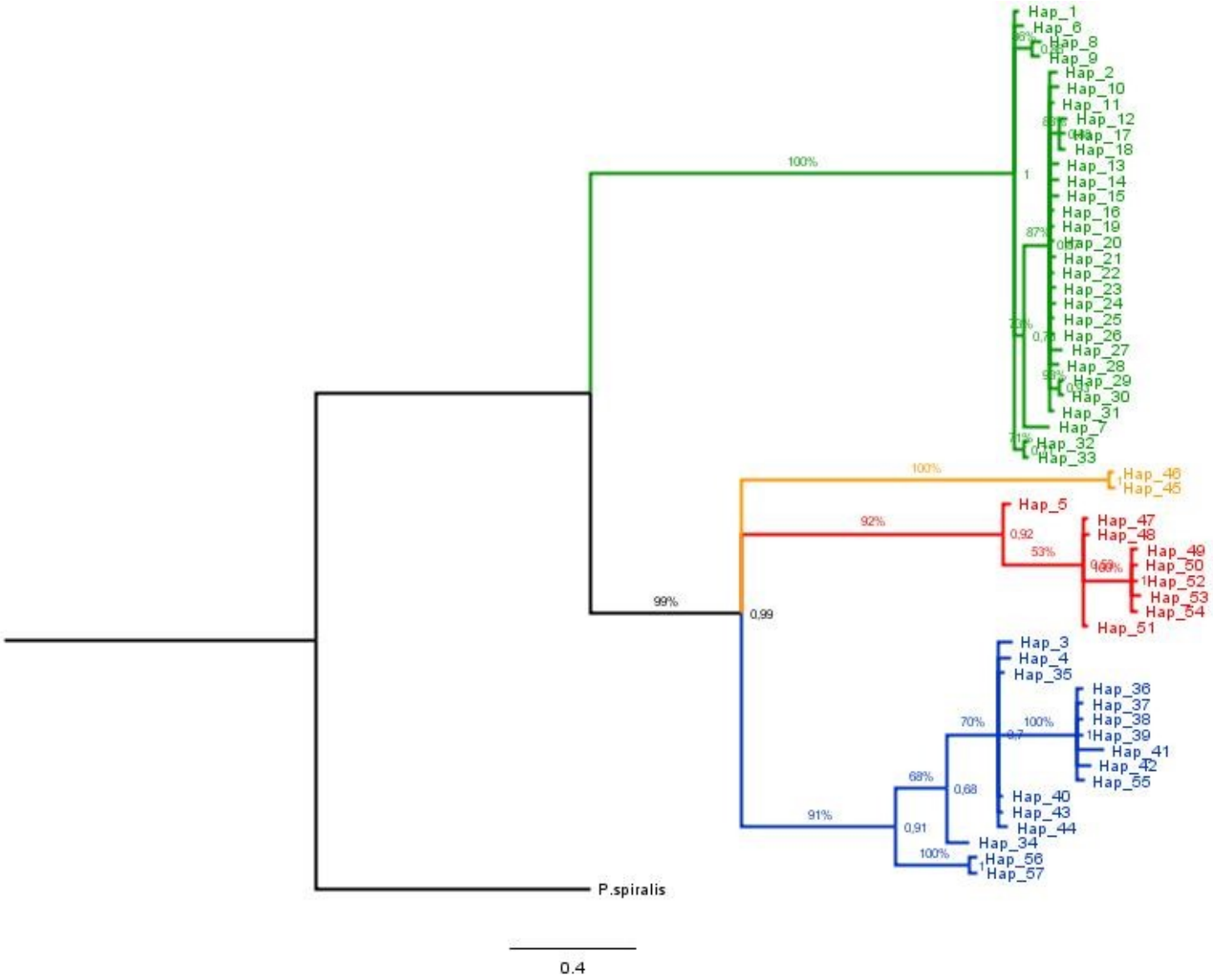

Figure C 6: Maximum-Likelihood tree based on HKY+G substitution model, with 662 COI sequences of *P. corethrurus* complex specimens.

Table C 3: Information on samples used in this study.

Biogeographic realm	Country or Island	Site	Nb of individuals	Nb of Sites	GPS information	GPS information	Date of sampling	Sampled by	Macro habitat	Micro habitat	AFLP (complex)	COI (*P. corethrurus* L1)
Ethiopian	Gabon	La Lopé	20	1	0°6'47,90" S	11°36'11,4" E	na	T. Decaens	Garden	soil	20	20
Ethiopian	Madagascar	Black soil in Mada-Lazaina	20	1	18°46'51,33" S	47°32'8,631" E	na	E. Blanchard	na	na	0	15
Neotropical	Brazil	Amapa	9	12	na	na	na	G. Brown, S. James	na	na	0	2
		Bahia (pasture)	15		15°15'22.9" S	39°05'50.4" W	09/11/2014	M. Bartz	na	na	5	13
		Bahia (cacao)	4		15°08'08.1" S	39°18'39.5" W	10/11/2014	M. Bartz, G. Brown	na	na	2	3
		Belém	14		1°27'04,34" S	48°26'39,7" W	04/05/2011	T. Decaens	na	na	21	15
		Caxiuana	14		1°44'15,70" S	51°27'19,05" W	30/04/2011	T. Decaens	na	na	18	12
		Embrapa	9		25° 18.777' S	49° 09.472' W	23/10/2015	S. Taheri, L. Cunha, E. Silva	Garden, grass	soil	0	5
		Joinville	17		26°13'11,89" S	48°51'19,43" W	na	na	na	na	22	17
		Muller's garden	3		26° 45.61'S	49° 02.65'W	12/11/2015	G. Brown, S. Taheri,, M. Bartz	Garden, grass	soil	0	1
		Orléans	16		28°22'49,8" S	49°14'53,2" W	2012	M. Bartz	na	na	24	13
		Rio Grande do Sul (deciduous forest)	10		29°47'38.7" S	51°09'27.1" W	31/07/2014	S. James, G. Brown, M. Bartz	Deciduous forest	na	2	9
		Sao Paolo (Alto da Serra)	8		na	na	12/03/2005	G. Brown, S. James	Primary forest	litters+soil	0	1
		Sao Paolo (Parque estadual Itaberá)	23		23°51' 01.31" S	49°08'15.7" W	05/02/2009	G. Brown, D. Baretta, S. James	Semi-Deciduous Forest	na	1	15
Neotropical	French Guiana	Cayenne	14	9	04°52.81' N	052°20.115' W	Jan. 2010	T.Decaens, E. Lapied	Garden	Soil	17	12
		Itoupé7	12		3°1'57,526"N	53°6'20,07"W	Jan. 2016	T. Decaens	Primary rainforst (477m elevation)	Decaying log	0	9
		Mitaraka	64		2°14.316'N	54°26.086'W	March 2015	T.Decaens, E. Lapied	Rocky savannah (401m elevation)	Soil	20	64
		Nouragues	66		4°05.154'N	52°40.483'W	Jan. 2011	T.Decaens, E. Lapied	Garden and drop zone	Soil	0	66
		Pararé A	16		4°02'25.2"N	52°40'29.5"W	June 2011	T.Decaens, E. Lapied	Lowland rainforest (62m elevation)	Soil	19	16
		Pararé B, CampI and II	26		4°02'19,3" N	52°40'22,9" W	June 2011	T.Decaens, E. Lapied	Garden and drop zone	Soil	8	16

		Pararé C	21		4°02'06.0" N	52°40'23.00" W	June 2011	T.Decaens, E. Lapied	Lowland rainforest (50m elevation)	Soil	13	15
		Paracou	3		4°2'16.08"N	52°40'20.28"W	June 2013	T.Decaens, E. Lapied	Garden	Soil	0	1
		Tirinité 5	13		4°2'16.08"N	52°40'20.28"W	April 2016	T.Decaens, E. Lapied	Lowland rainforest (121m elevation)	Decaying log	0	10
Neotropical	Mexico	Hayas	15	3	19°45'.730"N	96°35'.778"W	na	na	na	na	15	0
		Mancha	11		19°35'.624"N	96°22'.881"W	na	na	na	na	8	3
		Tlalcotlen	13		19°42'27" N	96°96'36"W	na	na	na	na	13	9
	Peru	Y29 C	15	1	5° 53' 13.1"S	76° 11' 23.5" W	na	P. Lavelle	(pasture land)	na	0	19
Neotropical	St. Lucia	na	2	1	13° 53' 23" N	61° 00' 16" W	27/09/1994	S. James	Anse la Raye parois, tete chemain Millet, Forest reserve	na	0	1
Neotropical	St. Vincent	Soufrieve volcano	2	1	13° 19' 34" N	61° 10' 21" W	25/09/1994	S. James	Pontoscolex corethrurus were found in a lower part of volcano	na	0	1
Neotropical	Trinidad & Tobago	Trinity hills	1	1	10° 10' N	61° 02' W	17/09/1991	S. James	Trinity Hills, *Pontoscolex corethrurus* everywhere.	na	0	1
Neotropical	Martinique	Schoelcher, plateau concorde	12	1	14° 40.748' N	61° 06.380' W	Oct.2017	M. Coulis	Primary forest, 582 m	soil	0	15
Oceanic	Hawai	Honolulu	7	1	na	na	01/05/2015	S. James	na	na	0	1
Oriental	Malaysia	Air putih, Pahang	3	1	3°50' N	103° 19.4' E	21/05/2011	na	na	na	0	1
Oriental	Philippines	Mt. Pugalungan	11	8	7° 50' N	124° 56' E	na	na	secondary forest	na	0	1
		Tawi Tawi Island	10		5° 02' N	119° 44' E	na	na	secondary forest	na	0	1
		Mt. Butig	2		7° 41' N	124° 18' E	na	na	secondary forest	na	0	1
		Mt. Katalung	3		7° 58.8' N	124°46' E	na	na	secondary forest	na	0	1
		Mt. Kimangkil	3		na	na	na	na	secondary forest	na	0	1
		PTAG	2		na	na	na	na	secondary forest	na	0	1
		Mt. Apo	5		7° 00' N	125° 16' E	na	na	secondary forest	na	0	2

		Mt. Parker	9		6 °5.5' N	124° 52' E	na	na	secondary forest	na	0	1
Oriental	Taiwan	National Taiwan university	37	1	25°00'52.4N	121°32'19.2'E	na	na	na	na	36	2
Oriental	Thailand	Pops	61	4	13°35'18.95" N	101°28'13.37" E	November 2014	T.Decaens	Hevea plantation (>25 years)	Soil	35	61
		Kuraburi (sud des ilesswim)	2		na	na	na	na	na	na	0	1
		Kuraburi (Morgan islands)	2		9 38' N	98 28' E	01/04/2014	S. James	secondary forest	na	0	1
		Uinversity Chulalongkorn	1		13 44.2' N	100 31.8 E	05/02/2012	S. James	university gardens	na	0	1
Palearctic	Azores	Pineapple plantation	4	1	37°45'12.5" N	25°24'18.3" W	01/01/2016	E. Da Silva	Plantation	na	0	3
Total	16	51	650	47	na	na	na	na	na	na	299	479

V. *<u>Chapter 4</u>: Ploidy degree of* Pontoscolex corethrurus *complex specimens*

V.1. Chapter's foreword

Knowledge of the ploidy degree and number of chromosomes of an organism is primordial before undertaking molecular and genomic analyses. This information directs us toward the adapted method of molecular analysis for the related species. For instance, for polyploid species, co-dominant markers are not advisable (e.g., microsatellites, Restriction Fragment Length Polymorphism (RFLP), Single-nucleotide Polymorphism (SNP), and restriction site-associated DNA sequencing (RADseq)), due to the difficulty for determination of the number of copies for each of the present alleles. Karyology and ploidy degree of several earthworm species are already known e.g., Bakhtadze et al., 2004; Kashmenskaya and Polyakov, 2008; Muldal, 1952; Vsevolodova-Perel and Bulatova, 2008. However, this information is still lacking for some species, in particular those used frequently as biological model for ecotoxicological, soil bioremediation, and bioindicator of soil quality, such as *Pontoscolex corethrurus* morphospecies. In the second chapter of my thesis, we showed that this morphospecies corresponds in reality to a complex of four cryptic species, and we assigned the lineage L1 to *P. corethrurus* sensu stricto. In the study presented in this (fourth) chapter, we aimed to develop a karyological protocol, functioning on *P. corethrurus* specimens; in order to investigate its ploidy degree and also the one of other specimens in the complex to examine whether different lineages have different ploidy degrees (i.e., polyploid complex). We were also interested to know if meiosis is retained. Due to the low number of samples that gave positive results, this chapter will not be published (at least 5 individuals with several chromosome counts are necessary for scientific publication). Through this study we established a vast collaboration with different laboratories. Firstly, this study was conducted in association with George Brown, Luis Cunha and Elodie Da Silva from the Brazilian Agricultural Research Corporation (Embrapa) who helped to collect alive specimens from Brazil and São Miguel Island. Some of my experiments were carried out in Brazil during a 2 month stay. Secondly, I worked in close collaboration for one year with Jean-Pierre Coutanceau and Chantal Guidi Rontani from the Systematic and Evolution department of Paris VI University in France. Table D 1 shows a brief summary of this chapter's results, collaboration and all the effort I have put in this study.

Table D 1: Chapter's summary

Date	Place of experimentations	Populations	Number of individuals	Organ	Protocol	Meiosis/Mitosis	Results	Lineage in complex	Collaboration
Oct.-Dec.2014	France (UPEC)[23]	Brazil	15[24]	Seminal vesicles or other organs on top of intestinal tube	1	Yes (meiosis)	not usable for two juvenile specimens	not sequenced	Brazil
Jan.-Mar.2015	France (UPEC)	Brazil	15	Tail	2	No	_	L1 and L4	Brazil
Oct.-Dec. 2015	Brazil (Parana university)	Brazil	68	Seminal vesicles or other organs on top of intestinal tube, and the tail	1 and 2	No	_	L1 and L4	Brazil
Jan.2016-Jan.2017	France (UPMC)[25]	Brazil	15	Seminal vesicles or other organs on top of intestinal tube	3	Yes (meiosis)	2n=70 for two adult specimens	L4	Paris VI for manipulations and Brazil for samples
	France (UPMC)	São Miguel	40	The tail and seminal vesicles or other organs on top of intestinal tube	2 and 3	No	_	L1 and L4	

[23] UNIVERSITÉ PARIS-EST CRÉTEIL

[24] THE SAME 15 INDIVIDUALS FROM EMBRAPA (BRAZIL) WERE USED IN DIFFERENT PERIODS. REDUCTION IN NUMBERS AFTER EXPERIMENTATIONS ARE NOT MENTIONED.

[25] UNIVERSITÉ PIERRE ET MARIE CURIE

V.2. Introduction

Chromosome biology of annelids, and particularly earthworms, have received limited attention (Venu et al., 2013). The karyology of earthworms has mainly been studied in the Lumbricidae family; Table D 2 shows the vast difference in ploidy degree which ranges from 2n to 10n within this family. In particular, different ploidy degrees exist within 6 morphospecies; *Aporrectodea caliginosa*, *Aporrectodea rosea*, *Dendrodrilus rubidus*, *Dendrobaena octaedra*, *Octodrilus transpadanus*, and *Eiseniella tetraedra*. These morphospecies could correspond to complexes of cryptic species with different ploidy degrees (i.e., polyploid complexes). For instance, evidence of cryptic species existence was suggested for *A. rosea* (Fernández et al., 2016). As different ploidy degrees are observed for this morphospecies, further studies are necessary to investigate if different species in this complex have different ploidy degrees (Table D 2). In literature, there is a big gap of knowledge on karyology of other families of earthworms, especially for those in tropical zone. It is worth noting that for cytogenetic and karyological studies, an optimized protocol based on each species physiology is necessary.

Table D 2: Earthworms karyology found in literature for Lumbricidae family

Species	Basic (n) chromosome number	Level of ploidy	References
Aporrectodea rosea	**18**	**2n, 3n, 4n, 5n, 6n, 8n, 10n**	(Vsevolodova-Perel and Bulatova, 2008)
Aporrectodea caliginosa	**18**	**2n, 3n, 4n**	(Kashmenskaya and Polyakov, 2008; Vsevolodova-Perel and Bulatova, 2008)
Bimastus tenuis	**16**	**3n**	(Muldal, 1952)
Dendrodrilus rubidus	**17**	**2n, 3n, 4n, 6n, 8n**	(Vsevolodova-Perel and Bulatova, 2008)
Dendrobaena octaedra	**18**	**5n, 6n, 8n**	(Hongell and Terhivuo, 1989; Vsevolodova-Perel and Bulatova, 2008)
Dendrobaena subrubicunda	**17**	**4n**	(Muldal, 1952)
Dendrobaena veneta	**18**	**2n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrobaena nassonovi nassonovi	**18**	**2n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrobaena hortensis	**18**	**2n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)

Dendrobaena pentheri	**18**	**2n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrobaena surbiensis	**18**	**2n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrobaena nassonovi adjarica	**18**	**2n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrobaena jaloniensis	**18**	**2n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrobaena marinae	**18**	**2n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrobaena tellermanica	**18**	**4n**	(Bakhtadze et al., 2004; Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrodriloides grandis perelae	**18**	**6n**	(Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrodriloides hydrophilica	**18**	**2n**	(Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrodriloides polysegmentica	**18**	**2n**	(Bakhtadze N.G., Bakhtadze G. I., 2003)
Dendrodriloides thamarae	**18**	**2n**	(Bakhtadze N.G., Bakhtadze G. I., 2003)
Eiseniella tetraedra	**18**	**3n, 4n**	(Muldal, 1952; Vsevolodova-Perel and Bulatova, 2008)
Eiseniella tetraedra hercynia	**18**	**4n**	(Muldal, 1952)
Eisenia atlavinyteae	**18**	**2n**	(Kashmenskaya and Polyakov, 2008)
Eisenia balatonica	**18**	**2n**	(Kashmenskaya and Polyakov, 2008)
Eisenia fetida	**11**	**2n**	(Bakhtadze et al., 2008)
Eisenia iverica	**18**	**2n**	(Bakhtadze et al., 2008)
Eisenia nordenskioldi	**18**	**2n**	(Kashmenskaya and Polyakov, 2008)
Elsenia rosea	**18**	**3n**	(Muldal, 1952)
Lumbricus rubellus	**18**	**2n**	(Bakhtadze et al., 2008)
Octodrilus transpadanus	**15**	**2n, 3n, 4n, 7n**	(Bakhtadze et al., 2008; Garbar et al., 2009)
Octodrilus complanatus	**18**	**2n**	(Vitturi et al., 2000)
Omodeoia byblica	**17**	**2n**	(Kvavadze et al., 2007)
Omodeoia arsianica	**17**	**2n**	(Kvavadze et al., 2007)
Octolasion lacteum	**18**	**3n**	(Kashmenskaya and Polyakov, 2008)
Octolasium cyaneum	**19**	**10n**	(Muldal, 1952)

Octolasion tyrtaeum	**18**	**3n**	(Vsevolodova-Perel and Bulatova, 2008)

It is recognized that animals with no sex determining chromosome, such as hermaphroditic earthworms, are particularly inclined to parthenogenetic reproduction (a form of asexual reproduction where the zygote is derived from an unfertilized female gamete; Lynch, 1984) (White, 1973). Parthenogenesis and polyploidy (i.e., having a chromosome set more than 2n) are considered to be related phenomena. Polyploidy is frequent in earthworms and ranges from tri to dodecaploidy (Diaz Cosin et al., 2011). Polyploidy is argued to confer a set of advantages such as larger cells, more gene expression, less sensibility to deleterious mutations and more evolutionary potential thanks to gene redundancy, which might provide an advantage in colonizing harsher environment (review in Tilquin and Kokko, 2016).

Some of the most widespread peregrine earthworm species of Lumbricidae, such as *Aporrectodea rosea* (Savigny), *Dendrobaena octaedra* (Savigny), *Eiseniella tetraedra* (Savigny), and *Octolasion cyaneum* (Savigny) are parthenogenetic polyploids (Table D 2). However, polyploid earthworms with an even degree of polyploidy that undergo sexual reproduction exist, such as sexual forms of *Eisenia nordenskioldi* with a ploidy level ranging from 2n to 8n (Stenberg and Saura, 2013). In parthenogenetic specimens, seminal vesicles, testes, spermathecae, genital setae, prostates, male pores and spermatophores are present, reduced or lacking (Diaz Cosin et al., 2011). Therefore, in earthworms, a "parthenogenetic polymorphism" or genital polymorphism" is often observed (see for instance Shen et al. 2011). For example, the parthenogenetic earthworm *Amynthas catenus* has various stages of degeneration in size, number and structure of reproductive organs. The athecal individuals (without spermathecae) of this species are; 2n, 3n, and 4n, while the sexthecals (with spermathecae) are 2n (Shen et al., 2011).

Relation between different ploidy degrees and species complex lineages have been rarely studied despite their connectivity and evolutionary importance. In the annelids, this question has been investigated for *Tubifex tubifex* complex (Müller, 1774). *T. tubifex* is a cosmopolitan annelid which is mostly found in the benthic freshwater communities, and can be found in 2n, 3n, 4n, and 5n forms (Marotta et al., 2014). This study was done in relation with cadmium resistance for different genetic lineages in this complex. Marotta et al. (2014) found that the different mitochondrial lineages within *T. tubifex* responded differently to cadmium resistance and these lineages corresponded to different ploidy degrees. The most cadmium-resistant lineages (I and III) corresponded to tetraploid lineages, while the least cadmium resistant lineage II, corresponded to triploid specimens (Marotta et al., 2014). In cases of species complex, different reproduction strategies per lineage may exist. For sexual reproduction species, meiosis cell division is obligatory to produce gametes. Retention of meiosis and spermatogenesis are also reported in some parthenogenetic species. For instance, in *Amynthas catenus* earthworm, species spermatogenesis for both, athecals and sexthecals, has been retained (Shen et al., 2011). This type of parthenogenesis with retention of meiosis is called 'automictic parthenogenesis' (Figure D 1) which

is very common for earthworms (Diaz Cosin et al., 2011). In this type of parthenogenesis, chromosome numbers are doubled prior to meiosis and genetically identical sister chromosomes are paired to form chiasmatic bivalents and regular meiosis is continued with the extrusion of two polar bodies (reviewed in Diaz Cosin et al., 2011). The genetic consequence of this cytological mechanism is similar to those of apomixis (i.e., the formation of clonal animal) however the heterozygosity is maintained (Stenberg and Saura, 2013). This kind of reproduction is compatible with different degrees of polyploidy, especially odd-numbered levels (Diaz Cosin et al., 2011; Terhivuo and Saura, 2006) i.e., the same number of chromosomes are obtained at the end of this type of meiosis (Figure D 1). *Dendrobaena octaedra* and *Dendrodrilus rubidus* are the only earthworms known to be apomictic parthenogens (suppression of meiosis during reproduction) (Diaz Cosin et al., 2011; Shen et al., 2011).

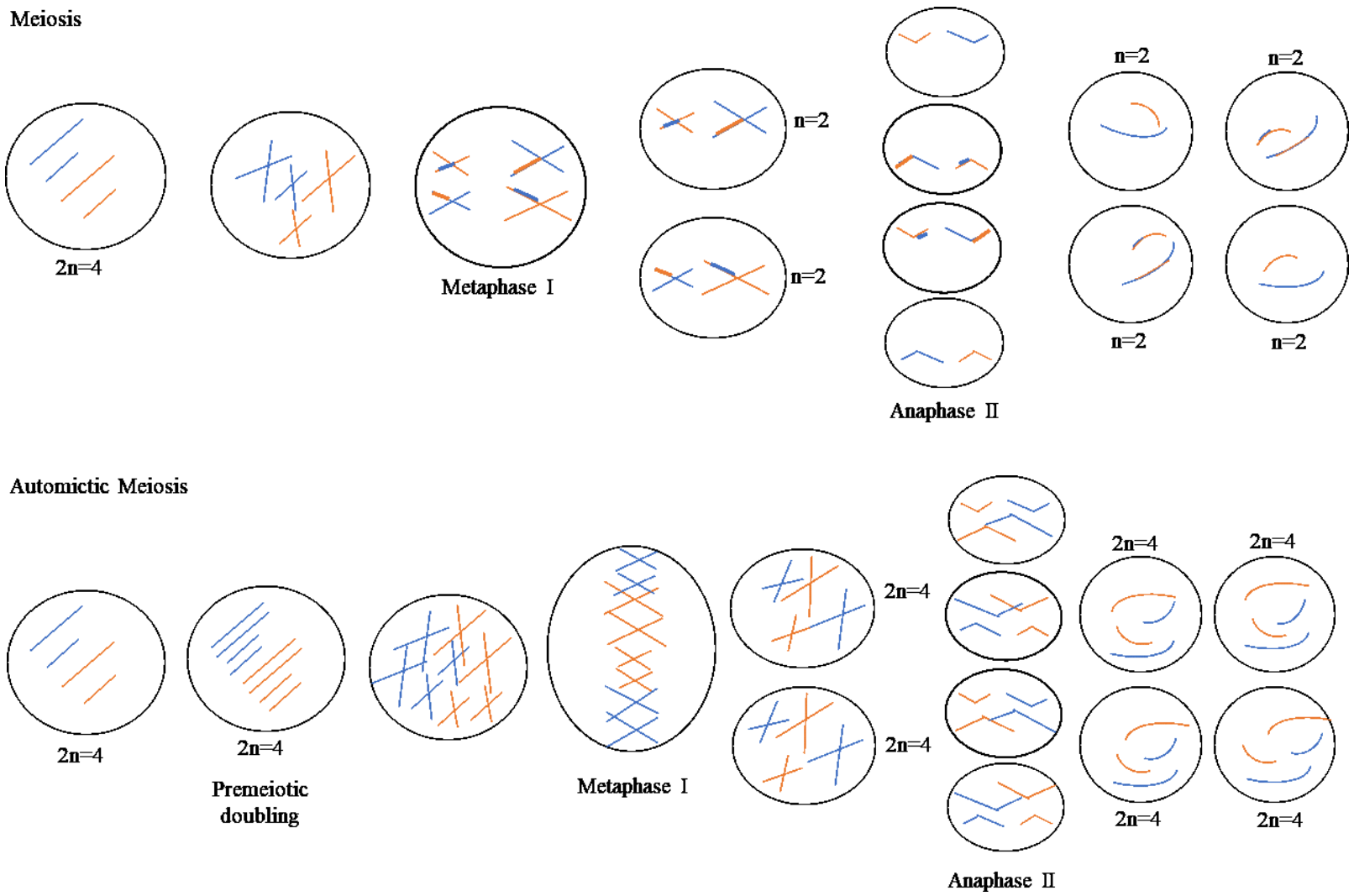

Figure D 1: The figure on top shows the stages of meiosis in sexual reproduction and the figure below shows meiosis stages in automictic parthenogenesis

Here, we were interested to do a karyological study on the peregrine earthworm species *Pontoscolex corethrurus*. Meanwhile, in chapter 2, we found a complex of four cryptic species for *P. corethrurus*. Therefore, we would like to know if a polyploid complex exists. *P. corethrurus* has been described as parthenogenetic but the possibility of sexual reproduction has been also suggested. Therefore, our objectives were; (i) to optimize an adapted protocol based on *P. corethrurus*, (ii) to find *P. corethrurus* ploidy degree, (iii) to investigate whether this complex of species corresponds to a polyploid complex, and (iv) to examine whether meiosis and spermatogenesis have been retained in *P. corethrurus* complex.

V.3. Materials and methods

Earthworms used for this study came from three locations; one site situated in Brazil (i.e., Embrapa's garden with 15 individuals, and during my two-month stay at Brazil I worked on a total of 68 individuals from the same location), and two sites, namely, Furnas volcano and pineapple plantation, from São Miguel Island of the Azores archipelago with 20 individuals from each population (Figure D 2). These specimens were kept at 24°C in boxes filled with tropical soil and horse dung as a source of nutrition. Water was added to these boxes every two weeks.

In this study, three different protocols were used. These protocols were tested in the following order: protocol 1 was tested on adults, juveniles, and cocoons of the Embrapa population in Brazil, protocol 2 and 3 were carried out on juveniles and adults coming from the three populations of this study i.e., Embrapa, pineapple plantation and Furnas volcano populations (protocols were done at different periods during three years). For the first protocol described by Gatti et al. (1994), we injected 0.03% colchicine (3mg/ml, 0.06 gr in 20 ml water) (a product which inhibits microtubule polymerization by binding to tubulin during metaphase stage of cell divisions) in the head of the animal and it was left for 15-20 hours. The day after, we dissected the worm after anesthetizing it with chloroform, and, if present, we took the seminal vesicles (i.e., the organ used for karyological studies because of high cell divisions) which are present between the 11th and the 17th segment. Otherwise, we took everything in the upper part of its gut. We put the organs in a 1.5 ml tube of 0.5% sodium citrate (0.5 gr of sodium citrate in 100 ml water), this solution is used as a hypotonic media which increases the cell volume by absorbing water in the cell to equilibrate the salt balance. At the same time, we smashed the organs in small pieces, and left it for 30 min, then replaced the supernatant with Carnoy solution (i.e., a fixative solution to fix and dehydrate chromosomes and cell nuclei after hypotonic shock; 1 ml of acetic acid/3 ml of methanol). After one hour, we spread the materials on slides and heated them at 24°C. Coloration was done with 3% Giemsa (i.e., 6 ml Giemsa + 6 ml PH7 + 188 ml water).

Figure D 2: Locations where samplings were done for this study. The figure in left shows the location used for sampling from Brazil (Embrapa's garden), and the image on the right shows the two locations of São Miguel Island; pineapple plantation and Furnas volcano (from left to right).

The second protocol followed Shen et al. (2011) procedure. The last five to six segments of earthworms' tail (without anesthetizing) were amputated. Twenty-four hours after the amputation, 0.05 ml of 0.3% (30 mg/ml, 0.6 gr in 20 ml water) colchicine was injected into the wound. After another 24h, the last five to six segments of the tail were amputated again. The regenerated tail was cut into fine pieces and immersed it in 0.56% colchicinized KCl solution (0.56 g KCl in 99.44 ml water + 1 ml 0.3% colchicine) for 4-5 hours. A few drops of Carnoy was added, then we centrifuged at 1000 rpm for 10 min. The supernatant was replaced by fixative and then re-centrifuged at 1300 rpm for 7 min. This step was repeated twice. The cell suspension was dropped on slides, which were put on heater for two hours, finally coloration was done with 3% Giemsa.

The last protocol which was a direct in vivo method according to the protocol of Bertollo (1978) (Figure D 3) with the following modifications: 0.04% colchicine (4mg/ml: 0.08 gr in 20 ml of water) at 24°C (tube held in 24°C bain-marie), was injected in the first 10 segments of the head (near the clitellum, if an adult) of specimens. The volume of injection was adjusted by the equation; 0.3 ml of colchicine for 100 grams of weighed animal. Then the animal was left in the soil (normal condition at 24°C) to avoid further stress for 15-20 hours. The following day, we dissected the specimens, after anesthetizing them by putting them in 70% of ethanol. If the worms were adult, we would take the seminal vesicles with testis sacs. Sometimes seminal vesicles were not observed even if the worms were adult, in this case, or if the worm was juvenile; all the other organs above the intestinal tube were taken. Tissues were rapidly dissociated with forceps, on 70-µm mesh cell strainers placed in small Petri dishes containing 2 ml hypotonic solution KCl (0.558% of 0.075M) at 24°C. The cell suspension was transferred to 15ml conical tubes containing 15ml of pre-warmed hypotonic solution. The resulting suspension was incubated for 20 min in a water bath at 24°C

with regular gentle shaking to provoke the hypotonic shock. At the last 5 minutes of the hypotonic shock we prepared the Carnoy (1 ml of acetic acid/3 ml of ethanol). We added 3 drops of Carnoy to stop the hypotonic shock and to fix the cells, then we mixed the liquid. Finally, we proceeded to centrifugation steps. The first centrifugation was for 10 minutes and 1500 rpm. After each centrifugation, the supernatant was taken and the materials were slowly homogenized with Carnoy at the bottom of the tube. We added again 6ml of Carnoy, while homogenizing. Then we left the tube for 10 minutes in a box filled with ice. The second centrifugation was done for 10 minutes and 1500 rpm. We proceeded exactly like the last stage, but we left the tube for 20 minutes on the ice. The last centrifugation was done for 10 minutes and 1500 rpm. The same procedure was repeated as the first and second centrifugations, but at the end we added 1 or 2 drops of Carnoy (depending on how much materials were left). Slide preparations were done by dropping 50µl of liquid material. Chromosome coloration was done by DAPI, i.e., 1 µg/ml DAPI (Sigma) in antifade solution (Vectashield Mounting Medium, Vector Laboratories, Peterborough, UK). Hybridized chromosomes were visualized under a Zeiss AXIO Imager M1 microscope, and images were captured with a CoolSNAPES camera. Photomicrographs were processed by the Genus software for karyotyping (Leica Microsystems).

At the end of the experimentation, we took a part of the specimens' tissue in order to verify to which phylogenetic lineage they correspond (we cannot distinguish the lineages morphologically) in *P. corethrurus* complex based on chapter 2 results (cytogenetic experimentations should be done on alive specimens, so sampling part of their tissue cannot be done before the manipulations).

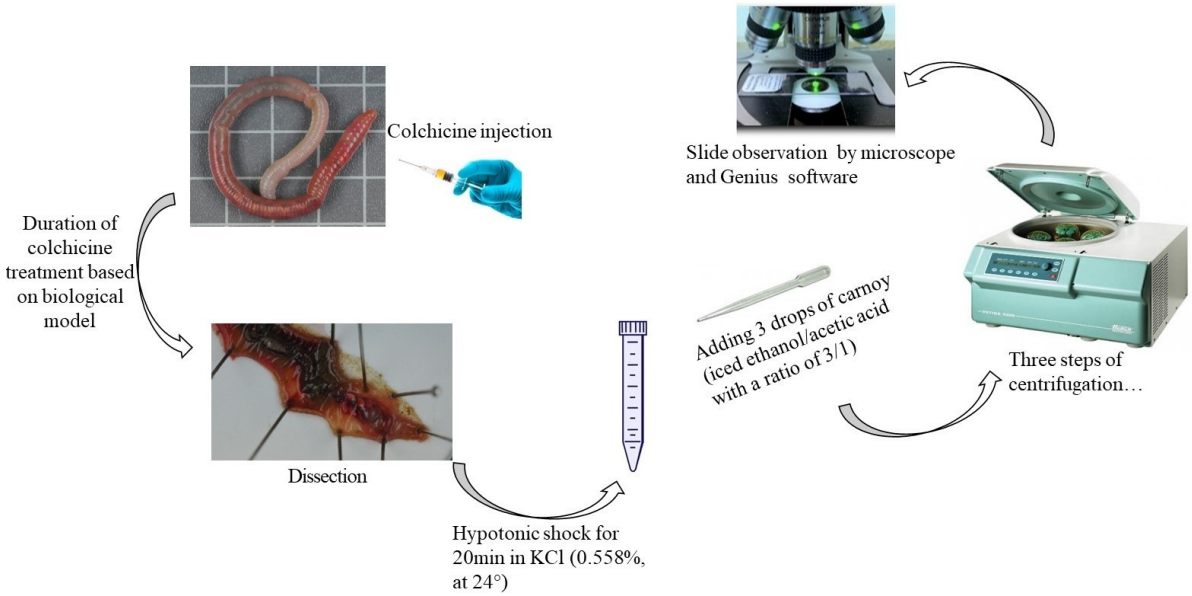

Figure D 3: The steps of protocol 3. The first step is colchicine injection (image on top left), the volume of injected colchicine depends on animal's weight i.e., 0.3 ml of 0.04% colchicine for 100 grams. Duration of colchicine treatment depends on the biological model used. KCl 0.558% at 24°C was used as hypotonic shock. Carnoy was added to stop the shock and to fix the chromosomes, then three steps of centrifugations were run. At the end, slides were observed under microscope to investigate chromosomes at metaphase stage.

V.4. Results and discussion

V.4.1. Problems encountered during experimentations

A total of 123 specimens (juveniles, adults and cocoons) were used for this study. During this study, we faced several problems. These problems were related to: limited access to alive specimens (tropical species), reproduction biology of *P. corethrurus* and the reproducibility of results.

First of all, in cytogenetics, we have to work on alive specimens, and because of international laws on alive specimen transportations prohibition, we couldn't have more specimens to work on than 15 individuals from Embrapa population of Brazil and 40 individuals from São Miguel Island. During my stay at Brazil, I had the opportunity to have access to specimens from Embrapa garden, but as I discuss later, the protocol used during this period (protocol 1) didn't work.

The protocol of tail regeneration (protocol 2) was tested on juveniles and adults, but we didn't obtain results. The main reason of low success of protocols 1 and 3 for most of the specimens, could be the lack of knowledge on reproduction biology and on physiology of this morphospecies (seminal vesicles are the organ used for both protocols). In many cases once the adult worms (i.e., clitellate specimens) were dissected, no seminal vesicles were found, in this case we took other organs above the gut, but no results were obtained. This observation reinforces our belief that these specimens reproduce parthenogenetically. At the other hand, when seminal vesicles were present, the experiments failed due to limited cell divisions (mostly related to lack of knowledge on periods of the year when spermatogenesis occurs) or technical problems e.g., duration of colchicine, hypotonic shock duration, etc. For instance, chromosomes in metaphase were found for two juveniles from Embrapa, based on protocol 1, but the results on the slides were not usable due to technical problems (Figure D 4). These problems were related to high hypotonic shock that made chromosomes spread a lot so that it was impossible to find the chromosome pairs, or weak hypotonic shock which made chromosomes overlap. Consequently, as results could not be produced regularly due to seminal vesicles absence or lack of observed cell divisions, modifications of the protocol based on few results obtained were useless (e.g., more or less hypotonic shock periods).

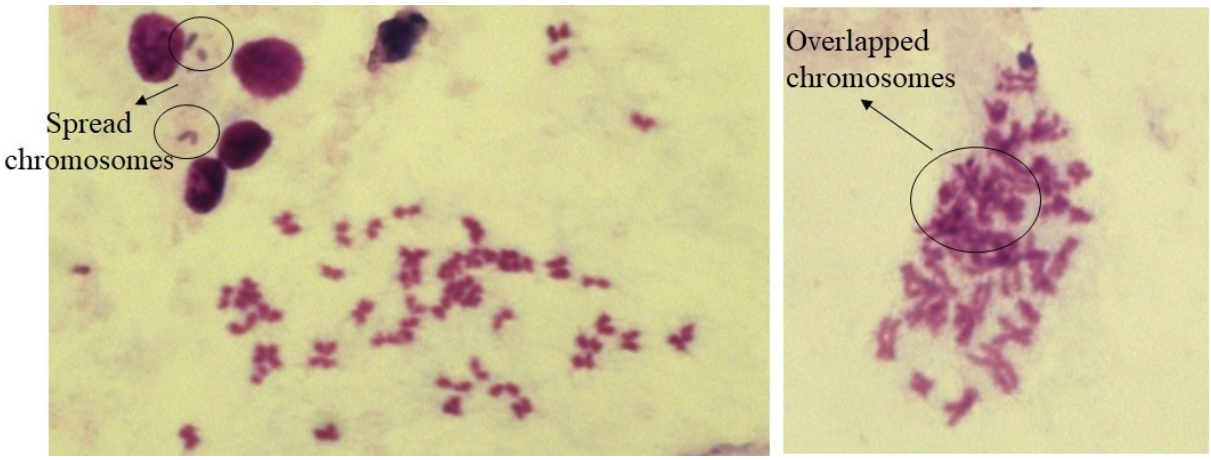

Figure D 4: Results obtained from protocol 1, taking seminal vesicules from juvenile specimens of Embrapa population. Left figure: chromosomes are too spread due to strong hypotonic shock, also long colchicine duration has caused the chromosome compaction. Right figure: chromosomes are overlapped, due to weak hypotonic shock.

V.4.2. First karyotype within Pontoscolex corethrurus *complex*

Out of 123 individuals tested during this study, only two adult individuals from Embrapa garden gave results based on protocol 3. In both cases, 2n=70 chromosomes were counted. Thus, haploid number of chromosomes equals 35, which is much higher than those already observed for Lumbricidae family (i.e., between 11 to 19 chromosomes). *Amynthas catenus* which is also present in tropical zone has high haploid chromosome number i.e., 56 (Shen et al., 2011).

Using COI sequences, we were able to assign the specimens used in this study to the lineages within the complex (Figure D 5). In the tree shown in Figure D 5, relationship between lineages L3 and L4 has changed in comparison to four genes tree (COI+16S+ITS2+28S), presented in chapter 2, mainly because this tree is done with one gene, consequently the relationships are not as complete as for the four genes tree. The specimens in Embrapa population (Brazil) corresponded to L1 and L4 lineages and the two populations of São Miguel Island corresponded also to L1 and L4 lineages, i.e., pineapple plantation (L1) and Furnas volcano (L4). Therefore, during this study, due to limited access to alive specimens, we just had specimens belonging to L1 and L4 lineages. The two specimens for which we obtained results belonged to Embrapa population. One of the adult specimens corresponded to L4 lineage (Figure D 5). While, for the other individual due to sequencing problem, we were unable to confirm to which lineage it corresponds (whether L1 or L4). The first karyotype within *P. corethrurus* complex belongs to L4 which is shown in Figure D 6.

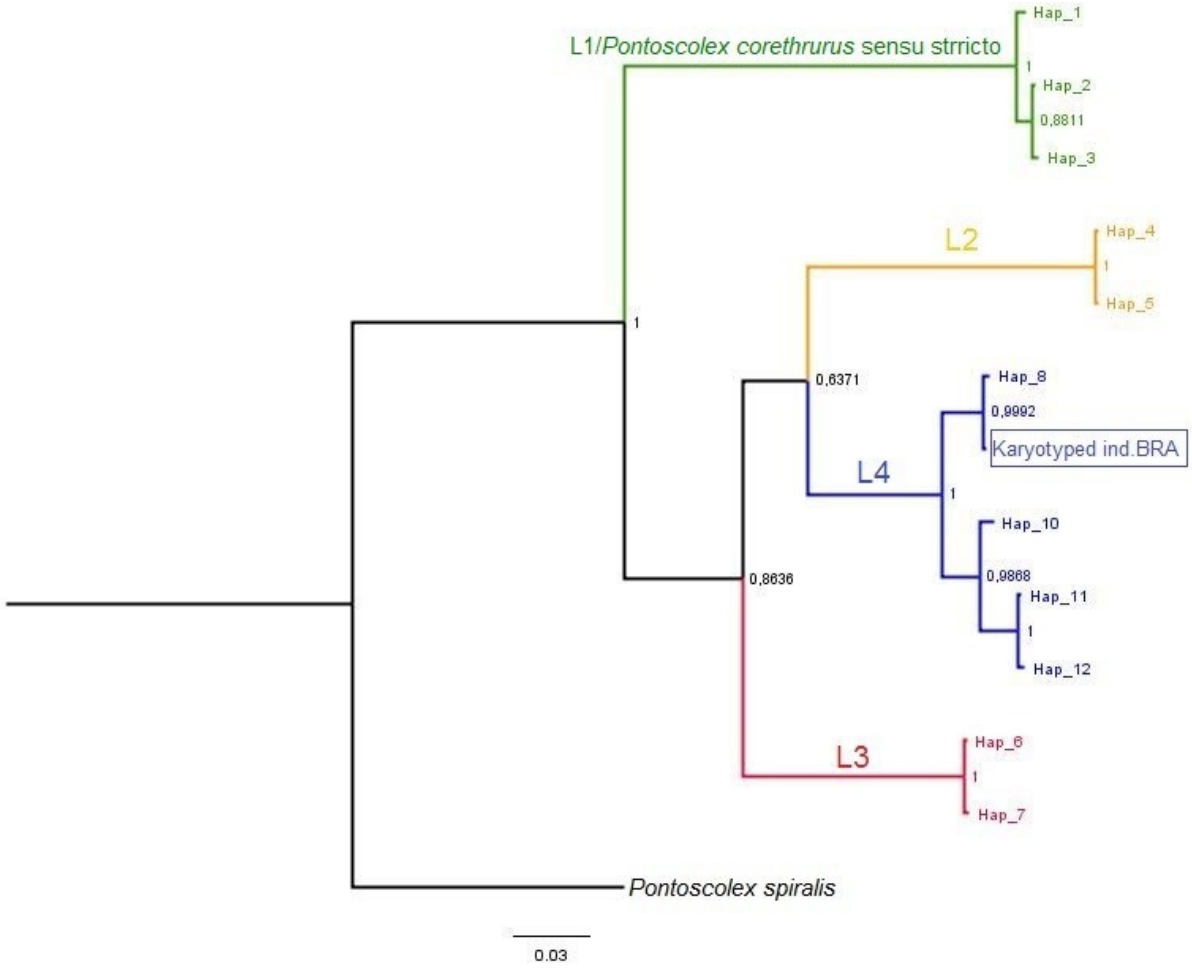

Figure D 5: Bayesian tree for COI gene of *Pontoscolex corethrurus* complex. The karyotyped individual (2n=70) from Embrapa's garden in Brazil, corresponded to L4 of this complex.

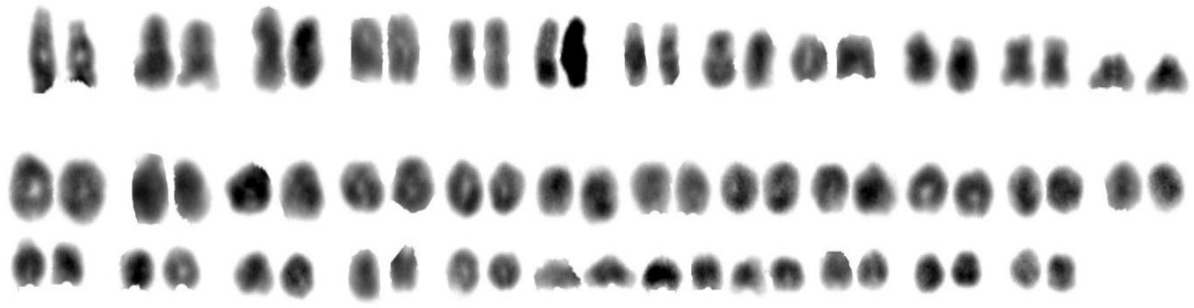

Figure D 6: First karyotype obtained based on protocol 3, from seminal vesicles of an adult specimen in L4 of *Pontoscolex corethrurus* complex within Embrapa population. The ploidy degree is diploid; 2n=70.

V.4.3. Sexually reproducing or parthenogenesis?

Evidence of meiosis during spermatogenesis was found for the two individuals for which we obtained results (Figure D 7). Based on our results, *P. corethrurus* L4 could be either a sexual reproducing species, or as spermatogenesis is retained for some parthenogenetic species (Cernosvitov, 1930; Shen et al., 2011), it could be an automictic parthenogen. Although it is not yet understood why meiosis and spermatogenesis are retained in parthenogenetic reproduction, Simon et al. (2003) highlighted that one of the origins of parthenogenesis in animals, including earthworms, is 'contagious parthenogenesis'. This type of origin is based on the hybridization of two closely related species where complete reproduction isolation between parthenogens and sexual ones have not yet occurred. Therefore, the sperm production of parthenogenetic lineage is necessary to fertilize females of the closely related sexual species. This hybridization engenders new parthenogenetic lineages or species. Thus, if we consider *P. corethrurus* L4 as an automictic parthenogen, the sperm production could be explained by this phenomenon.

In chapter 2 of my thesis, we found evidence of heteroplasmy for 3 individuals in one site (National Taiwan University) of Taiwan belonging to L4. Therefore, based on results obtained in chapter 2, and the proof of meiosis and spermatogenesis in this chapter, we can suggest that L4 reproduces sexually.

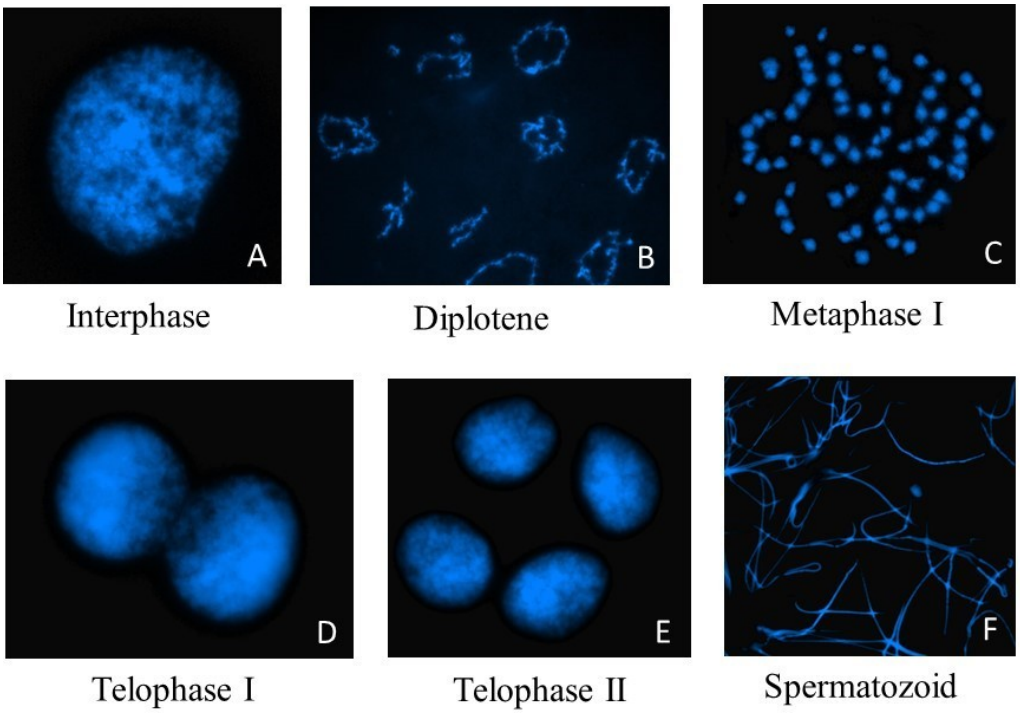

Figure D 7: Meiosis stages of two individuals in *Pontoscolex corethrurus* complex, one individual in L4 and the other one is unknown due to sequencing problem. These results were obtained on seminal vesicles organ (slide colorations are done by DAPI).

V.4.4. A polyploid complex for P. corethrurus*?*

As mentioned earlier, for species complex, we wonder if the speciation process could be related to different ploidy degrees among cryptic species, especially if they reproduce parthenogenetically. However, as in this study we didn't obtain results for other specimens in the complex, we could not answer this question. In the literature on earthworm's karyology, a lack of phylogenetic analysis alongside cytogenetic studies was observed. These two sciences are complementary and it is important to develop these kinds of studies in the future.

V.5. Conclusion and perspectives

To our knowledge, this is the first study investigating the ploidy degree within *P. corethrurus* complex. We found 2n=70 for L4 in the complex. Proof of spermatogenesis was also found for two specimens in L4. In this study, I have tested and tried every protocol known for earthworms karyotyping, but I didn't gain results as I hoped for. I think that classical cytogenetic protocols are not adapted for this complex as long as they are mostly parthenogens and even if seminal vesicles exist, the chance to gain desired results is low. Therefore, for an estimation of the ploidy degree, I propose flow cytometry to measure DNA content of somatic cells from coelomocytes in the future.

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VI. General Conclusions and Perspectives

VI.1. Speciation, hybridization and genetic variation within Pontoscolex corethrurus *complex*

VI.1.1. New species within the genus Pontoscolex *and cryptic diversity within the* Pontoscolex corethrurus *complex*

My thesis has been the first study on cryptic diversity discovery in a wide range of tropical and sub-tropical countries for the most invasive, peregrine earthworm species; *Pontoscolex corethrurus*. In my PhD, four new species within the genus *Pontoscolex* and four cryptic species within *P. corethrurus* complex were found. Analyses were done on 792 specimens from 25 countries in tropical and sub-tropical zones. These specimens were morphologically close to the morphospecies *P. corethrurus*, collected by soil scientists. Previously, Cunha et al. (2014) had found two cryptic species for *P. corethrurus* on Sao Miguel Island of Azores archipelago. High sampling effort (we have used the most extensive sampling done so far in this genus to date), could explain that two more cryptic species were found in *P. corethrurus* complex in this thesis.

Alongside cryptic diversity discovery, four new species were also found in the genus *Pontoscolex*. Through chapter two we highlighted an important aspect; which is the misleading morphological characters used to distinguish species within the genus *Pontoscolex. P. corethrurus* is mostly distinguished by the quincunx formation of the setae (brush tail) at the last quarter of its body. Three of the four newly found species in my PhD, had also quincunx formation (*P.* sp. 1, *P.* sp 2, and *P.* sp 3). Thus, as Moreno (2004) similarly highlighted, it is possible that in some studies due to identification error based on this trait, other species in the genus have been mistakenly used as *P. corethrurus*. Accordingly, we strongly suggest that this trait should not be used as a recognition characteristic for this morphospecies. It is also necessary that these new species be described in genus *Pontoscolex* in future, and samples from this thesis could be used as type species.

Morphological character examination had been the only method of species diagnosis for a long time. With the advent of molecular approaches, species identification became more accurate. In case of cryptic species where species morphology will not help us recognize different species, molecular approaches were a break through. In my thesis, we used two mitochondrial markers (COI and 16S) with two nuclear genes (28S and ITS2) on 792 samples. Three species delimitation methods of single-locus (ABGD, mPTP) and multi-locus (BPP) were applied. These methods showed congruent results, and confirmed the existence of four cryptic species. Phylogenetic results were confirmed with phylogenomics analyses by AHE method. Our study thus supports the value of molecular identification for species within the genus *Pontoscolex.* To date, this is the first study where cryptic species discovery in earthworms has been done based on single-locus and multi-

locus species delimitation methods, together with a phylogenomics study. However, the question remains for ecological aspect in an integrative taxonomy context. Are these species ecologically separate as well? Probably in future we could answer this question by investigating if the different phylogenetic species occupy different niches or not when they are found in sympatry.

For cryptic species discovery, it has been suggested to do single-locus and multi-locus, species delimitation analyses (Carstens et al., 2013). This is mostly due to contradictory results that different markers could have if they are interpreted separately. COI gene is the marker of choice for the majority of studies treating cryptic diversity discovery. In several cases, it has been observed that with different markers, different number of cryptic species could be found. For instance, for *Lumbricus rubellus* based on COI gene four cryptic species were found, while based on H3 gene, nine species (Martinsson and Erséus, 2017). The final number of cryptic species for *L. rubellus* was estimated based on the BPP multi-locus species delimitation method. This analysis, showed a total of seven cryptic species for this morphospecies. In our study number of cryptic species found based on COI gene (ABGD and mPTP for single-locus species delimitation analyses) were similar to those of multi-locus analysis (BPP). Results of the phylogenomic approach (AHE), which is based on 609 loci, also confirmed these results. The fact that independent single marker COI showed the same results as for four concatenated genes and full nuclear genomic DNA results, could be explained by the lack of introgression among cryptic species and long evolutionary periods of time. Meanwhile, as COI is only maternally inherited, the effective population size is small. These could be the reasons why COI alone permit us to distinguish the four cryptic species in *P. corethrurus* complex.

It has always been suggested that nuclear markers should be used together with mitochondrial genes for systematic studies (Song and Ahn, 2014). In our study, results of nuclear marker showed the evidence of four new species within the genus, but the cryptic lineages could not be identified by these markers. It has already been found that, phylogenetic reconstruction based on nuclear markers, within species or between closely related species, due to heterozygous sites, is problematic (Sota and Vogler, 2003). However, information of heterozygous sites for closely related or fast radiating taxa where diagnostic sites are rare, is particularly valuable (Lischer et al., 2014). In our study, heterozygous sites were observed in several sites for ITS2 and 28S genes. As more variation and more ambiguous base pairs were observed for ITS2 in the sequences, we did phasing analysis (a method of deriving information from heterozygous sites from a sequence, by dividing the sequence into two or more alleles) for ITS2 gene. Further analyses were done on phased sequences. ITS2 phased sequences showed the same results as for mitochondrial genes (i.e., four new species alongside four cryptic species in *P. corethrurus* complex). The ITS2 network haplotype done by median joining in chapter two was a proof of that. In fact, out of 27 polymorphic sites, only one site didn't have heterozygous sites. Meaning that, by replacing ambiguous bases for 26 sites with N (as missing information), the rest of ITS2 sequences were completely equal (except one site) for all the cryptic species in the complex. Thus, by ignoring heterozygous sites, only one big lineage with all the cryptic species mixed together (i.e., *P.*

corethrurus L1, L2, L3 and L4), was observed. Hence, this thesis highlights the importance of phasing of nuclear genes if ambiguous bases are found for them. This could be the case of studies on cryptic diversity for some other earthworm morphospecies. For instance, for *Aporrectodea longa* two divergent haplogroups were found based on COI gene, but not for ITS2 and H3 (Martinsson et al., 2015). Therefore, the hypothesis of two cryptic species existence was rejected for this morphospecies. However, we could possibly imagine that with phasing of ambiguous bases (if any were found), results could be different. At the other hand, in some studies congruent results between mitochondrial and nuclear markers, for cryptic diversity discovery, were found without nuclear genes phasing. For instance, five cryptic species were suggested for *Eisenia nordenskioldi pallida* based on ITS2 and COI, phylogenetic trees based on ML, BI, and ME methods (Shekhovtsov et al., 2016b). Although less variations were observed for ITS2, this gene corroborated the results of COI gene. The fact that, in Shekhovtsov et al. (2016b) study, ITS2 gene allowed to distinguish cryptic lineages is probably related to the high number of polymorphic sites within this gene. In ITS2 sequences, 112 polymorphic sites were found, while in our study 27 polymorphic sites out of 371 for *P. corethrurus* complex were observed.

As *P. corethrurus* morphospecies has been widely used as biological model, future studies working on *P. corethrurus* complex should consider that this complex of species consist of at least four cryptic species. We suggest researches to obtain COI sequences for their samples prior to any experimentations. Eventually, they could blast and verify to which one of the species in the complex their samples belong to, based on the COI barcodes on GenBank provided from this thesis. This is especially important for ecotoxicological experiments, as *P. corethrurus* has been the subject of some ecotoxicological studies. Different studies have shown that working on a complex of species for ecotoxicological questions could give biased results. For instance, diverse responses of the fungicide impact on the two lineages (LA and LB) of an amphipod complex of species, *Gammarus fossarum* (Feckler et al., 2012) and different adaptations of *Lumbricus rubellus* cryptic lineages (LA and LB) to soil contaminations (Kille et al., 2013) were observed.

VI.1.2. Reproductive isolation among species in the complex

In chapter two we showed that in only 7 sites out of 116, sympatry of different cryptic species was observed: L1/L3 within two sites in Mexico, L1/L3/L4 in Taiwan, and in Brazil L1/L3/L4, L1/L4 and within two sites for L1/L3. In three of them (two in Mexico and one in Taiwan) reproductive isolation within the complex was investigated. This question was examined between *P. corethrurus* L1 and *P. corethrurus* L3, based on AFLP markers (due to low number of individuals for *P. corethrurus* L2 and *P. corethrurus* L4 species, reproductive isolation could not be investigated for them). Results showed no mixture between these two species. As previously mentioned in the introduction, while researchers are confused about the number of species i.e., one versus two, we are in the 'grey zone' (De Queiroz, 2007). 'Grey zone' is in the path of speciation process, where reproductive isolation is gradually established among two species. Once, no gene flow occurs among species, the speciation process is complete. Based on our results, reproductive isolation has been established between *P. corethrurus* L1 and L3 as no evidence of hybridization among them was found. However, they still have the same morphological traits which shows that these species are still in the grey zone, and the speciation process has not been completed yet. Meanwhile, in chapter two of my thesis we raised the question of possible hybridization between *P. corethrurus* L3 and L4. This was because of observed heteroplasmy for COI gene in three individuals in one population of Taiwan, and shared ITS2 haplotypes among *P. corethrurus* L3 and L4. Unfortunately, because of low numbers of individuals for *P. corethrurus* L4, analysis based on AFLP profiles, couldn't prove the hybridization among *P. corethrurus* L3 and L4. Eventually, a more advanced molecular tool such as RADseq. (Baird et al., 2008) could probably help us determining with more accuracy the existence or absence of hybridizations among these two species within this complex.

One of the primordial information on any species is the knowledge on its ploidy degree. As shown in chapter four, the ploidy degree of earthworm species in Lumbricidae family is well-known in comparison to other families. Although, *P. corethrurus* has been the subject of a lot of studies, its ploidy degree has never been studied before. Through this thesis, we were interested to test the 'polyploidy complex' hypothesis for *P. corethrurus* complex. This hypothesis proposes that species within a complex have different ploidy degrees. Meanwhile, due to different ploidy degrees, these species are not compatible to reproduce with each other. Therefore, different ploidy degrees could be the reason of reproductive isolation among these species. In order to test this hypothesis, we needed to know the ploidy degree of each species in the complex. In chapter four of my thesis, we tried to answer this question by cytogenetics experimentations. *P. corethrurus* L4 came out to be diploid (2n=70). However, due to difficulties during the experimentations, we failed to answer this question by cytogenetics experimentations for other species in the complex. Reasons of failure were mainly due to limited access to alive specimens (tropical species) and lack of knowledge on reproduction strategy of these specimens (parthenogenesis or sexual), because the cytogenetics protocols are mainly based on seminal vesicles and in case of parthenogenesis, these organs don't exist. During my thesis, we collaborated with Luis Cunha, a post-doctoral student in

Embrapa (Brazilian Agricultural Research Corporation) of Brazil, under the supervision of George Brown. He was also interested in the ploidy degree of *P. corethrurus* complex, and used flow cytometry for determining it (Figure 4). In this approach, the ploidy degree of an organism is estimated based on the comparisons with 'standards' (organisms for which ploidy degrees are already known). Based on his analysis, *P. corethrurus* L1 from pineapple plantation of Azores Island, was also found to be diploid. Its genome size being estimated about 700 megabases (haplome). His analysis also confirmed the diploidy of *P. corethrurus* L4, as we found by cytogenetics.

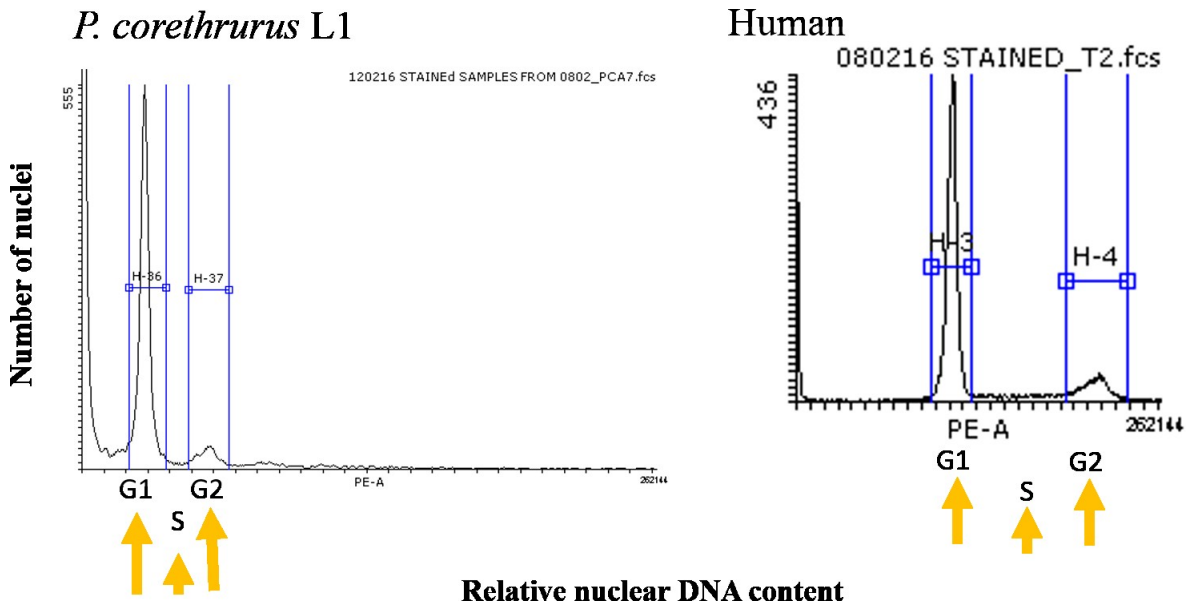

Figure 4: Flow cytometry results obtained by Luis Cunha, for pineapple plantation individuals of *P. corethrurus* L1, from Azores Island. Analyses were done based on coelomocytes cavity of specimens. Results suggested that *P. corethrurus* L1 is diploid.

The existence of 'polyploid complex' could be investigated for other complexes of earthworm species. For instance, *Aporrectodea rosea* could be a good candidate for future studies. In this case, access to alive specimens are not limited as long as this species is found in temperate zone. This species is polyploid and different ploidy degrees are already known for it; 2n, 3n, 4n, 5n, 6n, 8n, and 10n (Vsevolodova-Perel and Bulatova, 2008). The existence of cryptic species has also been suggested for this morphospecies (Fernández et al., 2016). As different ploidy degrees exist for *A. rosea*, an eventual question could be; Do different ploidy degrees correspond to different cryptic species within *A. rosea* complex?

VI.2. P. corethrurus *L1: the invasive species*

In chapter two, we were able to answer the question that has been rarely investigated. The questions were; when we have a case of a known peregrine and invasive morphospecies, for which we have found cryptic diversity with the help of molecular markers, can we assume that (i) one species in the complex is invasive, (ii) some of them are invasive or (iii) all of them? In case of *P. corethrurus* complex, we only found one of the species in the complex i.e., *P. corethrurus* L1, present in the majority of studied sites (100 sites out of 116). Thus, our hypothesis is that *P. corethrurus* L1 is the only invasive species in the complex. Eventually, we assume that most of the studies on this morphospecies have used *P. corethrurus* L1 as their biological model.

There are four main steps of invasion; introduction, colonization, establishment, and finally spread in new environments. Different ways of introduction of peregrine species throughout the world are known by anthropochory, hydrochory, and phoresy. It has been mentioned that the occurrence of the genus *Pontosocolex* (supposedly native in South America) in Caribbean Islands, could only be explained by human migration prior to European colonization (González et al., 2006). It has been suggested that some 2200 years ago, humans arrived in the Greater Antilles by island hopping from South America. Therefore, the vast distribution of *P. corethrurus* L1 is related to human activities rather than ecological factors. Facility of transportation and introduction of this species i.e., by attaching of cocoons to human boots, was also shown by a population genetics study on populations from French Guiana by AFLP markers (Dupont et al., 2012). Key traits of invasion success for the second step of invasion process, which is colonization, are r strategy and parthenogenetic reproduction. The r strategies of this species are related to continuous breeding with high fecundity rates, high hatching success and short development time. After the initial colonization, phenotypic plasticity and competitivity of *P. corethrurus* L1 are main characteristics of this species to become established in a new environment. The plasticity of this species is related to its adaptability in different soil types with varying pH degrees, adjusting cocoon production and incubation based on different environmental conditions and its fitness even in different degrees of contaminants in the soil. *P. corethrurus* L1 is highly competitive with other earthworm species, however evidence of exclusion by competition has never been reported for it. Competitivity of this species is mostly related to its presence in poor areas where no other species could occur. Based on the invasion step and thus reproduction strategy of this species, we could hypothesize that different levels of genetic diversity could be observed for different populations in different parts of the world. Consequently, studies taking this species as their biological model for ecotoxicological experimentations for instance, could have different results based on the invasion step of the specimens where samples come from. For instance, results of an ecotoxicological study on individuals in LOP population could vary from those in CAX population of Brazil due to weak genetic diversity in the former and higher genetic diversity in the latter. Thus, we might wonder if taking an invasive species is a good choice as a model species for ecological questions.

Another important reason to investigate the ploidy degree as mentioned above, for *P. corethrurus* L1 is that it could help us understand the invasion success of this species. Most of the invasive, peregrine species are polyploid. For instance, *Aporrectodea rosea* (2n, 3n, 4n, 5n, 6n, 8n, 10n) and *Octolasion tyrtaeum* (3n) (Vsevolodova-Perel and Bulatova, 2008), and *Dendrobaena subrubicun* (4n) (Muldal, 1952), are polyploid and peregrine earthworm morphospecies all over the world. The genetic consequences and advantageous of a polyploid species have already been discussed in the introduction. These advantageous could be related to larger cells, more gene expression, less sensibility to deleterious mutations and more evolutionary potential thanks to gene redundancy, which might provide an advantage in colonizing harsher environment (review in Tilquin and Kokko, 2016). Moreover, polyploidy and parthenogenetic reproduction are two related phenomena. Parthenogenesis is one of the main success traits for invasion success (Sakai et al., 2001). Probably, parthenogenesis is the key to maintain polyploidy. In this case, invasion success of parthenogenetic species could be explained in one hand by foundation of a population from one individual without the need to reproduce with another specimen, and at the other hand, it helps the maintenance of polyploidy which has several advantageous for peregrine species.

VI.2.1. Phylogeography and population genetics of the invasive species in the complex; **P. corethrurus *L1***

COI genetic diversity was investigated for *P. corethrurus* L1 populations. Thirteen COI haplotypes were found out of 479 sequences for *P. corethrurus* L1. In comparison with other studies for instance, De Sosa et al. (2017a) study on *Eiseniella tetraedra*, more COI genetic diversity was observed. Out of 92 sequences of the parthenogenetic species *E. tetraedra*, 46 haplotypes were discovered i.e., almost one haplotype every two sequences. Samples came from three main localities in Spain; Northern Spain, Central and Wales. Our study highlights low COI diversity of *P. corethrurus* L1 specimens.

This study has been the first on population genetics and phylogeography of an invasive, peregrine earthworm species, *P. corethrurus* L1, in a wide range of countries in the tropical and sub-tropical zones. Guayana shield has been suggested as the origin of genus *Pontoscolex* (Righi, 1984). This hypothesis was based on high species diversity observed in genus *Pontoscolex* in Guayana shield. However, based on AFLP markers analysis in this study, evidence of more genetic diversity in populations from Guayana shield in comparison with other parts of the world was not observed. Additionally, despite long distances among the studied populations; Brazil, Mexico, Caribbean islands, Gabon, India, Thailand, Taiwan, Madagascar, Philippines and Malaysia, one major COI haplotype (H1) was widespread in all of them. Lack of more genetic variation, in the supposed native zone in comparison with other areas, could be due to multiple re-introductions of individuals (genotypes) of *P. corethrurus* L1 in different parts of the world, so that the genetic variations throughout the world has become approximately equal. It could be possible that with more population samples for *P. corethrurus* L1, more information on the genetic variation among native and invaded zones of this species be found. However, our hypothesis is that due to several introductions and the long invasion history of *P. corethrurus* L1, it is almost impossible to trace back the zone of origin, and the invasion roads of this species. At the other hand, my thesis highlights the importance of using mitochondrial markers alongside nuclear genome information for population genetics and phylogeography studies. Due to invasion of one COI haplotype (H1) throughout the world, we could propose the invasion of a 'super-clone' for *P. corethrurus* L1. However, with AFLP markers more genetic variations were observed between populations, and no genotypes were shared between two individuals among populations in the world. Therefore, the hypothesis of the invasion of a 'super-clone' was rejected for *P. corethrurus* L1.

VI.2.2. An invasive species doesn't only have negative impacts

In my thesis, we considered invasive species as species with high competitive characteristics. These species following the disappearance of natural obstacles to their propagation in a new environment, are established and even spread into novel areas (Valéry et al., 2008). In many studies, invasive species are considered as those which have negative impacts where they inhabit. These negative impacts in case of an earthworm species, could be on the soil structure by soil compaction, on soil organisms with biodiversity reduction, and on plant growth reduction or even death due to more nutrient consumption by earthworms. *P. corethrurus* L1 has been considered for a long time as a compacting earthworm species by its activities. Soil compaction could lead to creation of an impermeable layer on the soil surface where water and air could hardly penetrate. Consequently, species of plant which are not resistant to lack of water could eventually die. However, in literature we found the evidence of re-structuration ability of this species for Oxisols. Meanwhile, the compaction impact of this species is mostly related to soil organic matter content of the soil. In low concentrations, this species has compacting impact especially due to absence of other de-compacting organisms in the soil. *P. corethrurus* is known to have positive impacts on plant growth, due to the existence of high concentration of minerals in their casts. High rates of mineralization for this species, starts from their gut, by the activation of soil organisms. This activation is related to the 'sleeping beauty' hypothesis (Lavelle et al., 1983), where microorganisms are highly activated by water and soluble-C in form of mucus in *P. corethrurus* intestinal tube. This species has been repeatedly reported as dominant where it is found. In chapter one we showed that this species could also co-occur with other native or exotic species. In some cases, the dominance of exotic and native species have been found over this species, for instance in one pasture of Cuba where exotic species were dominant. *P. corethrurus* is found in a wide range of habitats, form pastures, croplands and plantations to primary forests and tropical rainforests. This species could inhabit soils with poor or rich levels of organic matter. This suggests the high plasticity of this species. In any habitat where this species is found, based on the soil type, pH soil volume, degree of organic matter in the soil, species of plant present, presence or absence of other soil organisms, the impacts of *P. corethrurus* L1 varies; meaning that they could be positive or negative.

VI.3. Mixed reproductive strategy within a species complex and/or within a species

It is important to mention that, different species within a complex, could have different strategies of reproduction i.e., sexual reproduction or parthenogenesis. In general, the advantages of sexual over asexual reproduction are in 'mutational' and 'ecological' aspects (Simon et al., 2003). The mutational disadvantage of parthenogenetic reproduction is related to the fact that as long as recombination among homologous chromosomes doesn't exist, deleterious mutations could accumulate through time. These mutations are difficult to purge form asexual genomes. The ecological aspect is related to more resistance or adaptation of sexual genomes to environmental conditions. For instance, in cases of parasites or competition with relatives, sexually reproducing specimens due to higher genetic diversity could be advantageous over asexual ones. *P. corethrurus* L4 in the complex, due to observed spermatogenesis and diploid ploidy degree could be a sexually reproducing species. However, spermatogenesis has been also observed for parthenogenetically reproducing species. One of the origins of parthenogenesis in different organisms including earthworms, is 'contagious parthenogenesis'. In this type, sperms from parthenogenetic species would fertilize the females of a sister species, where reproductive isolation hasn't yet been established among them. Consequently, a new parthenogenetic lineage or species with a probable different ploidy degree than its parents is created. This type of strategy has also been observed for *Daphnia pulex* (Innes and Ginn, 2014). The asexual clones of this species have succeeded to invade the sexual populations, mostly by 'contagious parthenogenesis' where new parthenogenetic genotypes are formed. Despite the existence of different reproduction strategies in a species complex, some species could have mixed reproduction strategies. In mixed reproduction strategy, the species is a facultative parthenogenetic and has also sexual reproduction. This mixed reproduction strategy helps the species resistance. Resistance to environmental conditions due to mixed reproduction, in case of peregrine and invasive species could be very beneficial. For example, the earthworm morphospecies *Dendrobaena subrubicunda* which is widely present in the world has a facultative parthenogenetic reproduction alongside sexual reproduction (Muldal, 1952). An invasive species could be in different stages of invasion while present in an area. As mentioned above, in the first stages of invasion, for instance the colonization, the reproduction strategy could be parthenogenesis to enhance the probability of invasion success. Therefore, less genetic diversity is expected for populations which have newly colonized an area or are recently established. Through our population genetics study, low levels of genetic diversity have been observed in some populations. For example, based on AFLP markers, for CHG in Thailand and LOP in Gabon high levels of clones were observed with less genetic diversity. This could be due to parthenogenetic reproduction strategy and eventually conclude that these areas are newly invaded by *P. corethrurus* L1. Meanwhile, high genetic diversity was observed in some populations e.g., CAY, PARC, BEL, JNL, and TLC, which could be due to the accumulation of mutations through time because this species has invaded and established in these populations a long time ago. Another reason could be related to sexual reproduction of specimens within these

populations. Sexual reproduction strategy of *P. corethrurus* L1 was also suggested by variable levels of linkage disequilibrium and higher genetic diversities in some populations for this species. Therefore, we suggest a mixed strategy of reproduction for *P. corethrurus* L1; once it is in the first steps of invasion or has newly invaded an area, it has parthenogenetic reproduction, but while it has established in an environment long time ago, it could have sexual reproduction as well. In future, existence of sexual reproduction hypothesis for *P. corethrurus* L1 could be tested by controlled laboratory experimentations. This could be done by putting two or three earthworms per box and observe them regularly, if cocoons are deposited. In case of cocoons presence, we could genotype the two or three earthworms in the box and the cocoons. Eventually, we could examine to which specimens (in case of sexual reproduction) or specimen (in case of parthenogenesis), the cocoons belong to. At the other hand, in order to better understand the evolution of different reproduction strategies in *P. corethrurus* complex, we should probably gather the information on reproduction strategies of all the species in genus *Pontoscolex.*

VII. Thesis Appendix

VII.1. Appendix 1

Article on mitochondrial genome of *P. corethrurus* L1: In the context of our collaboration with Luis Cunha, we shared several unpublished information concerning the *P. corethtutus* complex and the occurrence of each cryptic species in different habitats. In this collaboration, I have participated in preparation of the paper entitled: The complete mitochondrial DNA sequence of the pantropical earthworm *Pontoscolex corethrurus* (Rhinodrilidae, Clitellata): Mitogenome characterization and phylogenetic positioning, which I have put in the Appendix of my thesis.

ZooKeys 688: 1–13 (2017)
doi: 10.3897/zookeys.688.13721
http://zookeys.pensoft.net

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The complete mitochondrial DNA sequence of the pantropical earthworm *Pontoscolex corethrurus* (Rhinodrilidae, Clitellata): Mitogenome characterization and phylogenetic positioning

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Academic editor: *F. Govedich* | Received 16 May 2017 | Accepted 17 July 2017 | Published 8 August 2017

http://zoobank.org/85EC475D-CAF6-455A-8B4F-A68EF853E5E9

Citation: Conrado AC, Arruda H, Stanton DWG, James SW, Kille P, Brown G, Silva E, Dupont L, Taheri S, Morgan AJ, Simões N, Rodrigues A, Montiel R, Cunha L (2017) The complete mitochondrial DNA sequence of the pantropical earthworm *Pontoscolex corethrurus* (Rhinodrilidae, Clitellata): Mitogenome characterization and phylogenetic positioning. ZooKeys 688: 1–13. https://doi.org/10.3897/zookeys.688.13721

Abstract

Pontoscolex corethrurus (Müller, 1857) plays an important role in tropical soil ecosystems and has been widely used as an animal model for a large variety of ecological studies, in particular due to its common presence and generally high abundance in human-disturbed tropical soils. In this study we describe the complete mitochondrial genome of the peregrine earthworm *P. corethrurus*. This is the first record of a mitochondrial genome within the Rhinodrilidae family. Its mitochondrial genome is 14 835 bp in length containing 37 genes (13 protein-coding genes (PCG), 2 rRNA genes and 22 tRNA genes). It has the same gene content and structure as in other sequenced earthworms, but unusual among invertebrates it has

several overlapping open reading frames. All genes are encoded on the same strand. Most of the PCGs use ATG as the start codon except for ND3, which uses GTG as the start codon. The A+T content of the mitochondrial genome is 59.9% (31.8% A, 28.1% T, 14.6% G, and 25.6% for C). The annotated genome sequence has been deposited in GenBank under the accession number KT988053.

Keywords
Pontoscolex corethrurus, mitochondria, mitochondrial genome, Rhinodrilidae, earthworm, Azores, peregrine species

Introduction

Excluding a few aquatic taxa, earthworms (Annelida: Clitellata) are mostly terrestrial and include ca. 5,500 species (Blakemore et al. 2006). Believed to have originated in the Guyana Shield (Righi 1984), the earthworm *Pontoscolex corethrurus* (Müller, 1857) is a globally distributed species found in most tropical regions. It mainly occurs in human-disturbed areas and can be used as an indicator of ecosystem disturbance (Brown et al. 2006), and is commonly used in ecotoxicological studies (e.g. Buch et al. 2011; Buch et al. 2013; Da Silva et al. 2016). The species formerly belonged in the Glossoscolecidae family, but was recently allocated to the Rhinodrilidae family by James (2012), following the phylogeny of James and Davidson (2012). It is also the most well-known earthworm species in the humid tropics, frequently used in ecological and agronomic studies (Bhattacharjee and Chaudhuri 2002; Buch et al. 2013; Chapuis-Lardy et al. 2010; Dupont et al. 2012; Hamoui 1991; Marichal et al. 2010). Being a geophagous endogeic species, *P. corethrurus* shows high plasticity regarding its tolerance to soil physicochemical characteristics, including variable moisture, high temperatures, exceptionally high carbon dioxide and low oxygen levels, and is capable of inhabiting nutrient-poor soils (Cunha et al. 2014; Hamoui 1991; Lavelle et al. 1987), as well as rotten logs (Buch et al. 2011).

Molecular data have become increasingly important in recent years. In animals, the mitochondrial DNA (mtDNA) typically contains 37 genes, encoding 13 proteins for the enzymes required for oxidative phosphorylation, the two ribosomal RNA units (rRNA), and 22 transfer RNAs (tRNAs) necessary for the translation of the proteins encoded by mtDNA (Anderson et al. 1981; Boore 1999; Zhao et al. 2015). Remarkable progress has been made over the past several years in the field of the molecular systematics of annelids. Compared with individual genes, the mitochondrial genome is still a promising tool for inferring phylogenetic relationships due to its high content of information, and has been applied in some phylogenetic studies involving earthworms (Zhang et al. 2015; Zhang et al. 2016b).

In this study, we sequenced the complete mtDNA sequence of *P. corethrurus* for the first time and analyzed its structure. Additionally, we conducted phylogenetic analyses based on the mitochondrial sequence data available elsewhere with the purpose of investigating the phylogenetic position of *P. corethrurus* within Clitellata. The information reported in this article will facilitate further investigations of phylogenetic relationships of different Annelida species.

Material and methods

Sample collection and DNA extraction

A group of clitellate (adult) *P. corethrurus* was collected in São Miguel Island (Azores, Portugal) inside pineapple greenhouses (Locality: Fajã de Baixo, 37°45'12.2"N, 25°38'21.3"W) during January 2015. Animals were euthanized in 10% ethanol and preserved in 96% ethanol for later work. A piece of body wall tissue was used for genomic DNA extraction using standard phenol/chloroform (Sambrook and Russell 2001) procedure followed by ethanol precipitation and kept at 4°C for subsequent use.

Mitochondrial DNA amplification

The complete *P. corethrurus* mitogenome was amplified using seven sets of primers (Table 1) designed based on sequences retrieved from a previous study (Cunha et al. 2014).

Long PCR targets were amplified using different combinations of the primer sets, and initially sequenced with the same forward or reverse primers. Subsequent primer walking method was used to close the sequencing gaps. To ensure the accuracy of the sequence, every two contiguous segments overlapped by at least 80 bp. PCRs were performed using ~40 ng of DNA and 0.4 µM forward and reverse primers, 0.2 mM dNTP mix and 1.25 U Platinum HiFi DNA polymerase buffered with 1X Mg-free buffer (Thermofisher Scientific, UK). PCR amplification buffer was supplemented with $MgCl_2$ to achieve a final concentration of 1.75 mM in a total volume of 25 µl reaction mixture. The reaction was denatured at 95°C for 3 min, cycled 35 times at 95°C for 30 s, 30 s at the required primer annealing temperature and 72°C for 1 min per 1000 bp depending on target fragment length. Negative controls were included in all PCR amplifications to confirm the absence of contaminants. Before sequencing, PCR cleanups were performed using Exo-SAP-IT (Amersham Pharmacia, UK) reagents. Exonuclease 1 (0.25 µl) and Shrimp Alkaline Phosphatase (0.5 µl) were mixed with the PCR product (10 µl) and incubated at 37°C for 45 min followed by 80°C for 15 min. DNA was sequenced using ABI PRISM® BigDye v3.1 Terminator Sequencing technology (Applied Biosystems, USA) on an ABI PRISM® 3100 DNA automated Sequencer.

Sequence editing and analysis

Sequence trace files were corrected and aligned with the MEGA v.7 (Kumar et al. 2016). The sequence overlap and mitogenome assembly were performed using CLC main workbench v.6 (Qiagen). The annotation of the 13 protein-coding genes and two rRNA genes were determined using the MITOS v.2 web server (Bernt et al. 2013) and

Table 1. Details of the primers used to amplify the mitochondrial DNA of *P. corethrurus*.

Primer code	Orientation	Annealing position (bp)	Nucleotide sequence (5'-3')	Melting Temperature (°C)
FP_1	Forward	2154..2175	CTCTACTATGTACCCAGGAGTG	57.46
RP_1	Reverse	2758..2775	GCGGCCAAGATAAAGCAC	57.67
RP_2	Reverse	3740..3762	TAGAGGCGGTAAGGAGAAAGTAT	58.61
RP_3	Reverse	5691..5708	CAGAGGCGAGGTAAATTC	53.85
RP_4	Reverse	6356..6373	TGTTCAGGGCTAGGATTG	54.99
FP_5	Forward	7983..8004	ACTAGTGTCACTTACAACAACC	57.16
RP_5	Reverse	8649..8670	TGATAAGGGGGAAAGTCTGATC	56.84
FP_6	Forward	8766..8787	AGTAGCCGCTATAATAGTCCTT	57.91
RP_6	Reverse	10328..10349	TGATTTGGGGTCAGAGCCGTAG	61.59
FP_7	Forward	10459..10478	AAAGCTTGGCGGTGCTTCAC	63.23
RP_7	Reverse	11242..11263	CCTAGTGTGTGTCAGGACGCTT	64.75

Table 2. Representative Clitellata species included in this study for comparison.

Species	Family	Length (bp)	GenBank accession number
Pontoscolex corethrurus	Rhinodrilidae	14,835	**Present study**
Tonoscolex birmanicus	Megascolecidae	15,170	KF425518
Amynthas gracilis	Megascolecidae	15,161	NC_027258
Duplodicodrilus schmardae	Megascolecidae	15,156	NC_029867
Metaphire guillemi	Megascolecidae	15,174	NC_029869
Perionyx excavatus	Megascolecidae	15,083	NC_009631
Lumbricus terrestris	Lumbricidae	14,998	NC_001673
Drawida japonica	Moniligastridae	14,648	NC_028050
Hirudo nipponia	Hirudinidae	14,414	NC_023776

manually curated using other published annelid mitogenomes as shown in Table 2, whereas the tRNA genes were identified using the program tRNAscan-SE 1.21 (Lowe and Eddy 1997). The annotated genome sequence was deposited in GenBank under accession number KT988053.

Phylogenetic analyses

To clarify the phylogenetic position of *P. corethrurus* within the Clitellata, the complete mitogenome sequences of eight representative Clitellata species (Table 2) were incorporated together with the presently obtained *P. corethrurus* mitogenome sequence for phylogenetic analysis. Phylogenetic analyses were based on 13 protein-coding genes and the two rRNA units, which were aligned separately using MEGA v.7 (Kumar et al. 2016) with minor manual adjustments and then concatenated. The possible bias of substitution saturation at each codon position of protein-coding genes and two rRNA genes was investigated using DAMBE v.4.5.57 (Xia and Xie 2001) and MEGA v. 7 (Kumar et al. 2016).

Two different methods, Bayesian inference (BI) and maximum likelihood (ML) were used to construct the phylogenetic tree. Bayesian analyses were undertaken with MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003) under the best-fit model of nucleotide evolution selected in MrModeltest v.2.3 (Nylander 2004), using the Assignment Index Criterion (AIC). Analyses were run for 1,000,000 generations, and sampled every 100 generations to assess convergence. Trees that produced non-stationary log-likelihood values were discarded as part of a burn-in procedure and combined the remaining trees that resulted in convergent log-likelihood scores from both independent searches. These trees were used to construct a consensus tree.

Maximum likelihood analysis (ML) was performed with MEGA v.7 (Kumar et al. 2016). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, alpha parameter = 0.9143)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 17.7596% sites). The analysis involved nine mitogenome sequences (Table 2). All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985).

Results and discussion

Mitochondrial genomic structure

The mitochondrial genome of *P. corethrurus* was determined to be 14 835 bp in length, comprising 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs), and one putative control region with a length of 318 bp (Figure 1).

The mitochondrial genome structure is detailed in Table 3. Gene order and orientation are similar to the previous earthworm mitochondrial genomes (Boore and Brown 1995; Zhang et al. 2015) but slightly smaller and more condensed (with several intergenic overlaps, see Table 3). The gene organization is similar to other earthworm species (e.g. *Lumbricus terrestris*: Boore and Brown 1995).

The nucleotide composition is asymmetric (31.9% A, 27.9% T, 14.9% G, and 25.3% for C) with an overall A+T content of 59.9%. One remarkable trait of metazoan mitogenomes is the strand-specific bias in nucleotide composition (Hassanin et al. 2005; Reyes et al. 1998). Such bias is measured as G/C-skew (G%-C%)/(G%+C%) and A/T-skew (A%-T%)/(A%+T%), respectively (Perna and Kocher 1995). The overall GC- and AT-skews of the H-strand of *P. corethrurus* mitogenome were -0.258 and 0.066, respectively, indicating a compositional bias associated with an excess of C over G nucleotides and a slight excess of A over T nucleotides on the H-strand.

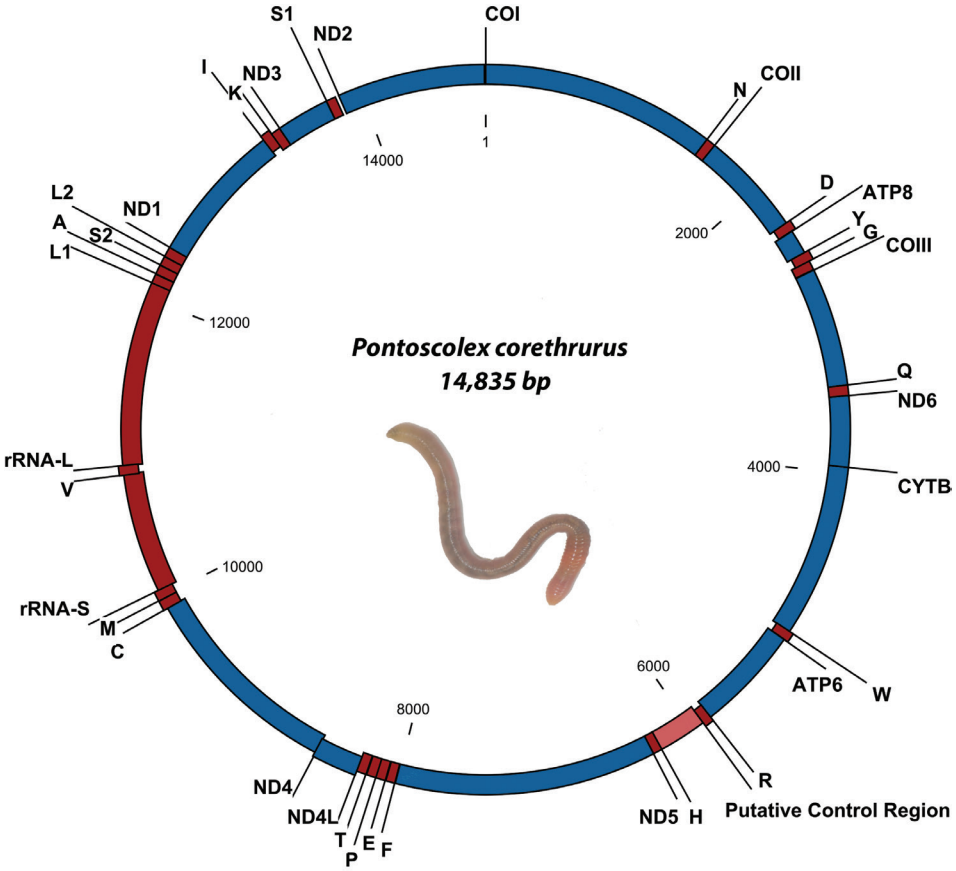

Figure 1. The mitochondrial genome of *Pontoscolex corethrurus* (Müller, 1857). Gene order and positions are shown, including the putative control region. IUPAC single letter codes are used to identify transfer RNA. The L1, L2, S1, and S2 transfer RNAs are differentiated on the basis of their anti-codons TAG, TAA, TCT, and TGA, respectively.

Protein-coding genes

The *P. corethrurus* genome contained the expected 13 protein-coding genes with a total of 11,131 bp in size, accounting for 75.03% of the whole mitogenome. Most of the PCGs are initiated with ATG codons, except for ND3 gene, which uses GTG as the initiation codon. Six PCGs (COX1, ATP8, COX3, ND6, ND3, and ND2) are terminated with an incomplete codon T or TA, which could be completed to TAA by polyadenylation post-transcriptionally (Ojala et al. 1981). COX2 and ND1 use TAG as a termination codon.

Nucleotide composition and codon usage frequencies were calculated from a concatenated sequence of all protein-coding genes on the H-strand. The base composition of protein-coding genes revealed a negative bias for A (14.4%), especially at second codon positions (12.9%, Table 4). For all protein genes, T was the most frequent nucleotide at the first and third positions whereas G was most frequent at the second position.

Table 3. Organisation and structure of the *P. corethrurus* mitochondrial genome.

Gene	Direction	From	To	Size (bp)	Start	Stop	Anti-codon	Intergenic bases (bp)
COX1	+	1	1540	1540	ATG	T--		0
tRNA-^{Asn}	+	1541	1602	62			GTT	0
COX2	+	1603	2289	687	ATG	TAG		-1
tRNA-^{Asp}	+	2289	2351	63			GTC	2
ATP8	+	2354	2513	160	ATG	T--		0
tRNA-^{Tyr}	+	2514	2576	63			GTA	-1
tRNA-^{Gly}	+	2576	2638	63			TCC	3
COX3	+	2642	3419	778	ATG	T--		0
tRNA-^{Gln}	+	3420	3488	69			TTG	0
ND6	+	3489	3954	466	ATG	T--		0
Cytb	+	3955	5094	1140	ATG	TAA		-2
tRNA-^{Trp}	+	5092	5154	63			TCA	1
ATP6	+	5156	5851	696	ATG	TAA		-2
tRNA-^{Arg}	+	5850	5910	61			TCG	0
Putative Control Region	+	5911	6228	318				
tRNA-^{His}	+	6229	6288	60			GTG	0
ND5	+	6289	8010	1722	ATG	TAA		3
tRNA-^{Phe}	+	8014	8073	60			GAA	4
tRNA-^{Glu}	+	8078	8141	64			TTC	0
tRNA-^{Pro}	+	8142	8204	63			TGG	4
tRNA-^{Thr}	+	8209	8272	64			TGT	0
ND4L	+	8273	8569	297	ATG	TAA		-7
ND4	+	8563	9921	1359	ATG	TAA		-2
tRNA-^{Cys}	+	9920	9986	67			GCA	1
tRNA-^{Met}	+	9988	10050	63			CAT	-1
s-rRNA	+	10050	10838	789				-7
tRNA-^{Val}	+	10832	10894	63			TAC	-2
l-rRNA	+	10893	12104	1212				0
tRNA-$^{Leu\ 1}$	+	12105	12166	62			TAG	2
tRNA-^{Ala}	+	12169	12230	62			TGC	1
tRNA-$^{Ser\ 2}$	+	12232	12293	62			TGA	1
tRNA-$^{Leu\ 2}$	+	12295	12360	66			TAA	0
ND1	+	12361	13290	930	ATG	TAG		-1
tRNA-^{Ile}	+	13290	13354	65			GAT	0
tRNA-^{Lys}	+	13355	13417	63			TTT	0
ND3	+	13418	13770	353	GTG	TA-		-1
tRNA-$^{Ser\ 1}$	+	13770	13832	63			TCT	0
ND2	+	13833	14835	1003	ATG	T--		0

Ribosomal and transfer RNA genes

Like other mitochondrial genomes (Inoue et al. 2000; Zardoya et al. 1995), twenty-two tRNA genes were identified (Supplementary figure 1). The tRNA genes were scattered throughout the mitochondrial genome and ranged in size from 60 to 67 bp

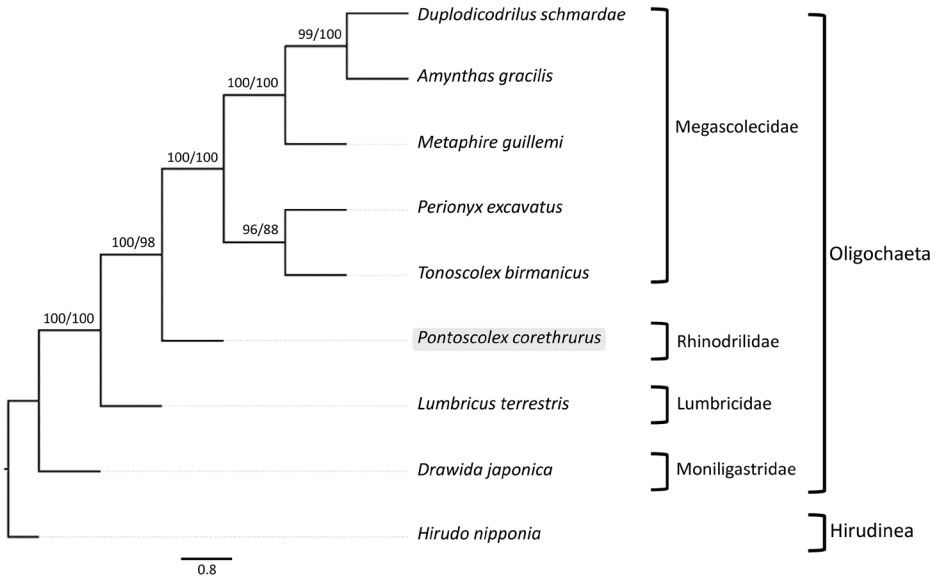

Figure 2. Phylogenetic relationships among phylum Annelida based on the combined 13,416 bp nucleotide positions. Total alignment length is greater than the combined *P. corethrurus* protein coding and rRNA sequence lengths due to overlapping protein coding sequences that are subsequently concatenated, and indel regions in the alignment. The posterior probability value of BI analyses and bootstrap support values of ML analyses (in the order: BI, ML) are indicated near the branches.

(Table 3). The *P. corethrurus* mitogenome also contained a small subunit of rRNA and a large subunit of rRNA, which were 789 bp and 1212 bp in length, respectively. As in other Clitellata genomes, these genes were located between the $tRNA^{Met}$ and $tRNA^{Val}$ genes and between $tRNA^{Val}$ and $tRNA^{Leu}$ genes, respectively (Zhang et al. 2016b).

Non-coding regions

As shown in Table 3, there are 22 intergenic spacer regions, ranging in size from -7 to 4 bp observed in *P. corethrurus*.

As in most Clitellata, the major non-coding region in *P. corethrurus* mitochondrial genome was located between tRNA-^{Arg} and tRNA-^{His}. It was determined to be 318 bp in length, less than other reported Clitellata species (Zhang et al. 2016a), and it had a base composition that was rich in A and T (A+T=67.6%).

Phylogenetic analyses within the Clitellata

The phylogenetic trees (the 50% majority-rule consensus tree is shown in Figure 2) were highly consistent regardless of the analytic method used, and were statistically

Table 4. Base composition for protein-coding, tRNA, and rRNA genes of *P. corethrurus* mitogenome.

Gene/Region	Base composition (%)				A+T (%)	Size (bp)
	T	C	A	G		
COX1	27.4	26.7	27.9	18.1	55.3	1,540
COX2	25.8	24.3	34.9	15.0	60.7	687
ATP8	24.4	26.9	36.9	11.9	61.3	160
COX3	27.3	27.5	25.7	19.5	53.0	778
ND6	28.3	26.0	30.3	15.5	58.6	466
Cytb	28.0	27.1	29.7	15.3	57.6	1,140
ATP6	29.6	29.2	30.6	10.6	60.2	696
ND5	27.7	26.7	31.7	13.9	59.4	1,722
ND4L	25.9	28.3	33.0	12.8	58.9	297
ND4	27.5	27.5	32.3	12.7	59.8	1,359
ND1	28.4	25.8	29.8	16.0	58.2	930
ND3	32.6	26.1	27.2	14.2	59.8	353
ND2	30.0	28.2	30.7	11.1	60.7	1,003
Protein Coding						
1st	30.2	25.4	17.4	27.0	47.6	3,710
2st	24.9	27.6	12.9	34.6	37.8	3,710
3st	36.1	27.9	13.8	22.3	49.8	3,710
Total	30.4	27.0	14.7	28.0	45.1	11,131
tRNA	30.5	17.6	34.9	17.0	65.5	1,391
rRNA	24.1	22.0	37.6	16.3	61.7	2,001
Putative Control Region	31.5	18.9	36.2	13.5	67.6	318
Overall	28.1	25.6	31.8	14.6	59.9	14,835

supported by high posterior probability and intermediate bootstrap values. This phylogenetic analysis represented the first investigation of *P. corethrurus* relationships within the Clitellata based on the complete mitogenome. As indicated by the tree, different species from the same family clustered together (Megascolecidae: *M. guillemi, D. schmardae, A. gracilis, P. excavatus and T. birmanicus*), and the species from Lumbricidae and the *P. corethrurus* formed a monophyletic group. The species *D. japonica* belongs to the Moniligastridae, the sister group to Crassiclitellata (earthworms), which explains its phylogenetic position. The Moniligastridae are not Crassiclitellata because they have a single cell layer in the clitellum.

Conclusion

For the first time, the sequencing, annotation and analysis of the mitochondrial genome of a member of Rhinodrilidae was completed. The mitogenome of *P. corethrurus* was found to be 14,835 bp in length and showed a similar composition in size, low GC content and gene order to earthworm mitogenomes already available. The complete mitogenome reported here is expected to allow for further studies of the *P. corethrurus* phylogeny and for analyses on the taxonomic status of the family Rhinodrilidae.

Declaration of interest section

The authors report no conflicts of interest and are responsible for the content and writing of the paper.

Acknowledgments

We thank Jose Talavera for the taxonomic identification of the studied specimen. This study was financially supported by the Portuguese Government (FCT project PTDC/AAC-AMB/ 115713/2009). Luis Cunha was supported by an EU Marie Curie fellowship - MSCA-IF-2014-GF-660378. George Brown, Elodie da Silva acknowledge fellowship support by CNPq. Ana Caroline Conrado was supported by a scholarship funded by CAPES. Sam James was supported by USA NSF award DEB-1136604. Dave Stanton was supported by a NERC grant (NE/M017656/1).

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Supplementary material 1

Inferred secondary structure of 22 tRNA genes in the mitochondrial DNA of the pantropical earthworm *Pontoscolex corethrurus* (Rhinodrilidae, Clitellata).

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Data type: molecular data

Link: https://doi.org/10.3897/zookeys.688.13721.suppl1

VIII. References (out of chapters)

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IX. List of figures and tables

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X. *Thèse en Français : Processus macro- et micro-évolutifs au sein d'un complexe d'espèces, cas d'étude de l'espèce tropicale et invasive de vers de terre ;* **Pontoscolex corethrurus**

Résumé :

Pontoscolex corethrurus est le ver de terre le plus répandu dans les zones tropicales et sub-tropicales ; il est par conséquent très étudié en science du sol. Il est présent dans toutes sortes d'habitats, des sols pauvres de prairie aux sols riches de forêt primaire, et ses caractéristiques écologiques sont bien connues. Ses caractéristiques biologiques ont été moins étudiées. Peu de données sur la variation génétique au sein de cette morphoespèce sont disponibles à l'exception de la découverte en 2014 de deux lignées génétiquement différentes dans l'île São Miguel des Açores. De plus, son degré de ploïdie n'est pas connu et sa stratégie de reproduction n'est pas bien décrite. L'un des objectifs de cette thèse était de comprendre les mécanismes et les caractéristiques qui font de *P. corethrurus* un envahisseur efficace. Notre deuxième objectif était de rechercher des lignées cryptiques dans le monde entier et de décrire leurs relations phylogénétiques. Un troisième objectif était d'identifier quelle lignée était invasive et de caractériser la structure génétique de ses populations dans les aires native et d'introduction. Le dernier objectif était de tester si les différentes espèces du complexe avaient différents degrés de ploïdie, ce qui pourrait expliquer l'isolement reproducteur entre ces espèces. Une synthèse bibliographique de 265 études couvrant tous les aspects des connaissances sur *P. corethrurus* a montré que la stratégie r et la plasticité de ce ver sont les caractéristiques clefs qui lui permettent d'envahir avec succès différents habitats. Afin d'étudier la diversité cryptique au sein de *P. corethrurus* à une échelle mondiale, j'ai examiné 792 spécimens collectés dans 25 pays et îles différents. Ces spécimens ont été analysés à l'aide de deux marqueurs mitochondriaux (COI et ADNr 16S), deux marqueurs nucléaires (internal transcribed spacers 2 et ADNr 28S) et une matrice de données de séquence multilocus obtenue à l'aide de la méthode AHE (Anchored Hybrid Enrichment). De plus, un total de 11 caractères morphologiques, internes comme externes, ont été étudiés dans toutes les lignées caractérisées génétiquement. Quatre espèces cryptiques (L1, L2, L3 et L4) ont été observées au sein du complexe d'espèces *P. corethrurus*. Elles ont été trouvées en sympatrie dans plusieurs localités et des analyses basées sur des marqueurs AFLP n'ont pas montré d'hybridation entre L1 et L3. La possibilité d'isolement reproducteur lié à des degrés de ploïdie différents a été évaluée à l'aide d'expérimentations de cytogénétique pour lesquelles plusieurs obstacles ont été rencontrés, à différentes étapes. Des résultats n'ont été obtenus que pour L4 (2n = 70). L'une des espèces du complexe, L1, était géographiquement répandue. Cette espèce correspondait aux spécimens topotypiques (échantillons provenant du jardin de Fritz Müller où *P. corethrurus* a été décrit en premier en 1856). Nous avons ciblé cette espèce invasive dans une étude de génétique des populations et de phylogéographie. En utilisant le gène COI et des marqueurs AFLP, nous avons révélé une faible diversité génétique dans la zone tropicale, probablement due à des évènements de colonisation récents et à une reproduction asexuelle. Cependant, la diversité génétique relativement élevée dans certaines populations, associée à un déséquilibre de liaison relativement faible, suggère aussi des évènements de reproduction sexuelle. A ce jour, c'est le premier travail réalisé à l'échelle mondiale sur la diversité génétique cryptique, la génétique des populations et la phylogéographie d'une espèce de vers de terre pérégrine dans la zone tropicale. J'ai produit la première revue complète des caractéristiques de *P. corethrurus*. De plus, son statut taxinomique a été clarifié ainsi que sa stratégie de reproduction qui est mixte (parthénogénèse et amphimixie). Ces informations seront utiles pour les expérimentations et les recherches futures sur les espèces du complexe *P. corethrurus*.

Contexte :

Les espèces invasives sont souvent définies comme des espèces qui franchissent une barrière géographique et colonisent des milieux en dehors de leur zone d'origine. Dans cette thèse, la définition d'une invasion biologique qui sera utilisée a été proposée par Valéry et al., (2008): "une invasion biologique consiste en l'acquisition d'un avantage compétitif par une espèce, suite à la disparition des obstacles naturels à sa prolifération, ce qui lui permet de se propager rapidement et de conquérir de nouvelles zones". Le processus d''invasion par une espèce peut se décomposer en trois étapes principales : 1) l'introduction d'une espèce dans un nouvel habitat, 2) la colonisation initiale et l'installation et 3) la dispersion et la propagation secondaire dans de nouveaux habitats. Au cours des différentes étapes de ce processus, la dispersion passive par différents vecteurs tels que l'homme, l'eau et les autres animaux joue un rôle important. La colonisation initiale par une espèce est favorisée par des types de reproduction uniparentale qui permettent l'établissement d'une nouvelle population sans avoir besoin d'un partenaire pour l'accouplement, comme l'autofécondation et la parthénogénèse. Une autre qualité importante d'une espèce invasive est la stratégie r (c'est-à-dire un temps de génération court, une fécondité élevée, et des taux de croissance élevés). De plus, le succès de l'installation d'une espèce dans une nouvelle aire géographique est étroitement lié à la plasticité phénotypique et à la capacité compétitive. Ces capacités sont importantes pour faire face aux différentes conditions environnementales et pour concurrencer d'autres espèces, en particulier les espèces natives.

Les invasions dans les milieux souterrains ont peu attiré l'attention, principalement en raison de la nature cryptique de cet environnement. Les vers de terre sont connus pour leur rôle d'ingénieurs du sol, c'est-à-dire qu'ils peuvent moduler directement ou indirectement la disponibilité des ressources des autres espèces de l'écosystème du sol, du fait de leurs activités. Lorsque des espèces de vers de terre exotiques sont introduites dans un nouvel habitat, elles peuvent altérer la structure du sol, la matière organique, le cycle des éléments nutritifs, les communautés végétales et animales du sol, et remplacer les espèces indigènes. La majorité des espèces de vers de terre pérégrines (espèces avec une répartition globale, ayant été dispersées par l'homme) connues appartient à la famille des Lumbricidae, qui compte 30 espèces pérégrines. Ces espèces sont principalement étudiées dans les régions tempérées. Malgré l'existence d'un plus grand nombre d'espèces en zone tropicale, la proportion de nos connaissances sur les espèces de vers de terre pérégrines dans les zones tempérées est beaucoup plus élevée (45 espèces pérégrines sur 500-600 espèces estimées) que dans les régions tropicales (51 espèces pérégrines sur plusieurs milliers d'espèces estimées). De plus, plusieurs espèces cryptiques (c'est-à-dire, des espèces morphologiquement indiscernables) ont été révélées par les approches moléculaires chez les vers de terre. Etant donné que la notion d'«espèce » est théorique et peut être parfois difficilement applicable, De Queiroz (2007) a suggéré que, lorsqu'il y a débat sur le dénombrement des espèces d'un même genre, nous sommes dans la « zone grise » du processus de spéciation (processus par lequel une espèce se divise en deux espèces ou plus). L'approche de la taxonomie intégrative permet de trouver la congruence entre différentes disciplines pour estimer le nombre d'espèces

dans la « zone grise ». Il est important de distinguer les espèces cryptiques, car elles influencent différemment le milieu et réagissent différemment aux pressions environnementales. Par exemple, chez les vers de terre, la tolérance à la pollution, la période de reproduction, et le régime alimentaire varient entre les espèces. Les vers de terre sont souvent utilisés comme modèles biologiques dans des études scientifiques (écotoxicologiques, bioindicateurs du sol, etc.), par conséquent, les résultats pourraient être biaisés si les expérimentations sont effectuées sur plusieurs espèces ou lignées sans que les chercheurs n'en aient conscience.

Modèle d'étude :

Pontoscolex corethrurus est l'espèce de vers de terre la plus répandue dans les zones tropicales et sub-tropicales. Elle est également l'une des plus étudiées en science du sol et il existe un grand nombre d'études dans différentes disciplines sur cette morphoespèce. *P. corethrurus* fait partie de la famille des Rhinodrilidae et appartient au genre *Pontoscolex* où 20 espèces ont été décrites. Le genre *Pontoscolex* proviendrait du plateau des Guyanes, où une grande diversité d'espèces a été observée. Cette morphoespèce est connue pour être présente dans un large éventail d'habitats, allant des sols de pâturage pauvres aux sols riches des forêts primaires. En 2014, deux lignées cryptiques ont été trouvées chez *P. corethrurus* sur l'île de São Miguel dans l'archipel des Açores. Une de ces lignées a été trouvée dans une plantation d'ananas et l'autre dans un environnement hostile sur une caldeira volcanique (Furnas). En tant qu'espèce invasive, aucune synthèse n'existe sur ses impacts sur l'environnement ou le type d'environnements qu'elle envahit. Au vu de la détection des lignées cryptiques et considérant que certaines différences pourraient potentiellement s'expliquer par l'existence de plusieurs espèces et non une seule, une telle synthèse apparaît nécessaire. De plus, malgré un nombre élevé d'études sur les aspects écologiques, peu d'études biologiques ont été réalisées à ce jour sur cette morphoespèce, donc aucune information n'est disponible sur le degré de ploïdie, la stratégie de reproduction et le placement phylogénétique de *P. corethrurus*. Cette thèse, ainsi que la publication du génome mitochondrial de *P. corethrurus* par Conrado et al. (2017 , Annexe 1), contribuent à combler les lacunes des connaissances sur cette morphoespèce.

Objectifs de thèse :

Dans cette thèse, j'ai cherché à comprendre quels mécanismes et caractéristiques sont responsables du succès de l'invasion de l'espèce de vers de terre invasive et pantropicale *Pontoscolex corethrurus.* Dans le même temps, comme deux lignées cryptiques ont déjà été trouvées chez *P. corethrurus,* j'ai cherché à savoir s'il existe encore plus d'espèces cryptiques dans la zone tropicale. Ensuite, j'ai cherché à déterminer si seulement une de ces lignées est invasive ou si plusieurs le sont. J'ai également voulu comprendre les routes d'invasion et la structure génétique de(s) l'espèce(s) invasive(s) dans le complexe *P. corethrurus* dans différentes

populations à travers le monde. Soulignons que ces questions ont rarement été étudiées pour les espèces de vers de terre pérégrines et tropicales. Enfin, je me suis intéressée à la question de l'existence d'un « complexe polyploïde », chez le complexe de *P. corethrurus,* c'est-à-dire un complexe d'espèces ayant des degrés de ploïdie différents. Ces objectifs ont été développés à travers quatre chapitres.

Résumés des chapitres de thèse :

Chapitre 1: Comment définir les impacts «positifs» et «négatifs» des espèces invasives?

Les vers de terre invasifs sont souvent considérés comme nuisibles pour l'environnement, en ayant des impacts négatifs sur l'écosystème et les espèces natives. Ce chapitre synthétise 265 études portant sur tous les aspects actuels de notre connaissance sur l'espèce de vers de terre invasive *P. corethrurus* depuis 1857 à 2017 : sa distribution, sa morphologie, ses caractéristiques biologiques et écologiques, ainsi que ses impacts sur les caractéristiques du sol et des communautés. Les résultats soulignent que les principaux traits écologiques expliquant le succès de l'invasion de *P. corethrurus* sont la reproduction par parthénogénèse et la stratégie r. Pour s'établir dans un nouvel environnement, les principaux atouts de cette espèce sont la plasticité phénotypique et la compétitivité. D'autre part, sa présence a des effets aussi bien négatifs que positifs sur l'écosystème qu'elle envahit en fonction des caractéristiques propres à l'environnent (pH du sol, espèces natives, espèces de plantes, et type du sol). Dans ce chapitre, nous soulignons le fait que les données sur les interactions de *P. corethrurus* avec la macrofaune du sol, autre que les vers de terre, sont rares. Enfin, les éléments disponibles n'ont pas permis de déterminer si les différentes études de la synthèse avaient pu être réalisées sur différentes lignées cryptiques. Ce chapitre a fait l'objet d'un article qui a été publié dans la revue Soil Biology and Biochemistry en janvier 2018.

Chapitre 2: Y a-t-il plusieurs espèces invasives dans un complexe d'espèces envahissantes?

Bien que les interactions écologiques de *P. corethrurus* avec son environnement soient bien documentées, le statut taxonomique de l'espèce reste incertain. Dans ce deuxième chapitre, nous avons étudié les relations phylogénétiques au sein du genre *Pontoscolex,* en se concentrant sur les lignées morphologiquement indiscernables (c'est-à-dire, cryptiques). Un total de 792 échantillons prélevés dans 25 pays et îles du monde ont été analysés en utilisant deux marqueurs mitochondriaux (COI et 16S ADNr) et deux marqueurs nucléaires (ITS2 et 28S ADNr). Onze caractères morphologiques internes et externes ont été étudiés dans toutes les lignées génétiquement caractérisées. Une matrice de données de séquences multi-locus à grande échelle a également été obtenue pour *Pontoscolex* spp., en utilisant la méthode «Anchored Hybrid Enrichment» (AHE). Les analyses phylogénétiques et phylogénomiques multi-locus, combinées à des analyses de délimitation d'espèces par les approches mono-locus (mPTP, ABGD) et multi-

locus (BPP), ont révélé des résultats congruents. Quatre espèces cryptiques ont été trouvées dans le complexe d'espèces *P. corethrurus*, ainsi que quatre espèces potentiellement nouvelles dans le genre *Pontoscolex*. Une lignée très répandue (L1), dans le complexe de *P. corethrurus,* a été observée dans la population actuelle du jardin de Fritz Müller où l'espèce a été décrite pour la première fois en 1856. Des lignées cryptiques ont été observées en sympatrie à plusieurs endroits. Ceci, en plus de l'hétéroplasmie observée dans le gène COI dans une population à Taïwan, soulève la question de l'isolement reproductif entre les espèces cryptiques. Ce chapitre a fait l'objet d'un article qui a été accepté pour publication sous réserve de révisions mineures dans la revue Molecular Phylogenetics and Evolution le 11 décembre 2017.

Chapitre 3: Phylogéographie et génétique des populations d'une espèce invasive de vers de terre pérégrine

Dans le chapitre 2, quatre espèces cryptiques ont été mises en évidence dans le complexe de *P. corethrurus* (L1, L2, L3 et L4). Dans 7 populations sur les 116 étudiées certaines de ces espèces ont été observées en sympatrie. De plus, le chapitre 2 a montré que *P. corethrurus* L1 est particulièrement répandu dans le monde, ce qui suggère de bonnes capacités d'invasion chez cette espèce. Dans ce chapitre 3, nos objectifs ont été d'étudier l'hybridation entre les espèces cryptiques vivant en sympatrie et de réaliser une étude phylogéographique à grande échelle pour *P. corethrurus* L1. Les hypothèses testées étaient que (i) un "super-clone" a colonisé une grande diversité de milieux dans le monde, (ii) la diversité clonale est plus faible dans la zone d'introduction que dans la zone native, et (iii) L1 a une stratégie de reproduction mixte. Cette étude a été menée à partir d'échantillons provenant de 16 pays de la zone (sub)tropicale: Brésil, Pérou, Guyane française, Trinité-et-Tobago, Mexique, Sainte-Lucie, Saint-Vincent, Martinique, Açores, Gabon, Madagascar, Thaïlande, Malaisie, Taïwan, Philippines et Hawaï. L'admixture génétique au sein de trois populations où L1, L3 et L4 ont été détectées en sympatrie, a été analysée en utilisant 57 profils AFLP. De plus, des analyses de la variation génétique des populations L1 de *P. corethrurus* ont été effectuées en utilisant à la fois des informations génétiques nucléaires (226 profils d'AFLP) et mitochondriales (479 séquences COI). Aucune preuve d'hybridation entre les espèces n'a été obtenue. Au sein de l'espèce L1, une faible diversité génétique a été révélée pour COI, avec un haplotype majeur (H1) partagé par 72% des spécimens. Une longue histoire d'invasion et de multiples réintroductions de spécimens provenant de différentes parties du monde peuvent expliquer le niveau de diversité génétique similaire entre les régions d'introduction et la région d'origine. Bien que les AFLPs aient permis d'identifier un nombre significatif de clones dans certaines populations, aucun clone n'a été trouvé dans plusieurs populations. Le niveau relativement élevé de diversité clonale a donc permis de rejeter l'hypothèse d'invasion par un « super-clone ». L'absence de clones, la diversité génétique élevée et de faibles valeurs de déséquilibre gamétique ont été observées dans plusieurs populations. Ces résultats suggèrent des évènements de recombinaison dans certaines populations et par conséquent une stratégie de

reproduction mixte (parthénogénèse et reproduction sexuée) semble probable chez *P. corethrurus* L1.

Chapitre 4: Degré de ploïdie des spécimens du complexe *Pontoscolex corethrurus*

Connaître le degré de ploïdie et le nombre de chromosomes d'un organisme est indispensable avant d'entreprendre des études empiriques en écologie et en biologie. Le caryotype et le degré de ploïdie sont déjà connus pour plusieurs espèces de vers de terre. Cependant, cette information manque encore pour certaines espèces, en particulier celles utilisées fréquemment comme modèles biologiques en écotoxicologie, biorémédiation du sol, ou comme bioindicateur de la qualité du sol, comme c'est le cas pour la morphoespèce *Pontoscolex corethrurus*. Dans le deuxième chapitre, nous avons montré que cette morphoespèce correspond en réalité à un complexe de quatre espèces cryptiques. L'étude présentée dans ce quatrième chapitre s'est attachée à développer un protocole de caryotypage fonctionnant sur des spécimens du complexe *P. corethrurus*, afin d'étudier les degrés de ploïdie des espèces du complexe et de tester l'hypothèse d'un complexe polyploïde. En effet, des degrés de ploïdie différents parmi les espèces d'un même complexe pourraient expliquer l'isolement reproductif entre ces espèces. Nous avons déterminé le degré de ploïdie de *P. corethrurus* L4, avec 2n = 70 chromosomes. La spermatogenèse a également été observée pour cette espèce du complexe, suggérant la reproduction sexuée chez cette espèce. En raison de différents problèmes rencontrés lors des expérimentations cytogénétiques, nous ne sommes pas parvenus à déterminer le degré de ploïdie des autres espèces du complexe. Ce chapitre, bien qu'il soit structuré comme un article scientifique, ne sera pas soumis à publication, à cause du peu de résultats positifs.

Conclusion et perspectives générales :

Cette thèse constitue le premier travail alliant phylogénétique, phylogéographie, génétique des populations et cytogénétique pour étudier une espèce de vers de terre invasive et pérégrine sur un grand nombre de pays dans la zone tropicale et subtropicale. Elle a permis de mettre en évidence l'existence de quatre nouvelles espèces dans le genre *Pontoscolex*, ainsi que l'existence de quatre espèces cryptiques dans le complexe de *P. corethrurus* (L1, L2, L3 et L4). L'isolement reproductif dans le complexe a été étudié entre *P. corethrurus* L1 et L3 (en raison d'un manque d'individus, il n'a pas été possible de l'étudier pour *P. corethrurus* L2 et L4). Les résultats ont montré l'absence du flux de gènes, indiquant qu'il n'y a pas d'hybridation entre ces deux espèces. On peut donc supposer qu'elles ont passé la « zone grise » dans le processus de spéciation, ce qui confirme la distinction en deux espèces. Dans cette thèse, on a également pu répondre à une question rarement posée : chez une morphoespèce pérégrine, où des espèces cryptiques ont été trouvées grâce aux outils moléculaires, est-ce qu'une seule espèce est invasive, plusieurs ou toutes ? Dans le cas présent, *P. corethrurus* L1 semble la seule espèce invasive dans le complexe. Les raisons du succès

de son invasion pourraient être la plasticité, la compétitivité, la stratégie r et un régime mixte de reproduction. En effet, la forte diversité génétique observée dans certaines populations et les valeurs faibles de déséquilibre de liaison ont suggéré la reproduction sexuée chez cette espèce invasive. Elle serait donc capable de reproduire da façon sexuée et par parthénogénèse. On peut formuler l'hypothèse que, dans les premières étapes de la colonisation, la stratégie de reproduction employée serait la parthénogénèse, alors que dans les populations installées depuis longtemps, on peut proposer que la reproduction soit mixte, avec une parthénogénèse facultative.

Un autre objectif a été de tester l'hypothèse de « complexe de polyploïdie », mais divers obstacles rencontrés lors des expérimentations cytogénétiques n'ont pas permis de confirmer cette hypothèse. La seule espèce dont le degré de ploïdie a pu être déterminé est *P. corethrurus* L4, pour lequel il est égal à 2n=70. De plus, chez cette espèce, la preuve de spermatogénèse suggère la reproduction sexuée. La question reste donc ouverte, et il serait intéressant d'examiner plus de spécimens pour vérifier cette hypothèse. Un de nos collaborateurs au Brésil, Luis Cunha, s'est aussi intéressé au degré de ploïdie dans le complexe de *P. corethrurus* et, par la méthode de cyrtométrie en flux, il a confirmé la diploïdie de *P. corethrurus* L4. Il a aussi mis en évidence que *P. corethrurus* L1 est diploïde, Ses résultats ont été obtenus sur des spécimens provenant des populations d'une plantation d'ananas et de la caldera du volcan Furnas, aux Açores.

Dans la littérature, il est suggéré que le plateau des Guyanes soit l'aire native du genre *Pontoscolex*. En revanche, dans notre étude de génétique des populations, des niveaux similaires de diversité génétique ont été observés dans les populations de l'aire native et celles introduites, ne permettant pas de confirmer cette hypothèse. La difficulté à distinguer aire native et aire introduite à partir de données génétiques pourrait également s'expliquer par l'histoire ancienne de colonisation de cette espèce dans le monde entier, avec de nombreuses réintroductions par l'homme. De ce fait, les routes d'invasion de l'espèce sont impossibles à retracer.

En comparant un plus grand nombre de populations et de spécimens, on pourrait imaginer trouver plus d'espèces cryptiques, et de trouver plus de variations génétiques entre les populations à travers le monde entière.

Abstract

Pontoscolex corethrurus is the most widespread earthworm species in the tropical and sub-tropical zones, it is hence one of the most studied earthworm in soil science. Ecological aspects of *P. corethrurus*, which is known to be present in a wide range of habitats from poor soils of pasture to rich soils of primary forest, were intensively investigated but biological aspects are less addressed. In particular, information on the genetic variation within the morphospecies is scarce except for the finding of two genetically differentiated lineages in São Miguel Island of Azores archipelago in 2014. Moreover, the ploidy degree of the morphospecies is not yet known and its reproduction strategy is not well understood. One of the objectives of this thesis was to understand the mechanisms and characteristics which make *P. corethrurus* a successful invader. Our second objective was to look for cryptic lineages in the whole world and to describe the phylogenetic relationships between them. A third objective was to identify which lineage was invasive and to characterize its population genetic structure in the native and the introduced ranges. The last objective was to test if the different species of the complex have different ploidy degrees (polyploid complex). This could eventually explain the reproductive isolation among these species. A bibliographic synthesis of 265 studies covering all subjects of knowledge on *P. corethrurus* showed that the r strategy and plasticity of this earthworm are the key characteristics which make it a successful invader in different habitats. In order to investigate the cryptic diversity within *P. corethrurus* in a world-wide scale, I examined 792 specimens collected from 25 different countries and islands. These specimens were analyzed using two mitochondrial (COI and 16S rDNA) and two nuclear (internal transcribed spacers 2 and 28S rDNA) markers and a large-scale multilocus sequence data matrix obtained using the Anchored Hybrid Enrichment (AHE) method. In addition, a total of 11 morphological characters, both internal and external, were investigated in all genetically characterized lineages. Four cryptic species (L1, L2, L3 and L4) were found within the *P. corethrurus* species complex, and four potentially new species within the genus *Pontoscolex*. The cryptic species were observed in sympatry at several localities, and analyses based on AFLP markers showed no hybridization among L1 and L3. The possibility of reproductive isolation among species of the complex because of different ploidy degrees was investigated by cytogenetic experimentations. Due to different obstacles encountered at different steps of the experimentations, results were just obtained for L4 (2n=70). One of the species belonging to the complex, L1, was particularly widespread per comparison with the others. This species corresponded to topotype specimens (samples from Fritz Müller's garden where *P. corethrurus* was first described in 1856). Thus, we focused on this invasive species in a population genetics and phylogeography study. Using COI gene and AFLP markers, we revealed low genetic diversity through the tropical zone, probably due to recent colonization events and asexual reproduction type. Meanwhile, due to weak linkage disequilibrium and relatively high genetic diversity in some populations, sexual reproduction was suggested for L1. To date, this is the first study investigating at a world-wide scale, cryptic species diversity, population genetics and phylogeography of a peregrine earthworm species throughout tropical zone. I produced the first comprehensive review of all ecological and biological aspects of *P. corethrurus*. Moreover, the taxonomic status of *P. corethrurus* was clarified as well as its reproduction strategy which is mixed (parthenogenetic and sexual). All these findings represent potentially useful information for future experimentations and researches on species of *P. corethrurus* complex.