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Effects of the Extreme Environment of Volcanic Origin in Organisms

Using Earthworms as Biological Models

Dissertação apresentada na Universidade dos Açores para obtenção do grau de Doutor em Biologia na especialidade de Biologia Celular e Molecular

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By

Luís Cunha

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Governo dos Açores SRCTE DRCTC PROEMPREGO FCT Fundação para a Ciência e a Tecnologia

"It may be doubted whether there are many other animals which have played so important a part in the history of the world, as have these lowly organized creatures."

In the “The Formation Of Vegetable Mould”

by Charles Darwin 1881

Para ti Mia…

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É com enorme prazer que vejo este trabalho chegar ao fim, com ele encerro um grande capítulo da minha vida e com certeza um dos mais importantes. Dado o momento não poderia deixar de sublinhar umas breves palavras de agradecimento a todos aqueles que pelo seu destino se cruzaram no meu caminho e ajudaram sempre e quando foi necessário. Agradeço aos meus país Ruben e Maria de Lurdes Cunha por me terem dado o possível e o impossível e por me terem apoiado sempre. Aos meus irmãos Ruben, Mário e Sara e ao resto da família em geral por todos os momentos em que estivemos juntos, mas ainda mais pelos momentos em que estive distante mas que sempre senti o carinho e apoio familiar. Ao meu afilhado Gonçalo pela sua energia e boa disposição, e ao qual desejo que cresça e tenha as mesmas ou melhores oportunidades que eu. Ao Doutor Armindo Rodrigues pela sua dedicação e espirito sempre curioso o qual despertou em mim a vontade de agarrar o tema desta tese desde o primeiro minuto. Ao Rafael Montiel por ter sido a pessoa que me possibilitou crescer de forma rigorosa na área da biologia molecular e acima de tudo por me ter brindado com a sua amizade. A todas as pessoas que me ajudaram no decurso dos trabalhos experimentais, ao Ricardo Camarinho, Sué Vázquez, Ana Valentim, Mitzi Flores, Lianet Noda, Carolina Parelho, José Talavera, Gustavo Hernandez, Patrícia Garcia, Isabell Barois, Paula Aguiar, Daniel Pass, Craig Anderson e à Ceri Morris. Aos eternos Tunídeos por todos os momentos de loucura saudável nos quais me perdi e me encontrei vezes sem conta, principalmente quando a mente precisava de distracção. Gostava de realçar, sem dúvida alguma, o papel preponderante do Professor John Morgan e do Professor Peter Kille que foram, simplesmente, as pessoas mais marcantes na minha carreira científica. O John por ser um ser humano excepcional e o verdadeiro cientista que eu sonhava ser na minha

infância, ao qual elevo o meu mais profundo agradecimento. O Pete por ser simplesmente o Pete, a sua genialidade, criatividade e racionalidade servem de base para uma fonte de inspiração inesgotável que me cativaram mesmo perante os resultados de mais difícil “digestão”. Ambos fizeram-me sentir membro de uma grande equipa de investigação mas acima de tudo, uma grande família unida. A todos os meus amigos, cuja lista só cabe no meu coração. Por último, e sem dúvida a pessoa a quem tudo quero dar, á Raquel por ter entrado na minha vida na hora certa e me ter preenchido com o seu amor e paixão que nem a matemática pode mensurar.

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SUMMARY

Understanding how living processes persist under the challenges of intense physical or chemical stress emanating from diverse geogenic sources, such as those originated by volcanic activity have increasingly gained importance mainly compelled by biotechnology imperatives. Volcanically active regions, such as Furnas, a geothermal field located in São Miguel Island (Azores), often have a high-density of human inhabitants due to the elevated natural fertility of its soils, despite of creating significant risk scenarios of exposure to a wide range of chemical substances. The volcanic gases at the Furnas caldera create a hypoxic and hypercapnia environment combined with 10ºC elevated soil temperatures and acidic conditions promoting bioavailability of heavy metals. These tripartite stress factors combine to create an in-hospitable challenge to the resident biota; therefore, it is surprising that this extreme soil environment supports a viable earthworm population. In fact, the epidermis of *Amynthas gracilis* resident on Furnas soil is ~50% thinner than the respiratory exchange surface in conspecifics resident on inactive volcanic soils. This was also found in resident *Pontoscolex corethrurus.* This leads to the plausible conclusion that the earthworm's responses to the multi-stressor challenges in active volcanic soils are, like the adaptations of arthropods and vertebrates to hypoxic conditions, multifactorial and involve integrated modifications ranging from genetic and biochemical, to cellular and physiological levels of organization. Analyses of mitochondrial data using different approaches corroborate the existence of two different genetic lineages living in São Miguel Island. Furnas population showed lower genetic diversity when compared to the populations living within pineapple plantations. Molecular markers included the mitochondrial regions of the cytochrome c oxidase subunit I gene (COI), small ribosomal unit (s- rRNA), the NADH desidrogenase subunit 2 and 3. The importance of genetic reduction in the

population genetic structure of earthworms living under the stress of the volcanic environment is further discussed. Analysis using AFLP markers showed *Pontoscolex corethrurus* to be a genetically heterogeneous complex with direct association with the previous results of mitochondrial divergence. The complete congruence between molecular markers suggests that cryptic speciation is a plausible explanation for the deep mitochondrial divergence in *P. corethrurus* in São Miguel Island. Four pairs of primers generated 425 loci. The average ratio of polymorphic loci among the studied populations was of 84%. Shannon information index was 0.28 with a higher value of 0.3 in Furnas. These results show that the genetic diversity detected with AMOVA was mainly caused by individual differences within a population. In fact, three different ancestral clusters were identified among populations. One cluster showed to be almost exclusive to Furnas individuals showing and confirming the genetic differentiation of this apparently isolated geographic group.

On the one hand it is plausible to consider that the homogenizing effect of selection on genomic diversity would intensify in populations successfully inhabiting intensely stressful environmental conditions, such as actively volcanic soils. In contrast, an intriguing alternative scenario may pertain where chemical contaminants increase genetic diversity by causing genomic mutation which could explain why Furnas population showed the highest number of private bands.

Microbial populations associated with the earthworms revealed some conspicuous results. Some bacteria were found in both earthworm populations as resident microbial flora (e.g. Nitrobacter, Serratia, Bradyrhizobium, and Methylobacterium) while others seem to be restrict to one of the studied populations. The Azorean *P. corethrurus* has some conspicuous genera such Anaeromyxobacter and Desulfovibrio that may result of adaptations to the environment in which the host lives. This is also the first report of

Verminephrobacter phylotypes within the *Pontoscolex* genus. With this project was possible to elucidate some of functional mechanisms employed by annelids that allow it to maintain viable populations in soil exhibiting elevated heavy metal availability, low oxygen/high CO2 content and a high ambient temperature (~37ºC) as well the consequences of living under such environment revealed at genetics level from the individual to the population.

Chapter 1

Challenges and opportunities presented by active volcanism in the Azores

"The very geographical position of the Azores... poses important dispersal questions and makes these islands a natural laboratory. Midway between the European and the North American continents and born out of the attrition of three tectonic plates, the indigenous fauna and flora associations of the various islands... are expected to improve our understanding of colonization strategies. The conspicuous variability of external morphology or the concealed divergence in molecular structures from population to population (indicate that evolution) can be caught red-handed in the Azores."

Frias Martins, A.M. (1993)

Challenges and opportunities presented by active volcanism in the Azores

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THE ENVIRONMENTAL SCENARIO

Oceanic archipelagos provide an excellent setting to study the evolutionary forces that drive speciation and diversification. A reliable time frame for geological events, superimposed on patterns of molecular and/or morphological variation, allows inferences about allopatric differentiation, the predominant mode by which new species arise (Emerson, 2002). The Azores archipelago (Figure 1) consists of nine volcanic islands near the Mid-Atlantic Ridge, spanning an area of 530 (latitudes 36°55'–39°43' N) by 320 km (longitudes 25°–31°17'). There are three groups of islands: a western group (Flores and Corvo), a central group (Terceira, Graciosa, Faial, Pico and São Jorge) and an eastern group (Santa Maria and São Miguel). The largest island, São Miguel, was formed by two separate islands that coexisted for about 0.550 My and that merged about 50 000 years ago.

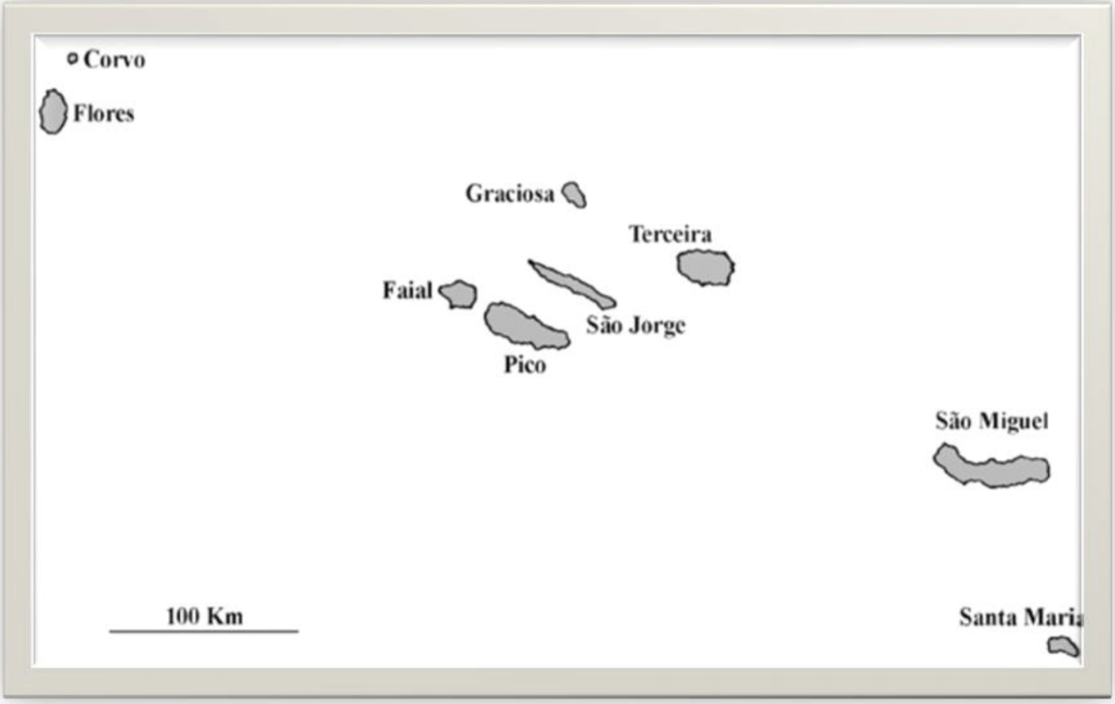

Figure 1. The Azores archipelago (Forjaz 1997).

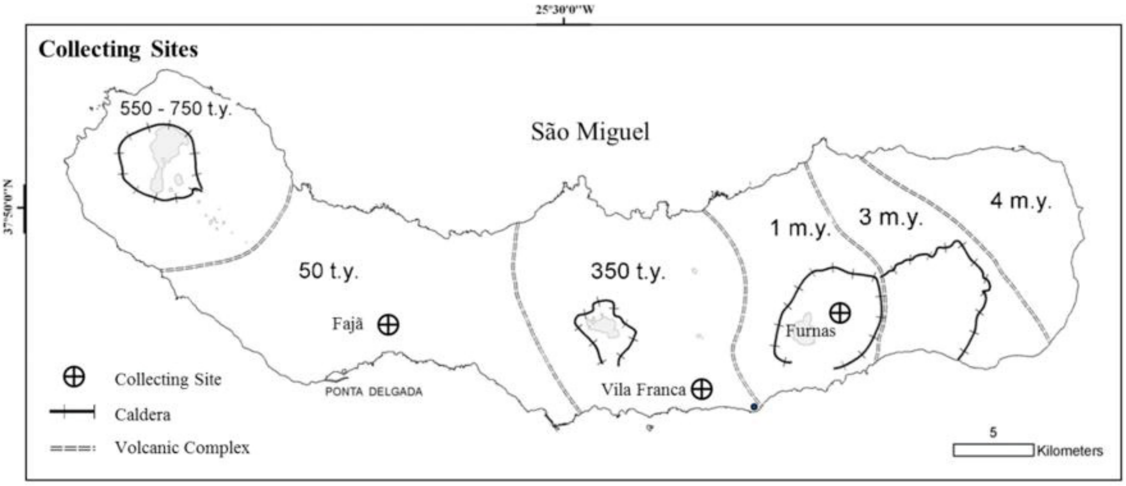

Figure 2. Collecting sites in São Miguel Island. M.y. million years; t.y.,thousand years.

The extant volcanic activity in certain terrestrial locations on São Miguel, one of the nine islands comprising the Azores archipelago, provide good "field-laboratories" for investigating aspects of the capacity of soil-dwelling macroinvertebrates to inhabit natural habitats continuously receiving potentially toxic chemical inputs in a milieu conducing bioavailability (Cunha *et al.* 2011). Furnas, the most easterly of the three active volcanoes on the island of São Miguel in the Azores, consists of a steep-sided caldera complex 8×5 km across (Figure 2) formed about 30,000 years BP, with historical eruptions occurring during the early occupation of the area at about 1440 AD and another in 1630 AD (Guest *et al.* 1999). The geological history of the Azores (See Figure 2) is relatively well documented and offers a wide range of ages (0.04–8 My) for the different islands (Morton *et al.* 1998).

COMMUNITY VULNERABILITY TO VOLCANIC HAZARD CONSEQUENCES

The high fertility of volcanic soils often attracts high-density human habitation, thus creating important risk scenarios in such regions (Baxter *et al.* 1999). Geothermal biotopes are reducing environments with certain unique features, mainly characterised by elevated soil, water, and atmospheric elemental composition, together with constant diffuse degassing and high temperatures (Cruz *et al.* 1999). Volcanic gases typically comprise water vapour, carbon dioxide, sulphur dioxide, hydrogen sulphide, and hydrogen chloride with lesser amounts of hydrogen fluoride (Ferreira and Oskarsson, 1999). Rocks and volatiles of volcanic origin are enriched with metals/metalloids such as Al, As, Cu, Hg, Pb, Rb, Sr, and Zn. Moreover, the elevated temperatures, as well as relatively anoxic combined with acidic conditions represent further inhospitable challenges to the resident biota. Thus, deriving a mechanistic understanding of the adaptation of ecologically relevant soil-dwelling organisms to cocktails of physical and chemical stressors of non-anthropogenic origin undoubtedly represents a major technical and conceptual challenge. A primary task of ecophysiology is to understand how organisms evolve adaptations to cope with the site-specific challenges posed by stressful environments. Ecotoxicology seeks easily interpretable exposure (‘environment’) versus response (‘phenotype’) relationships analogous to those that underpin classical toxicology, but a serious potential confounder in field-based ecotoxicology is that locally-adapted populations may express responses (in terms of accumulated levels of metals in their tissues, for example, or in terms of diverse functionality parameters) that deviate markedly from those predicted from combining environmental measurements with response amplitudes derived from laboratory tests on naive organisms lacking multi-generational exposure history to the stressor(s) (Morgan *et al.* 2007).

THE ROLE OF EARTHWORMS

“The plough is one of the most ancient and most valuable of mans inventions; but long before he existed the land was in fact regularly ploughed, and still continues to be thus ploughed by earth-worms.”

In the “The Formation Of Vegetable Mould” by Charles Darwin in 1881.

Earthworms are detritivorous macroinvertebrates conferred with the status of ‘ecological engineers and it is known that from the exact moment that soil is in contact with the earthworm body, either superficially or internally, physicochemical and microbiological changes take place (Brown & Doube 2004). These changes are mainly revealed by the modification of the distribution of nutrients and their availability to other organisms, whilst the earthworm behaviour and the biogenic structures that they build profoundly affect soil physical properties such as aeration, water-holding capacity or aggregate stability (Brown 1995; McLean & Parkinson 1997; Jouquet *et al.* 2006). Earthworms also play a significant role in decomposition of organic material and thus in nutrient cycling (Edwards & Bohlen 1996). Such ability can be very valuable for soil fertility establishment in young volcanic soils (Yamaguchi & Haruki 2003). In fact earthworm inoculations have been used in pastures (Stockdill 1982), cultivated lands (Springett *et al.* 1992) rehabilitated peat (Curry & Boyle 1995) and coal mines (Scullion & Malik 2000) of temperate soils.

AMYNTHAS GRACILLIS *AND* PONTOSCOLEX CORETHRURUS

We have previously collected and identified two earthworm species, *Amynthas gracilis* (Kinberg 1867) (Figure 3A) and *Pontoscolex corethrurus* (Müller, 1856) (Figure 3B), from the study site at Furnas .

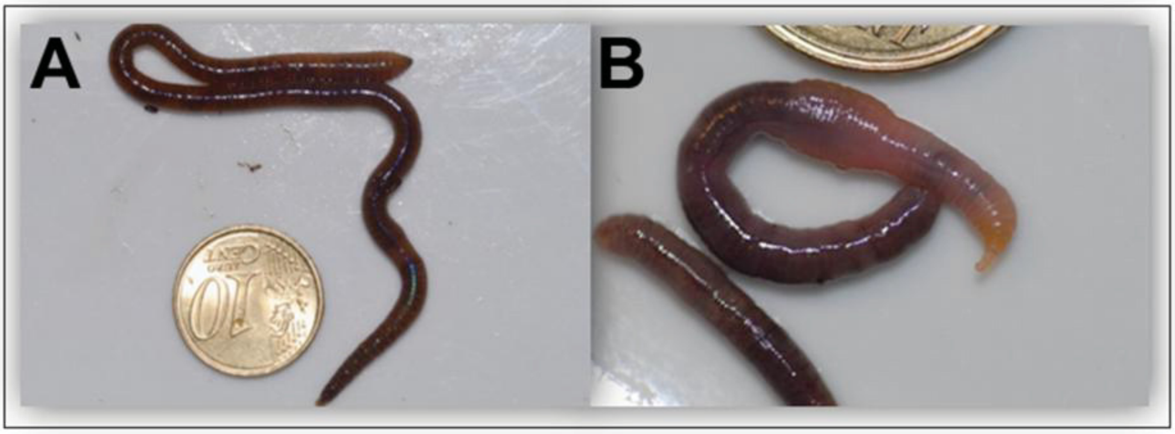

Figure 3. A, *Amynthas gracillis* and B, *Pontoscolex corethrurus.*

Amynthas gracilis shows a wide distribution within São Miguel and elsewhere in the Azores (Cunha *et al.* 2011). Originally from Asia, *A. gracilis* is considered a cosmopolitan, pantropical, peregrine and invasive species favouring disturbed, human-influenced, habitats including arable land, pastures, and second-growth forests. It appears as an exotic on many islands, including the Canary Islands, Channel Islands, and the Isle of Man. It belongs to the epigeic eco-physiological category, where it actively burrows to depths of 20 cm or near the surface under litter or other decomposing organic material. *A. gracilis* is probably parthogenetic and can enter diapause in dry soils, and its activities may promote plant growth (Fragoso *et al.* 1997).

Originally from northeast South America, the Glossoscolecidae family member *P. corethrurus* apparently was very successful in colonizing the inhospitable active volcanic soil of Furnas. *P. corethrurus* is a well-known invasive species of most tropical regions and its distribution seems to be only limited by temperature around the globe (Lavelle *et al.* 1987). *P. corethrurus* is a well-known invasive species of most tropical regions and its distribution seems to be only limited by temperature around the globe. Actually, being a geophagous endogeic species (Lavelle *et al.* 1987; Buch *et al.* 2011), *P. corethrurus* shows high plasticity which accordingly with Lavelle et al. (1987) is related to its wide range tolerance to soil physico-chemical characteristics, to moisture and temperature conditions, a very efficient assimilation of low-quality soil organic matter allowing it to survive even in very poor soils (Lavelle *et al.* 1987; Lavelle *et al.* 1995). The presence and high abundance of this species in the geothermal field may indicate its relative tolerance to the multifactorial stress challenge posed by the active volcanic environment. In São Miguel Island, the exotic earthworm *Pontoscolex corethrurus* is found to also habit pineapple greenhouses probably due to the high and constant temperature. In fact, their distribution around São Miguel seems to be temperature limited as it is only found in Furnas hot soils or inside such greenhouses.

EXPERIMENTAL HYPOTHESIS

The present research project will attempt to elucidate the influence of long-term mixed stress in a geogenic habitat on the local organisms such the earthworms. The core concerns in the project will be pursued via a robust series of field-based experiments designed to test four distinct hypotheses:

Hypothesis 1: Survival in active volcanic soils requires specific biochemical, structural and cellular changes that impart tolerance to multiple stressors, including elevated metal availability, hypoxia, hypercapnia and increased temperature. This should be in agreement for two ecological distinct earthworm species such the epigeic Amynthas gracilis and the endogeic Pontoscolex corethrurus.

Hypothesis 2: A local organism displays a low background degree of genetic diversity on the geographically delimited geothermal field when compared with possible adjacent populations;

Hypothesis 3: Locally adapted populations within volcanically active soils are genetically distinct from the 'background';

Hypothesis 4: Microbial populations associated with the *drilosphere* flora in volcanic earthworm's populations should be remarkably different from those living in non-volcanic areas..

The focal point of the study will be the active volcanic site at Furnas, São Miguel.

REFERENCES

Baxter P., Baubron J. & Coutinho R. (1999). Health hazards and disaster potential of ground gas emissions at Furnas volcano, Sao Miguel, Azores. *J. Volcanol. Geotherm. Res.*, 92, 95-106.

Brown G. (1995). How do earthworms affect microfloral and faunal community diversity? *Plant and Soil*, 170, 209-231.

Brown G. & Doube B. (2004). Functional interactions between earthworms, microorganisms, organic matter, and plants. *Earthworm ecology. 2nd edn. CRC Press, Boca Raton, FL*, 213–239.

Buch A.C., Brown G.G., Niva C.C., Sautter K.D. & Lourencato L.F. (2011). Life cycle of *Pontoscolex corethrurus* (Muller, 1857) in tropical artificial soil. *Pedobiologia*, in press.

Cruz J., Coutinho R., Carvalho M., Oskarsson N. & Gislason S. (1999). Chemistry of waters from Furnas volcano, Sao Miguel, Azores: fluxes of volcanic carbon dioxide and leached material. *J. Volcanol. Geotherm. Res.*, 92, 151-167.

Cunha L., Campos I., Montiel R., Rodrigues A. & Morgan A.J. (2011). Morphometry of the epidermis of an invasive megascoelecid earthworm (*Amynthas gracilis*, Kinberg 1867) inhabiting actively volcanic soils in the Azores archipelago. *Ecotoxicol. Environ. Saf.*, 74, 25-32.

Curry J. & Boyle K. (1995). Restoring soil fauna in reclaimed land, with particular reference to earthworms in cutover peat. *Acta Zoologica Fennica*, 371-375.

Edwards C. & Bohlen P. (1996). *Biology and ecology of earthworms*. Springer.

Forjaz V. (1997). Alguns vulcões da Ilha de S. Miguel. I Parte. In. Observatório vulcanológico e geothérmico dos Açores Ponta Delgada, Açores, Portugal.

Fragoso C., Brown G., Patrón J., Blanchart E., Lavelle P., Pashanasi B., Senapati B. & Kumar T. (1997). Agricultural intensification, soil biodiversity and agroecosystem function in the tropics: the role of earthworms. *Appl. Soil Ecol.*, 6, 17-35.

Frias Martins A.M. (1993). The Azores-Westernmost Europe: where evolution can be caught red-handed.

Guest J., Gaspar J., Cole P., Queiroz G., Duncan A., Wallenstein N., Ferreira T. & Pacheco J. (1999). Volcanic geology of Furnas Volcano, Sao Miguel, Azores. *J. Volcanol. Geotherm. Res.*, 92, 1-29.

Jouquet P., Dauber J., Lagerlöf J., Lavelle P. & Lepage M. (2006). Soil invertebrates as ecosystem engineers: intended and accidental effects on soil and feedback loops. *Appl. Soil Ecol.*, 32, 153-164.

Lavelle P., Barois I., Cruz I., Fragoso C., Hernandez A., Pineda A. & Rangel P. (1987). Adaptive strategies of *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta), a peregrine geophagous earthworm of the humid tropics. *Biol. Fertil. Soils*, 5, 188-194.

Lavelle P., Lattaud C., Trigo D. & Barois I. (1995). Mutualism and biodiversity in soils. *Plant and Soil*, 170, 23-33.

McLean M. & Parkinson D. (1997). Changes in structure, organic matter and microbial activity in pine forest soil following the introduction of *Dendrobaena octaedra* (Oligochaeta, Lumbricidae). *Soil Biology and Biochemistry*, 29, 537-540.

Morgan A.J., Kille P. & Stürzenbaum S.R. (2007). Microevolution and ecotoxicology of metals in invertebrates. *Environ. Sci. Technol.*, 41, 1085-1096.

Morton B., Britton J.C. & de Frias Martins A.M. (1998). *Coastal ecology of the Açores*.

Scullion J. & Malik A. (2000). Earthworm activity affecting organic matter, aggregation and microbial activity in soils restored after opencast mining for coal. *Soil Biology and Biochemistry*, 32, 119-126.

Springett J., Gray R. & Reid J. (1992). Effect of introducing earthworms into horticultural land previously denuded of earthworms. *Soil Biology and Biochemistry*, 24, 1615-1622.

Stockdill S. (1982). Effects of introduced earthworms on the productivity of New Zealand pastures. *Pedobiologia*, 24, 29-35.

Yamaguchi T. & Haruki M. (2003). Developing soil from nutrient-poor volcanic ash using earthworms and leaf litter. *Journal of the Japanese Society of Revegetation Technology*, 28, 417-425.

Chapter 2

Morphometry of earthworm epidermis as a biomarker of actively volcanic soil quality – the terrestrial equivalent of hydrothermal vents

Morphometry of earthworm epidermis as a biomarker of actively volcanic soil quality – the terrestrial equivalent of hydrothermal vents

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ABSTRACT

For the first time, the structure, dimensions, and composition of earthworm epidermal surfaces were used as a biomarker under exposure to actively volcanic soil. The epidermis of earthworm’s resident at the reference site was found to be approximately twice the thickness of the epidermis of conspecifics resident in volcanically-active Furnas soil. Reference worms transferred to Furnas soil for 14 days experienced an epidermal thinning of approximately 51%. In comparison, when the “volcanic” earthworms were transferred to microcosms at the volcanically inactive Fajã soil the epidermal thickness increased by approximately 21% over a 14 day exposure period. Earthworm’s resident on Furnas soil had higher goblet cell counts than the residents on volcanically inactive S. Maria soil. Transferring worms from both populations to microcosms of active Furnas soil induced a strong and significant increase in goblet cell counts. Metal composition analysis showed similar concentrations in both experimental soils; however, inoculated earthworm showed temporal differences in accumulated tissue metal concentrations. The active volcanic environment at Furnas poses a multifactorial stress challenge to the local soil-dwelling organisms, including the earthworm.

Keywords: *Amynthas, earthworm, volcanic soil, morphometry, epidermis, Azores*

INTRODUCTION

The high fertility of volcanic soils often attracts high-density human habitation, thus creating important risk scenarios in such regions (Hall-Spencer et al., 2008; Ribeiro et al., 2008; Viveiros et al., 2008; Viveiros et al., 2009). Geothermal biotopes are reducing environments with certain unique features, mainly characterised by elevated soil, water, and atmospheric elemental composition, together with constant diffuse degassing and high temperatures (Cruz et al., 1999; Viveiros et al., 2008; Viveiros et al., 2009). Volcanic gases typically comprise water vapour, carbon dioxide, sulphur dioxide, hydrogen sulphide, and hydrogen chloride with lesser amounts of hydrogen fluoride (Ferreira and Oskarsson, 1999). Rocks and volatiles of volcanic origin are enriched with metals/metalloids such as Al, As, Cu, Hg, Pb, and Zn (Cruz et al., 1999; Ferreira and Oskarsson, 1999; Aiuppa et al., 2000), with the diffusion of acidic volcanic gases through the rocks mobilizing the metals in associated soils and water bodies (Cruz et al., 1999; Bagnato et al., 2007).

A primary task of ecophysiology is to understand how organisms evolve adaptations to cope with the site-specific challenges posed by stressful environments. The extant volcanic activity in certain terrestrial locations on São Miguel, one of the nine islands comprising the Azores archipelago, provide good "field-laboratories" for investigating aspects of the capacity of soil-dwelling macroinvertebrates to inhabit natural habitats continuously receiving potentially toxic chemical inputs in a milieu conducing bioavailability (Amaral et al., 2006a; Cunha et al., 2008; Rodrigues et al., 2008).

Earthworms are detritivorous macroinvertebrates conferred with the status of 'ecological engineers' because they modify the distribution of nutrients and their availability to other organisms, whilst the biogenic structures that that they build profoundly affect soil physical properties (Jouquet et al., 2006). A number of criteria support the use of earthworms as sentinel species for soil quality assessment (Morgan and Morgan, 1998; Morgan and Morgan, 1999; Suthar et al., 2008) including: ecological functions; ability to inhabit extremely contaminated metalliferous soils (Morgan and Morgan, 1993); well-defined soil/tissue relationships for a number of metals (Peijnenburg, 2002); established acute and chronic toxicity tests (Kula and Larink, 1998); and a burgeoning body of ecotoxicogenomic protocols and observations (Spurgeon et al., 2008). Whilst the fate of inorganic contaminants in earthworm tissues is still a focus of ecotoxicological research (Cotter-Howells et al., 2005), the main thrust of the field is to assess the toxic effects of soil contaminants on the receptor organism, from the molecular-genetic and cellular levels of organisation to the physiological and demographic levels (Spurgeon et al., 2004; Bundy et al., 2008). Indeed, morphometric and complimentary cellular assays have been performed to determine the effects of volcanic soils in the Azores on the alimentary epithelia and chloragocytes of a non-resident earthworm species (*Lumbricus terrestris*) exposed under laboratory conditions (Amaral et al., 2006a). No comparable studies have to date been performed on indigenous species exposed in situ to volcanic soils.

The earthworm epidermis consists of supporting and basal cells, plus at least two types of mucus-secreting goblet cells with overlying cuticle (Jamieson, 1981). The integument is highly permeable, and not only serves as the sole respiratory surface but may also be the predominant uptake route for metals such as Cd, Cu, and Pb (Vijver et al., 2003). The structure, dimensions, and composition of epidermal surfaces have been widely used for assessing the effects of xenobiotics on molluscs (Gomot-de Vaufleury and Pihan, 2002), fish (Lamche and Burkhardt-Holm, 2000), amphibians (Fenoglio et al., 2009), and mammals (Fullerton and Hoelgaard, 1988; Iwata et al., 1999) but, surprisingly in view of its anatomical features and direct interfacing with soil, not in earthworms. Thus, the main objective of the present investigation was to determine morphometrically the effect of a volcanic soil on the epidermis of a cosmopolitan endogeic earthworm species (*Amynthas gracilis* Kinberg, 1867; Megascolecidae) resident in the Azores by comparing populations with different exposure histories confined within microcosms in field locations with and without volcanic activity. The amount of ecophysiological and ecotoxicological observations on this species is very limited.

MATERIALS AND METHODS

Earthworm sources and exposure sites

The Azores archipelago comprises nine islands and is located in the North Atlantic Ocean, between 36º45’–39º43’N and 24º45’–31º17’W, at the triple junction of Eurasian, African and North American plates, characterized by a complex tectonic settlement, where seismic and volcanic phenomena are common (Booth et al., 1978). São Miguel and Santa Maria are the two most eastern islands of the archipelago, and the latter is the oldest of all nine. São Miguel is the largest island (757 km^2), which presents several active volcanic spots including fumarolic fields, cold and thermal springs and soil diffuse degassing (Viveiros et al., 2008). Santa Maria (92 km^2), is also of volcanic origin but no volcanic activity has occurred there for >3 million years (Feraud et al., 1981). Two field sites on São Miguel, differing in their contemporary volcanic activity (thermal and degassing outputs), were selected for microcosm exposures: (a) Furnas, which displays the most conspicuous degassing and geothermal activity in the entire Azores archipelago, and (b) Fajã, which does not presently display any thermal and degassing phenomena (Table I).

Table I. Physical properties and concentrations of metals (µg g^{-1} dry weight) in soils of Furnas, Fajã (São Miguel) and Ribeira (Santa Maria).

Physical properties

	Site	Soil CO_2 (% vol.)	Soil O_2 (% vol.)	CO (ppm)	Soil Temp. (ºC)	Surface Temp. (ºC)	Moisture (%)	pH (H_2O)	Clay-silt (%)	OM (%)
São Miguel										
	Furnas	54.45	9.7	0.5	37	17	25	5.8	76	34
	Faja	0	21.1	0	18	18	34	7.8	75	34
Santa Maria										
	Ribeira*	-	-	-	-	-	17.72	6.9	80	2

Metal Content

	Site	Cu	Pb	Zn	K	Ca
São Miguel						
	Furnas	42	81	225	32909	9149
	Faja	41	90	195	21261	22516
Santa Maria						
	Ribeira*	66	74	197	83700	14600

* source: (Amaral et al., 2006b)

A group of adult (clitellate) *A. gracilis* from Furnas, 37º 46’ 24.6’’ N 25º 18’ 10.3’’ W (São Miguel) and another group from Ribeira 36º57’04.6’’N 25º05’38.9’’W (Santa Maria) were collected by digging and hand-sorting during Spring 2008, and were assigned to factorial-design treatments (with earthworm sources and exposure sites as factors, and exposure time as a covariable) within 24 h of collection (Figure 1). Twelve individual worms were placed in perforated, cube-shaped, plastic boxes (volume 20 L) covered with a permeable mesh. Ten boxes were used per site, with five boxes per 'treatment' (Furnas- or S. Maria-derived worms, respectively). Soil from the given exposure site was used to which approximately 120 g of re-wetted, oven-dried (at 140 ºC), urine-free horse manure (produced from a single known horse not subject to any medication) was deposited on the soil surface at the beginning and after 14 days exposure. For t_0 analysis, five earthworms from S. Maria and five from the two Furnas (S. Miguel) populations were randomly chosen.

In the Furnas (volcanically active site) exposures, five individuals (one from each of the appropriate replicated box) from both original sources (i.e. Furnas and S. Maria) were randomly sampled for analysis at 4, 8, and 14 days. In the Fajã 37º45'12.2N 25º38'21.3''W (S. Miguel, volcanically inactive site) exposures, five earthworms from both of the original populations (Furnas and S. Maria) were randomly sampled for analysis at 4, 8, 14, and 28 days. Figure 1 provides a schematic representation of the experimental design. After sampling, the earthworms were immediately transferred to the laboratory, where they were depurated of gut contents by placing them on moistened paper for 36 h. A fresh piece of tissue located 3-4 segments posterior to clitellum was excised from each individual earthworm for use in light microscopy and morphometry, the rest of the earthworm body was used for metal analysis.

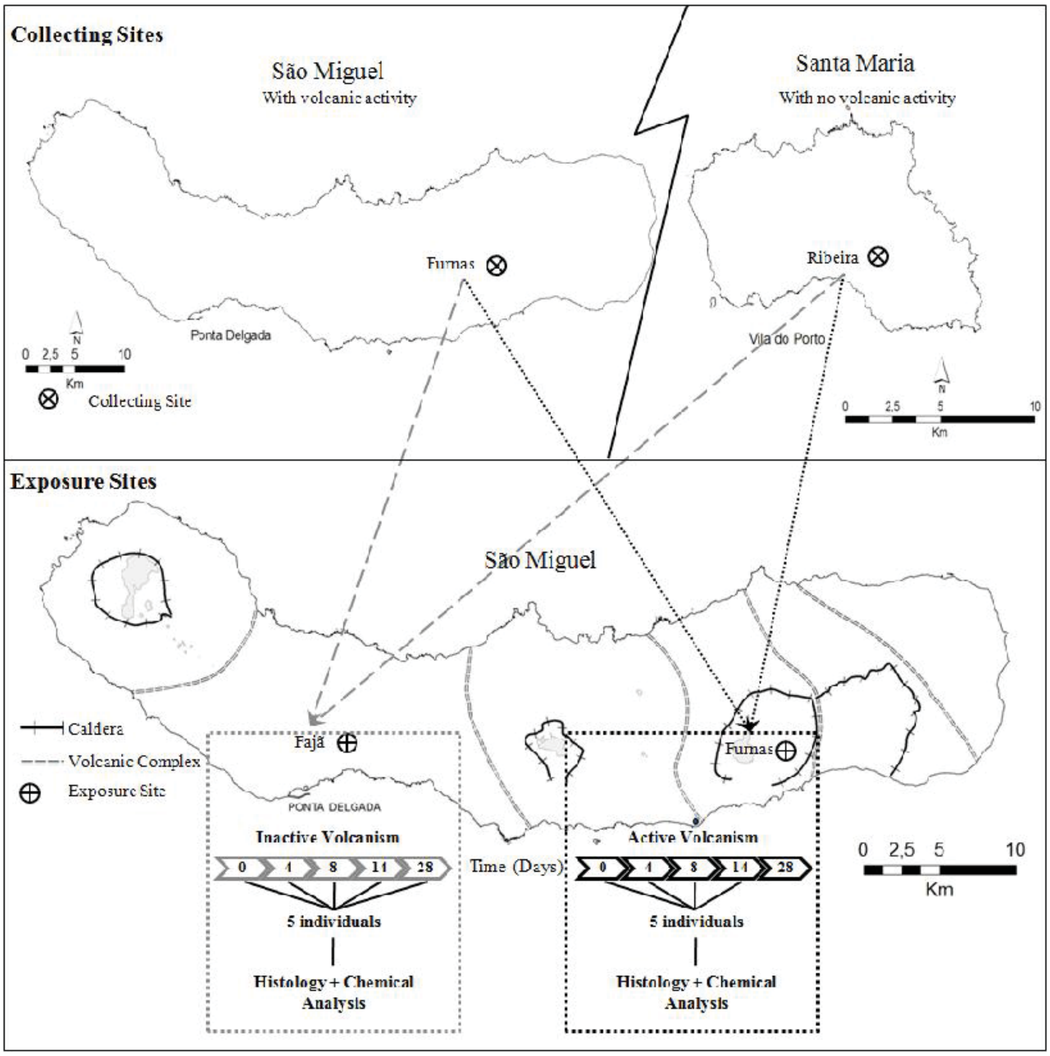

Figure 1. Schematic representation of the experimental design.

Metal analyses

The soft tissues of *A. gracilis* were dried (130 °C) for 48 h, digested in aqua regia at 95°C for 2h, and then microwave-digested inside closed vessels for 2min. Digests were diluted and analyzed for Ca, Cu, K, Pb and Zn on a Finnegan Mat Element 2 High Resolution ICP/MS (Actlabs, Canada). Soil samples at 10 cm were thoroughly homogenised, air-dried, and gently crushed prior to aqua regia digestion and ICP/MS metal analyses as for worm tissues. A quality control was implemented that included reagent blanks and reference materials. The accuracy, was always higher than 95% for the analyzed elements, and was assessed through the analysis of eight standard reference materials GXR-1, GXR-2, GXR-4, GXR-6, OREAS-13P, SDC-1, SCO-1, NIST694 and DNC-1 (Actlabs, Canada). Ultrapure water was used to prepare blanks and calibration standards.

Histological processing

Each fresh piece of tissue was fixed in neutral-buffered formaldehyde for 5h, dehydrated in graded ethanol series, and embedded in paraffin wax. Histological sections (4 µm thickness) were cut on a Leitz 1512 microtome (Leica Microsystems, Wetzlar, Germany), mounted on albumin- coated slides (Menzel-Glaser, Braunscheig, Germany), dried at 40°C for 24 h, and stored at room temperature until staining.

Morphometry

Sections were stained with haematoxylin–eosin (Martoja and Martoja-Pierson, 1970). Epidermis thickness was measured in 4 sections (4 fields per section), 28µm apart, in each individual worm. Images were captured using a CoolSNAP-cf camera (Photometrics GmbH, Munich) coupled to a light microscope, and analyzed with Image Pro-Plus 5.0 software (Media Cybernetics, Silver Springs). Thus, for statistical analysis the average value from16 measurements per individual earthworm were considered the true replicates (n = 5 per treatment).

Goblet cell counts

Another set of sections was stained with PAS-Alcian Blue (pH 2.5) to distinguish neutral and acidic mucopolysaccharides (Martoja and Martoja-Pierson, 1970), so that the blue-stained goblet cells containing acid mucins could be located. Counts were made in four microscope fields on two different sections 45 µm apart. Goblet cell numbers were expressed both as cells μm^{-1} and cells μm^{-2} (by dividing the cells μm^{-1} by the cross-sectional area of the epidermis).

Statistical analyses

Epidermal thickness measurements and goblet cell counts, respectively, were analysed (with or without $\log_e$ transformation, as appropriate) by two-way ANOVA and Tukey *post hoc* pair-wise comparisons, with $p \leq 0.05$ considered the level of significance.

RESULTS

Soil and earthworm metal analyses

Cu, Pb and Zn concentrations were very similar in the soils from the volcanically active (Furnas) and inactive (Fajã) sites (Table I). However, Ca concentration appeared to be appreciably higher and K concentration was appreciably lower in Furnas soil. Other noteworthy differences between the two soils are the lower pH (5.8 compared with 7.8), and exceptionally high CO_2 content (with accompanying lower O_2 and higher CO content), in Furnas soil (Table I). The Ribeira (S. Maria), site from which 'reference' worms were derived, had similar metal composition (apart from much higher K concentration) to Furnas and Fajã soils, and a neutral pH (Table I).

Cu, Pb and Zn concentrations were higher in earthworms maintained for a comparable exposure period of 14 days within microcosms containing actively volcanic Furnas soil compared with inactive Fajã soil, irrespective of the source of the earthworms (Figure 2) and despite the similarities of the metal contents of the soils at the exposure sites (Table I). In fact, Cu, Pb and Zn showed an accumulation pattern in both populations exposed to Furnas environment, with the highest concentration values found at 14 days of exposure when compared with t_0, revealing differences in the bioavailability of metals. Tissue K concentrations did not differ significantly amongst the earthworm 'treatment' groups; however, Ca concentrations tended to be lower in earthworms maintained in the relatively Ca-impoverished, acidic, Furnas soil compared with their counterparts maintained on Fajã soil (Figure 2).

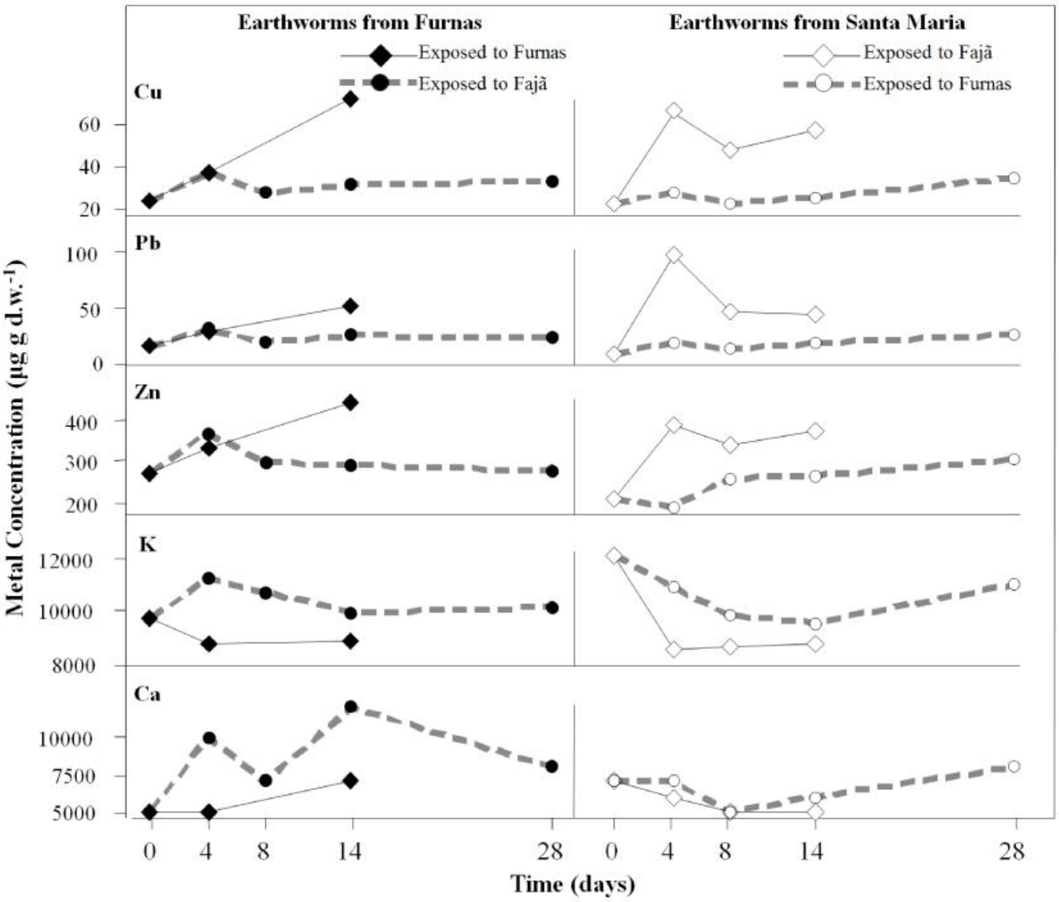

Figure 2. Concentrations of metals (μg element g of dry weight $^{-1}$) measured by HR-ICP/MS in tissues of *Amynthas gracilis* colected in Furnas, S. Miguel and S. Maria islands which were exposed to, Furnas a site with volcanic activity and other with no volcanic activity, Fajã.

Epidermal structure

Representative histological and histochemical (i.e. PAS/Alcian Blue and HE-stained sections) of the epidermis of earthworms from the two source populations before and after 14 days exposure to active (Furnas) and inactive (Fajã) volcanic soils are given in Figure 3.

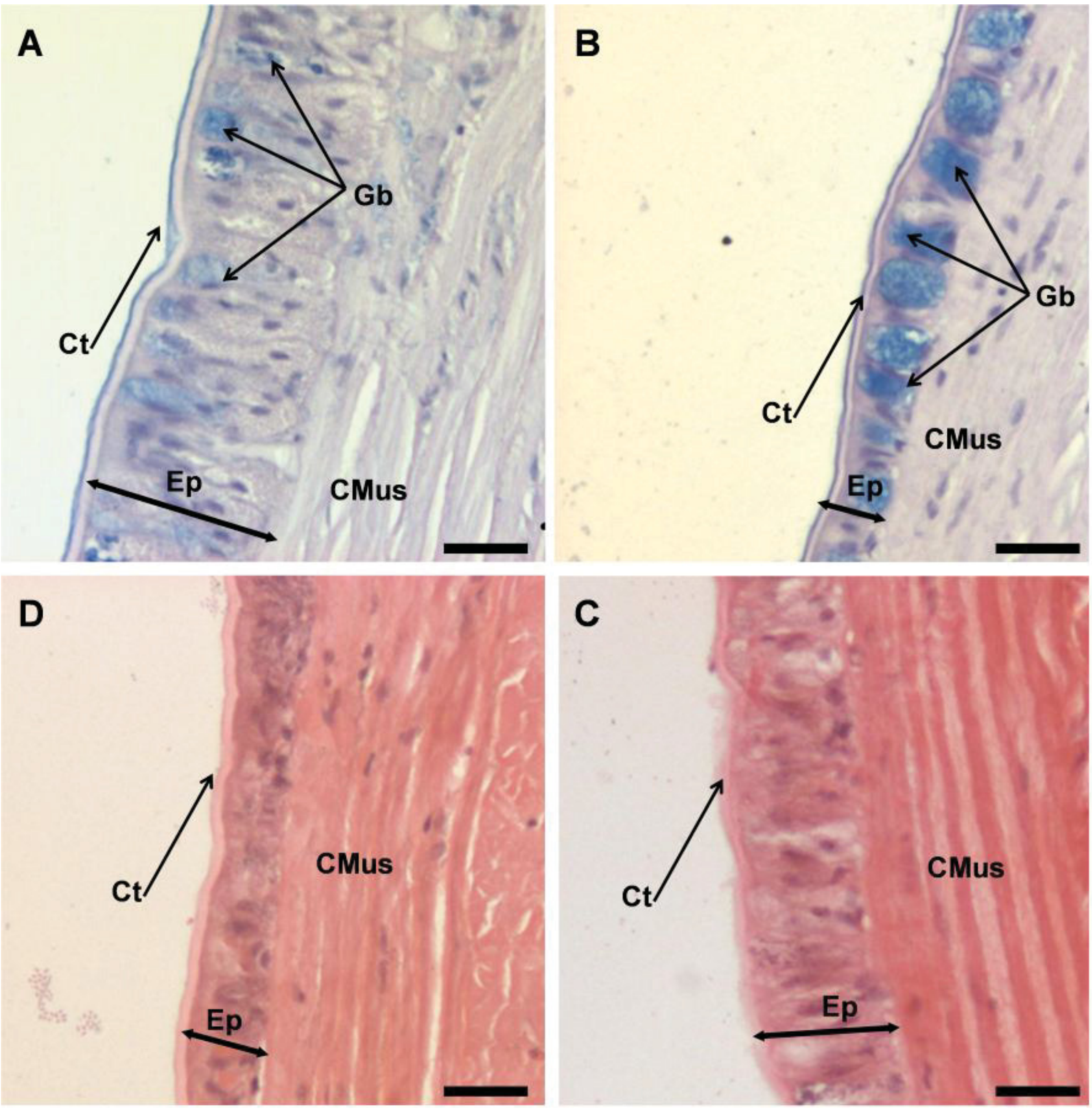

Figure 3. Micrographs of epidermal epithelia of *Amynthas gracilis* from Ribeira, Santa Maria before exposure (A) and after exposure (B) in Furnas and Furnas earthworms before exposure (C) and after exposure (D) to the non volcanic Fajã Soil. Scale bars = 25 µm. Ct, cuticle;Gb, Goblet cells; Ep, epidermis; CMus, cicular muscle; LgMus, longitudinal muscle.

Epidermal thickness

The epidermis of earthworm's resident at the reference site (Ribeira) on S. Maria was approximately twice the thickness of the epidermis of conspecifics resident in volcanically-active Furnas soil (Figure 3). When the reference earthworms were transferred to microcosms at the volcanically inactive Fajã soil the epidermal thickness decreased by approximately 30% over a 14 day exposure period. In comparison, S. Maria worms transferred to Furnas soil for 14 days experienced an epidermal thinning of approximately 51% (Figure 4).

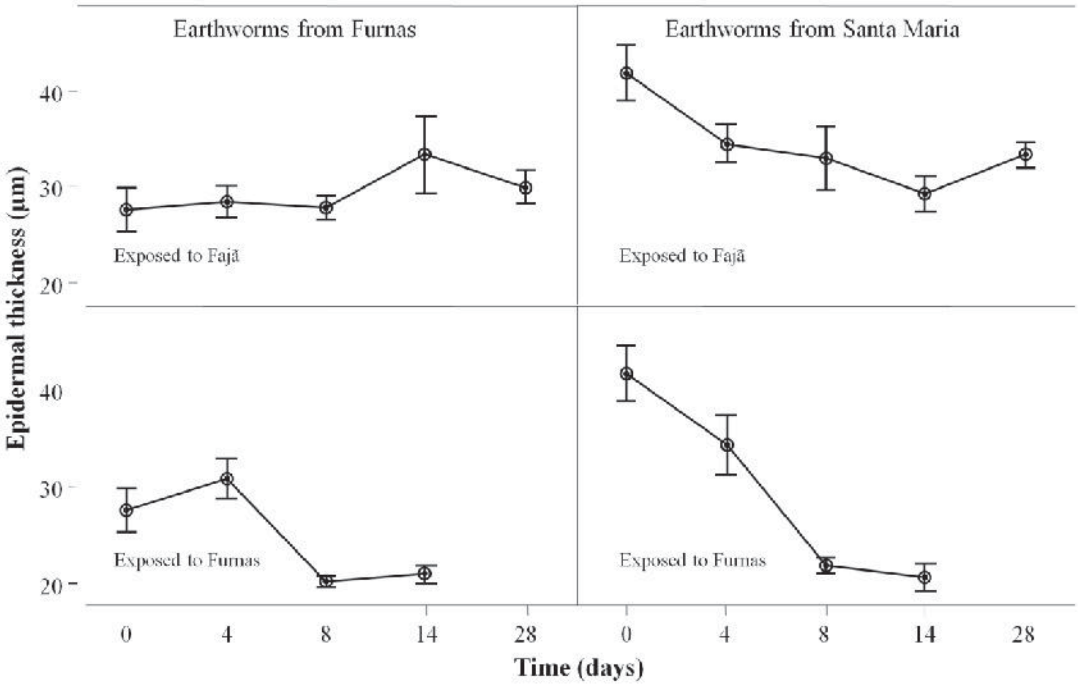

Figure 4. The epidermal thickness (μm) variation with time (days) and respective standard error bars in *Amynthas gracilis* collected in Furnas, S. Miguel and Ribeira, S. Maria, which were exposed to the active volcanic environment (Furnas) and to place without volcanic activity (Fajã).

Earthworms resident at Furnas experienced a modest epidermal thickening (approx. 21%) after 14 day maintenance in Fajã soil, but a progressive thinning of the epidermis (approx. 24%) when confined within microcosms of their 'own' soil (Figure 4).

Correlation analysis between earthworm tissue heavy metal concentrations and epidermis thickness (Table II) indicated significant negative relationships in the cases of

Furnas and S. Maria worms maintained on Furnas soil. There were no observable significant correlations between heavy metal burden and the morphometric parameter in either earthworm population transferred to microcosms containing Fajã soil.

Table II. Pearson's correlation coefficients (r^2) between epidermis thickness and concentrations of the analyzed elements in tissues of *A. gracilis* from Furnas and S. Maria exposed in microcosms to the active volcanism of Furnas and to a site without volcanic activity, Fajã.

	Earthworms from Furnas, Sâo Miguel		Earthworms from Ribeira, Santa Maria	
Elements	Furnas - active volcanism	Fajã - inactive volcanism	Furnas - active volcanism	Fajã - inactive volcanism
Cu	-0.614*	0.125	-0.432*	-0.095
Pb	-0.585*	0.144	-0.166	-0.241
Zn	-0.575*	-0.067	-0.522*	-0.187
Total Heavy Metal load	-0.738*	0.268	0.678*	0.279

* Significant correlations at $p \leq 0.05$

Goblet cell counts

The epidermis of earthworms resident on Furnas soil had higher goblet cell counts (expressed either as number per unit epidermis length or per unit epidermis area) than the residents on volcanically inactive S. Maria soil (Figure 5). Transferring worms from both populations to microcosms of inactive Fajã soil (14 and 28 days) or active Furnas soil (14 days) tended to induce an increase in goblet cell counts. The two modes of expressing the cell counts gave some inconsistencies. It is our view that expressing cells per unit epidermal area (cf. Figure 5C and 5D) rather than per linear dimension yielded more easily interpretable observations (cf. Figure 5A and 5B); if true, then it is evident that transferring worms to microcosms of Furnas soil induced goblet cell accretion more strongly than transfers to Fajã soil for the same exposure period.

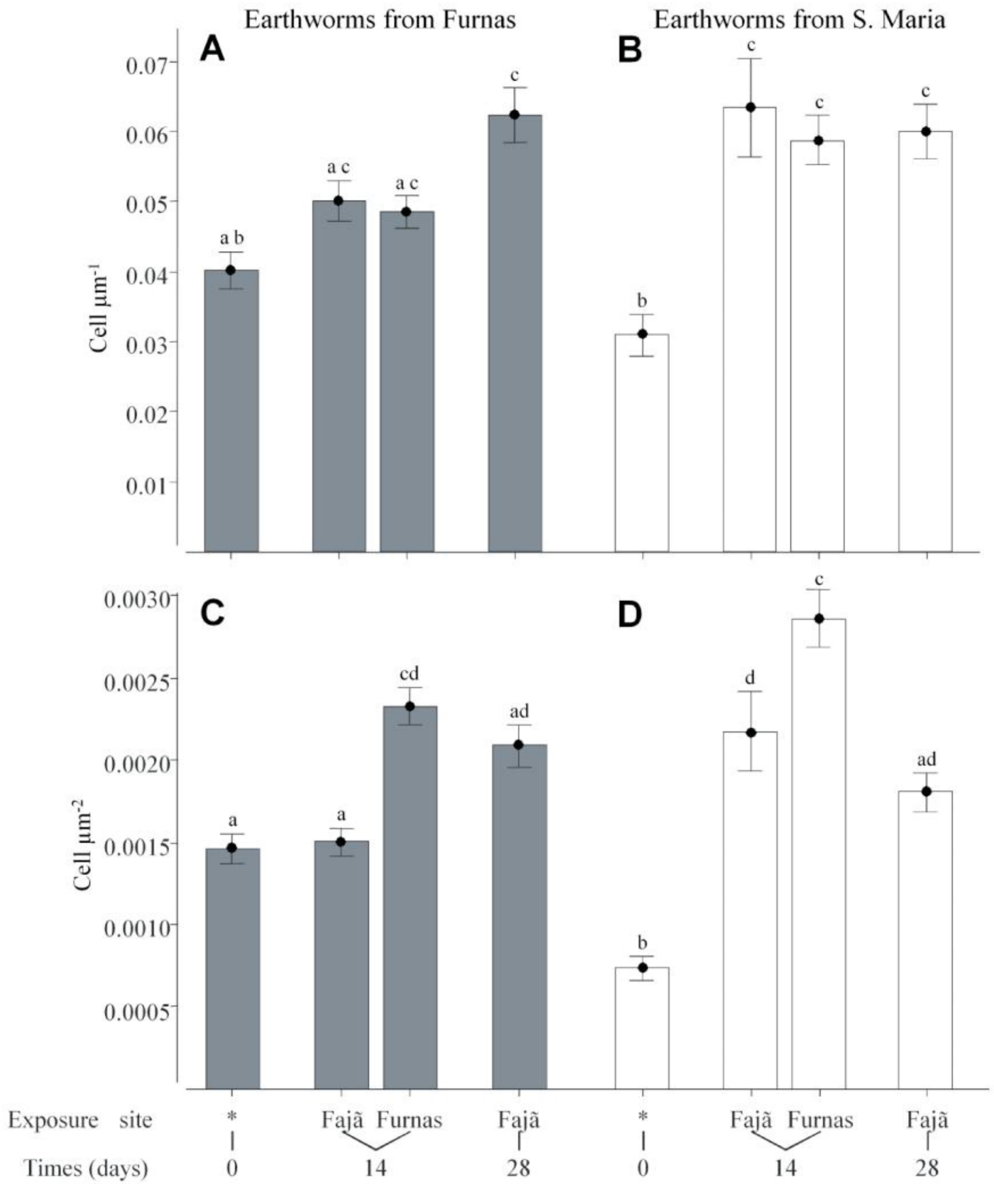

Figure 5. A and B, Goblet cells numbers per epidermis length (Cell µm^{-1}) and C an D per epidermis cross-sectional area (Cell µm $^{-2}$) in *Amynthas gracilis* collected in Furnas, S. Miguel and Ribeira, S. Maria which were exposed to Furnas, a volcanic environment and to place

without volcanic activity, Fajã. Different letters above and below bars mean significant differences at $p \leq 0.05$.

Certainly, Furnas and (particularly) S. Maria worms enclosed in Furnas soil for 14 days had significantly elevated goblet cell counts.

DISCUSSION

Chemical analysis

Most of the analysed elements showed similar concentrations in both experimental soils; however, inoculated earthworm showed temporal differences in accumulated tissue metal concentrations. It has been recorded previously that the high mineralisation and trace element bioavailability in Furnas soils and water together, together with diffuse degassing phenomena, do influence the tissue accumulation of metals by representatives of the local fauna (Cruz et al., 1999; Cunha et al., 2008; Rodrigues et al., 2008; Viveiros et al., 2009). Tissue metal concentrations (especially Zn and Cu) in earthworms at the end of the experimental exposure to Furnas soil were found to be almost two-fold higher than the soil concentrations and even similar to what was found in earthworms living in, or inoculated into, polluted sites (Ruiz et al., 2009). Moreover, it is widely known that most soil physical traits, such as pH, temperature and % clay-silt exert modulating influences on metal bioavailability (Barrow, 1986a; Barrow, 1986b; Spurgeon and Hopkin, 1996). Thus, the low pH and high temperatures that characterise the Furnas volcanic soils promote the mobility and the bioavailability of metals to soil-dwelling organisms.

Morphometry and cell composition

Conspicuous volcanic stress factors such as temperature, hypoxia, pH, and heavy metal presence, are known to cause changes in the phasic activity, atrophy and cellular composition of several animal tissues (Amaral et al., 2006a; Andersen et al., 2006; Hourdez and Lallier, 2007; Cunha et al., 2008). Such structural changes can be assessed morphometrically, a technique that is sufficiently sensitive to detect epithelial thinning due to environmental stress in the digestive epithelia and reproductive tissues of several invertebrates (Vega et al., 1989; Marigomez et al., 1998; Siekierska and Urbanska-Jasik, 2002). Morphological alterations are not uncommon among organisms exposed to extreme environments. A clear example is the case of the hydrothermal tubeworm (*Ridgeia piscesae*) which shows a 'short-fat' morphotype that lives in a microhabitat of high temperature but low O_2 concentration, whilst the 'long skinny' morphotype of the same species lives in a cooler, more O_2-rich, microhabitat (Andersen et al., 2006). It is noteworthy that the branchial surface area (i.e. essentially the respiratory gas exchange surface) is similar in both morphotypes, but the branchial thickness, thus the diffusion distance, is lower in the 'short-fat' compared with the 'long-skinny' worms (Andersen et al., 2006; Hourdez and Lallier, 2007).

In earthworm *A. gracilis* inhabiting volcanic soils, it is plausible to consider epidermal thinning as a physiological adaptation (probably plastic rather than constitutive) for reducing the O_2 diffusion distance across its integumentary respiratory surface. By such a structural modification the earthworm can obtain the O_2 that it requires for metabolism from its hypoxic native (actively volcanic) Furnas soil. The high soil temperature at Furnas also presumably increases metabolic O_2 consumption and demand, adding a premium to improved trans-epidermal diffusion efficiency. The epidermis tissue interface is known to act as the primary barrier protecting earthworms from potential bacterial infections (Fischer and Horváth, 1977). Since the epidermis also plays functional roles in ion transport, it is unsurprising that non-essential metal ions and xenobiotics enter the organism through the it (Dietz, 1974; Clauss, 2001). It is noteworthy that there is evidence that the dermal route is the predominant route for metal uptake by earthworms (Vijver et al., 2003). Reducing epidermal thickness and increasing body surface humidity by goblet cells secretion confers respiratory advantages, but could increase exposure to metals, particularly in acidic environments such as pertain at Furnas. To counter this, the worms may upregulate continuous mucus production for cation trapping and shedding; to this extent, increasing goblet cell counts would be beneficial. This notion is consistent with the observations that secreted mucopolysaccharides on the dermal surface trap heavy metals (Fischer and Horváth, 1977; Back, 1990), whilst the amount of surface-adsorbed Cd and Zn at any given time is minimal compared with the amount of internally sequestered metal (Vijver et al., 2005).

CONCLUSION

In conclusion, the active volcanic environment at Furnas poses a multifactorial stress challenge to the local soil-dwelling organisms, including the earthworm *A. gracilis*. In normal circumstances it would be deemed imperative to attempt to dissect the effects of the individual chemical and physical stressors by performing robust, controlled, laboratory exposures and monitoring the induced changes with a suite of morphological, physiological, and molecular observations. However, active volcanic soils comprise a suite of dynamic and, in some cases, volatile constituents that are more-or-less continuously expelled. To this extent, such sites are terrestrial equivalents of marine hydrothermal vents, and cannot easily be replicated in the laboratory. The recommendation from the 3rd Workshop on Earthworm Ecotoxicology that “more attention should be given to field testing in general” and “there is a need to explore the baseline variables influencing biomarker responses (confounding factors such as drought, temperature) and the linkage between biomarker and physiological responses" (Van Gestel and Weeks, 2004) is particularly apposite for active volcanic soils.

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REFERENCES

Aiuppa, A., Allard, P., D'Alessandro, W., Michel, A., Parello, F., Treuil, M., Valenza, M., 2000. Mobility and fluxes of major, minor and trace metals during basalt weathering and groundwater transport at Mt. Etna volcano (Sicily). Geochim. Cosmochim. Acta. 64**,** 1827-1841.

Amaral, A., Soto, M., Cunha, R., Marigómez, I., Rodrigues, A., 2006a. Bioavailability and cellular effects of metals on *Lumbricus terrestris* inhabiting volcanic soils. Environ. Pollut. 142**,** 103-108.

Amaral, A., Cruz, J., Cunha, R., Rodrigues, A., 2006b. Baseline levels of trace metals in volcanic soils of the Azores (Portugal). Soil Sediment Contam. 15**,** 123-130.

Andersen, A., Flores, J., Hourdez, S., 2006. Comparative branchial plume biometry between two extreme ecotypes of the hydrothermal vent tubeworm *Ridgeia piscesae*. Can. J. Zool. 84**,** 1810-1822.

Back, H., 1990. Epidermal uptake of Pb, Cd, and Zn in tubificid worms. Oecologia. 85, 226-232.

Bagnato, E., Aiuppa, A., Parello, F., Calabrese, S., D'Alessandro, W., Mather, T., McGonigle, A., Pyle, D., Wängberg, I., 2007. Degassing of gaseous (elemental and reactive) and particulate mercury from Mount Etna volcano (Southern Italy). Atmos. Environ. 41, 7377-7388.

Barrow, N., 1986a. Testing a mechanistic model. II. The effects of time and temperature on the reaction of zinc with a soil. Eur. J. Soil Sci. 37, 277-286.

Barrow, N., 1986b. Testing a mechanistic model. IV. Describing the effects of pH on zinc retention by soils. Eur. J. Soil Sci. 37, 295-302.

Booth, B., Croasdale, R., Walker, G., 1978. A quantitative study of five thousand years of volcanism on Sao Miguel, Azores. Philos. Trans. R. Soc. London, A. 271-319.

Bundy, J., Sidhu, J., Rana, F., Spurgeon, D., Svendsen, C., Wren, J., Stürzenbaum, S., Morgan, A., Kille, P., 2008. 'Systems toxicology' approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm *Lumbricus rubellus*. BMC Biol. 6, 25.

Clauss, W., 2001. Epithelial transport and osmoregulation in annelids. Can. J. Zool. 79, 192-203.

Cotter-Howells, J., Charnock, J., Winters, C., Kille, P., Fry, J., Morgan, A., 2005. Metal compartmentation and speciation in a soil sentinel: The earthworm, *Dendrodrilus rubidus*. Environ. Sci. Technol. 39, 7731-7740.

Cruz, J., Coutinho, R., Carvalho, M., Oskarsson, N., Gislason, S., 1999. Chemistry of waters from Furnas volcano, Sao Miguel, Azores: fluxes of volcanic carbon dioxide and leached material. J. Volcanol. Geotherm. Res. 92**,** 151-167.

Cunha, L., Amaral, A., Medeiros, V., Martins, G., Wallenstein, F., Couto, R., Neto, A., Rodrigues, A., 2008. Bioavailable metals and cellular effects in the digestive gland of marine limpets living close to shallow water hydrothermal vents. Chemosphere. 71**,** 1356-1362.

Dietz, T., 1974. Active chloride transport across the skin of the earthworm, *Lumbricus terrestris* L. Comp. Biochem. Physiol. A. 49**,** 251-258.

Fenoglio, C., Grosso, A., Boncompagni, E., Gandini, C., Milanesi, G., Barni, S., 2009. Exposure to heptachlor: Evaluation of the effects on the larval and adult epidermis of *Rana kl. esculenta*. Aquat. Toxicol. 91**,** 151-160.

Feraud, G., Gastaud, J., Schmincke, H., Pritchard, G., Lietz, J., Bleil, U., 1981. New K-Ar ages, chemical analyses and magnetic data of rocks from the islands of Santa Maria (Azores), Porto Santo and Madeira (Madeira Archipelago) and Gran Canaria (Canary Islands). Bull. Volcanol. 44**,** 359-375.

Ferreira, T., Oskarsson, N., 1999. Chemistry and isotopic composition of fumarole discharges of Furnas caldera. J. Volcanol. Geotherm. Res. 92**,** 169-179.

Fischer, E., Horváth, I., 1977. Cytochemical studies on the cuticle and epidermis of Tubifex tubifex Müll. with special regard to the localization of polysaccharides, heavy metals and the DAB-reactivity. Histochem. Cell Biol. 54**,** 259-271.

Fullerton, A., Hoelgaard, A., 1988. Binding of nickel to human epidermis in vitro. Br. J. Dermatol. 119**,** 675-682.

Gomot-de Vaufleury, A., Pihan, F., 2002. Methods for toxicity assessment of contaminated soil by oral or dermal uptake in land snails: Metal bioavailability and bioaccumulation. Environ. Toxicol. Chem. 21**,** 820-827.

Hall-Spencer, J., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S., Rowley, S., Tedesco, D., Buia, M., 2008. Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. Nature 454**,** 46-47.

Hourdez, S., Lallier, F., 2007. Adaptations to hypoxia in hydrothermal-vent and cold-seep invertebrates. Rev. Environ. Sci. Biotechnol. 6**,** 143-159.

Iwata, M., Takebayashi, T., Ohta, H., Alcalde, R., Itano, Y., Matsumura, T., 1999. Zinc accumulation and metallothionein gene expression in the proliferating epidermis during wound healing in mouse skin. Histochem. Cell Biol. 112**,** 283-290.

Jamieson, B., 1981. The ultrastructure of the Oligochaeta. Academic Press, London.

Jouquet, P., Dauber, J., Lagerlöf, J., Lavelle, P., Lepage, M., 2006. Soil invertebrates as ecosystem engineers: intended and accidental effects on soil and feedback loops. Appl. Soil Ecol. 32**,** 153-164.

Kula, H., Larink, O., 1998. Tests on the earthworms *Eisenia fetida* and *Aporrectodea caliginosa*. In: H. L. C. A. M. V. Gestel, (Ed.), Handbook of soil invertebrate toxicity tests. Wiley, New York, pp. 95-111.

Lamche, G., Burkhardt-Holm, P., 2000. Changes in apoptotic rate and cell viability in three fish epidermis cultures after exposure to nonylphenol and to a wastewater sample containing low concentrations of nonylphenol. Biomarkers. 5**,** 205-218.

Marigomez, I., Kortabitarte, M., Dussart, G., 1998. Tissue-level biomarkers in sentinel slugs as cost-effective tools to assess metal pollution in soils. Arch. Environ. Contam. Toxicol. 34**,** 167-176.

Martoja, R., Martoja-Pierson, M., 1970. Técnicas de histología animal. Toray-Masson, Barcelona.

Morgan, J., Morgan, A., 1998. The distribution and intracellular compartmentation of metals in the endogeic earthworm *Aporrectodea caliginosa* sampled from an unpolluted and a metal-contaminated site. Environ. Pollut. 99**,** 167-175.

Morgan, J., Morgan, A., 1999. The accumulation of metals (Cd, Cu, Pb, Zn and Ca) by two ecologically contrasting earthworm species (*Lumbricus rubellus* and *Aporrectodea caliginosa*): implications for ecotoxicological testing. Appl. Soil Ecol. 13**,** 9-20.

Morgan, J. E., Morgan, A. J., 1993. Seasonal changes in the tissue-metal (Cd, Zn and Pb) concentrations in two ecophysiologically dissimilar earthworm species: pollution-monitoring implications. Environ. Pollut. 82**,** 1-7.

Peijnenburg, W., 2002. Bioavailability of metals to soil invertebrates. In: H. E. Allen, (Ed.), Bioavailability of metals in terrestrial ecosystems: importance of partitioning for bioavailability to invertebrates, microbes, and plants. SETAC, Pensacola, Florida, pp. 89–112.

Ribeiro, D. C., Martins, G., Nogueira, R., Cruz, J. V., Brito, A. G., 2008. Phosphorus fractionation in volcanic lake sediments (Azores - Portugal). Chemosphere. 70, 1256-1263.

Rodrigues, A., Cunha, L., Amaral, A., Medeiros, J., Garcia, P., 2008. Bioavailability of heavy metals and their effects on the midgut cells of a phytopaghous insect inhabiting volcanic environments. Sci. Total Environ. 406, 116-122.

Ruiz, E., Rodriguez, L., Alonso-Azcarate, J., 2009. Effects of earthworms on metal uptake of heavy metals from polluted mine soils by different crop plants. Chemosphere. 75, 1035-1041.

Siekierska, E., Urbanska-Jasik, D., 2002. Cadmium effect on the ovarian structure in earthworm *Dendrobaena veneta* (Rosa). Environ. Pollut. 120, 289-297.

Spurgeon, D., Hopkin, S., 1996. Effects of variations of the organic matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*. Pedobiologia. 40, 80-96.

Spurgeon, D., Morgan, A., Kille, P., 2008. Current research in soil invertebrate ecotoxicogenomics. In: K. P. Hogstrand C, (Ed.), Comparative toxicogenomics. Elsevier, Amsterdam, pp. 133–164.

Spurgeon, D., Stürzenbaum, S., Svendsen, C., Hankard, P., Morgan, A., Weeks, J., Kille, P., 2004. Toxicological, cellular and gene expression responses in earthworms exposed to copper and cadmium. Comp. Biochem. Physiol. Part C Toxicol. Pharmcol. 138, 11-21.

Suthar, S., Singh, S., Dhawan, S., 2008. Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu, Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological category? Ecol. Eng. 32, 99-107.

Van Gestel, C., Weeks, J., 2004. Recommendations of the 3rd International Workshop on earthworm ecotoxicology, Aarhus, Denmark, August 2001. Ecotoxicol. Environ. Saf. 57, 100-105.

Vega, M., Marigomez, J., Angulo, E., 1989. Quantitative alterations in the structure of the digestive cell of *Littorina littorea* on exposure to cadmium. Mar. Biol. 103, 547-553.

Vijver, M., Vink, J., Miermans, C., van Gestel, C., 2003. Oral sealing using glue: a new method to distinguish between intestinal and dermal uptake of metals in earthworms. Soil Biol. Biochem. 35, 125-132.

Vijver, M., Wolterbeek, H., Vink, J., van Gestel, C., 2005. Surface adsorption of metals onto the earthworm Lumbricus rubellus and the isopod *Porcellio scaber* is negligible compared to absorption in the body. Sci. Total Environ. 340, 271-280.

Viveiros, F., Ferreira, T., Cabral Vieira, J., Silva, C., Gaspar, J. L., 2008. Environmental influences on soil CO2 degassing at Furnas and Fogo volcanoes (São Miguel Island, Azores archipelago). J. Volcanol. Geotherm. Res. 177, 883-893.

Viveiros, F., Ferreira, T., Silva, C., Gaspar, J., 2009. Meteorological factors controlling soil gases and indoor CO2 concentration: A permanent risk in degassing areas. Sci. Total Environ. 407, 1362-1372.

Chapter 3

The relevance of Pontoscolex corethrurus as a bioindicator of soil quality under geogenic stress conditions

The relevance of Pontoscolex corethrurus as a bioindicator of soil quality under geogenic stress conditions

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ABSTRACT

Furnas volcanic soils exhibit elevated heavy metal availability, low oxygen/high CO_2 contents and a high temperature (~37^{o}C), being therefore under permanent conditions of geogenic stress. In such particular soils, the use of an appropriated bioindicator species could be routinely used to obtain and evaluate consistent evidence on the past, present and future situation of the environment and therefore enable an effective management of soil quality. The presence and high abundance of this species in Furnas geothermal field may indicate its relative tolerance to the multifactorial stress challenge posed by the active volcanic environment. Furthermore, the amount of ecophysiological and ecotoxicological observations on this species is very recent and limited. Most of the analysed elements in the soils were also present in earthworm's tissues; however, the proportion between concentration in soil and earthworm tissues varied greatly among the analysed chemical elements. In fact, the presence of some elements in the body tissue of *P. corethrurus* that were not detected in the soil reveals that species can be highly sensible to detect these elements even at very low concentrations. The epidermis of earthworm's from the geothermal field in Furnas was significantly 30% thinner than the conspecifics at the pineapple culture houses. Despite the soil they were collected from, both earthworm populations of P. corethrurus showed similar proportions of coelomocytes, indicating that the immune response of their circulating coelomocytes is similar. This result reveals that even under stressful environmental conditions, such the ones present in the geothermal field, the earthworm population is able to maintain the immune response in a level similar to the observed in its con-specifics clearly demonstrated a good correlation between the concentrations of distinct chemical elements in the soil and earthworm tissues for a widely spread pantropical species. Thus we propose that *Pontoscolex corethrurus* should be considered as a relevant bioindicator species for soil quality, since it reflects with accuracy the composition of a given soil, with a valuable independency of the surrounding environmental factors due to its environmental tolerance and plasticity.

Keywords: *Pontoscolex corethrurus*, geogenic stress, morphometry, chemical composition, soil quality, volcanogenic pollution, heavy metals

INTRODUCTION

Understanding how living processes persist under the challenges of intense physical or chemical stress emanating from diverse geogenic sources, such as those originated by volcanic activity have increasingly gained importance mainly compelled by biotechnology imperatives. Volcanically active regions, such as Furnas, a geothermal field located in São Miguel island (Azores), often have a high-density of human inhabitants due to the elevated natural fertility of its soils, despite of creating significant risk scenarios of exposure to a wide range of chemical substances (Hall-Spencer *et al.* 2008; Ribeiro *et al.* 2008; Viveiros *et al.* 2008; Viveiros *et al.* 2009; Amaral & Rodrigues, 2011). Volcanic gases in Furnas geothermal field typically comprise water vapour, carbon dioxide, sulphur dioxide, hydrogen sulphide, and hydrogen chloride with lesser amounts of hydrogen fluoride (Ferreira & Oskarsson 1999). These rocks and volatiles of volcanic origin are enriched with metals/metalloids such as aluminium (Al), arsenic (As), copper (Cu), mercury (Hg), lead (Pb), and zinc (Zn) (Cruz *et al.* 1999; Ferreira & Oskarsson 1999; Aiuppa *et al.* 2000), with the diffusion of acidic volcanic gases through the rocks mobilizing the metals in associated soils and water bodies (Cruz *et al.* 1999; Bagnato *et al.* 2007). Cunha et al. (2011) showed that Furnas volcanic soils exhibit elevated heavy metal availability, low oxygen/high CO_2 contents and a high temperature (~37^{o}C), being therefore under permanent conditions of geogenic stress. In such particular soils, the use of an appropriated bioindicator species could be routinely used to obtain and evaluate consistent evidence on the past, present and future situation of the environment and therefore enable an effective management of soil quality. A number of criteria support the use of earthworms as sentinel species for soil quality assessment (Morgan & Morgan 1998, 1999; Suthar *et al.* 2008; Cunha *et al.* 2011), since these organisms are capable to inhabit extremely contaminated metalliferous soils

(Morgan & Morgan 1993), and thus can reveal well-defined soil/tissue relationships for a wide number of metals (Peijnenburg 2002). However, according to Suthar et al. (2008) the patterns of metal bioaccumulation in soil dwelling earthworms are affected by species-specific feeding behaviour, earthworm niche structure, ecological category and even horizontal distribution of contaminants in soil layers, and thus the selection of an appropriate bioindicator should be preferentially done considering these factors

Amynthas gracilis (Kinberg, 1867) and *Pontoscolex corethrurus* (Müller, 1856) are two earthworm species that were identified and collected from Furnas geothermal field. We have elected to focus this study on *P. corethrurus* because of its ecological features and its success in colonizing inhospitable active volcanic soil, as well the ease of rearing in the laboratory (Buch et al., 2011). This earthworm is a well-known invasive species of most tropical regions and its distribution around the globe seems to be only limited by soil temperature. Actually, being a geophagous endogeic species (Lavelle *et al.* 1987; Buch *et al.* 2011), *P. corethrurus* shows high plasticity which accordingly with Lavelle et al. (1987) is related to its wide range of tolerance to soil physico-chemical characteristics, to moisture and high temperature conditions, and, a very efficient assimilation of low-quality soil organic matter allowing it to survive even in very poor soils (Lavelle *et al.* 1987; Lavelle *et al.* 1995). The presence and high abundance of this species in Furnas geothermal field may indicate its relative tolerance to the multifactorial stress challenge posed by the active volcanic environment. Furthermore, the amount of ecophysiological and ecotoxicological observations on this species is very recent and limited (Buch *et al.* 2011; Huynh *et al.* 2011; Liang *et al.* 2011). Thus, the main objective of the present investigation was to determine whether a native earthworm to the volcanic soils, the geophagous *P. corethrurus* could be used to get valuable information about the relationships between soil chemical contents and earthworm tissues , by comparing two populations with different exposure histories to soil pollutants (volcanogenic *vs* anthropogenic polluted soils).

MATERIALS AND METHODS

Earthworm collection and sampling sites

The Azores archipelago comprises nine islands and is located in the North Atlantic Ocean, between 36º45'–39º43'N and 24º45'–31º17'W, at the triple junction of Eurasian, African and North American plates, characterized by a complex tectonic settlement, where seismic and volcanic phenomena are common (Booth et al., 1978). São Miguel is the largest island (757 km^2), which presents several active volcanic spots including fumarolic fields, cold and thermal springs and soil diffuse degassing (Viveiros et al., 2008). *Pontoscolex corethrurus* in the Azores seems to be delimited to the hot soils of the degassing fields in Furnas and inside greenhouses for pineapple production around the island. The following sampling sites, differing in their contemporary soil traits, were selected for earthworm capture: (a) Furnas, which displays the most conspicuous degassing and geothermal activity in the entire Azorean archipelago, and (b) pineapple greenhouses located in Fajã de Baixo (Faja), an agricultrual area about 3 Km apart from Ponta Delgada. A group of sexually mature (clitellated) *P. corethrurus* from the geothermal field (Table 1), 37º 46' 24.6'' N 25º 18' 10.3'' W (Furnas), and another group from a pineapple greenhouse (Table 1), 37º45'12.2N 25º38'21.3''W (Fajã de Baixo), were randomly collected from the soil by digging and hand-sorting during winter of 2011. A brief description of each soil main physic-chemical parameters is given in Table I. After sampling, the earthworms were immediately transferred to the laboratory, where they were depurated of gut contents by placing them on moistened paper for 36h.

Table I. Physical properties in soils of Furnas and pineapple greenhouses in Fajã de Baixo (São Miguel) .

	Physical properties of soils						
Site	Soil CO_2 (% vol.)	Soil O_2 (% vol.)	CO (ppm)	Soil Temp. (ºC)	Surface Temp. (ºC)	pH (H_2O)	OM (%)
Furnas	54.45	9.7	0.5	37	17	5.8	34
Fajã de baixo	0	21.1	0	18	18	5.2	25

Metal analyses

The soft tissues of *P. corethrurus* were dried (130 C) for 48h, digested in *aqua regia* at 95°C for 2h, and then microwave-digested inside closed vessels for 2min. Digests were diluted and analysed for thirty different elements on a Finnegan Mat Element 2 High Resolution ICP/MS (Actlabs, Canada). Soil samples were thoroughly homogenised, air-dried, and gently crushed prior to *aqua regia* digestion and ICP/MS metal analyses. A quality control was implemented that included reagent blanks and reference materials. The accuracy was always higher than 95% for the analysed elements, and was assessed through the analysis of eight standard reference materials: GXR-1, GXR-2, GXR-4, GXR-6, OREAS-13P, SDC-1, SCO-1, NIST694 and DNC-1 (Actlabs, Canada). Ultrapure water was used to prepare blanks and calibration standards. A ranking criteria, described in Table II was assigned in order to assess the proportions between the presence of the chemical element in the soil and in earthworm tissues.

Table II. Description of the criteria used to rank the relation between metal presence/concentration in soil and body tissue of *Pontoscolex corethrurus*.

Rank	Increase of element concentration in tissues	Definition
0	0-fold	Detectable in soil but not in tissue
1	between 0 and 0.8-fold	Detectable in tissue and soil
2	between 0.8 and 1-fold	Similar concentration in both tissue and soil
3	higher than 1-fold	Accumulates in tissue and it is detectable in soil
4	Only detected in tissue	Accumulates in tissue but it is not detectable in soil

Cohen kappa statistic was used as a measure of concordance between the ranks assigned to each sampling site. For $P<0.05$, the null hypothesis (kappa=0) was rejected. Kappa values of 0.01 to 0.20, 0.21 to 0.40, 0.41 to 0.60, 0.61 to 0.80, and 0.81 to 1.0 represent "marginal," "fair", "moderate", "substantial," and "almost perfect" agreement, respectively (Vieira et al., 2005). On the other hand, for $P>0.05$, the null hypothesis was accepted, concluding that the consistency between ranks from each sampling site is no better than would be expected by chance alone.

Histological and cytological markers

Coelomocyte Extrusion and counts

Coelomocytes were extruded via dorsal pores by electrical stimulation using a modified protocol (Homa *et al.* 2005). Briefly, the worms were immersed in 2 ml of PBS solution

(Phosphate Buffered Saline, Ca^{2+} and Mg^{2+} free) and exposed to a 9V electrical current during 30 seconds. The resultant mixture was recovered to 1 ml tubes and centrifuged at 1500 rpm during 5 minutes. The *pellet* was re-suspended in 100 µl of PBS solution and immediately utilized for cytologic staining of coelomocyte.Proportion of different coelomocytes types was assessed using suspension smears. Briefly, slides were air-dried at room temperature, fixed in methanol at 0ºC during 25 seconds, hydrolysed at room temperature in 5 M hydrochloric acid for 12 minutes. After hydrolysis, the slides were immersed, in the dark, into Schiff's reagent for 20 minutes, rinsed with water and counterstained with light green for 45 seconds dehydrated with ethanol, cleared with xylene and finally mounted (Martoja & Martoja-Pierson 1970; Soames *et al.* 1995). *Pontoscolex corethrurus* coelomocytes were identified using the classification criteria proposed by Adamowicz and Wojtaszek (2001).

Morphometry: Thickness of the epidermis

A fresh piece of tissue located 3-4 segments posterior to clitellum was excised from each earthworm for use in light microscopy morphometrical observations. The rest of the earthworm body was used for metal analysis, as described in 2.2. Each fresh piece of tissue was fixed in neutral-buffered formaldehyde for 12h, dehydrated in graded ethanol series, and embedded in paraffin wax. Histological sections of 4 µm thickness were stained with haematoxylin–eosin (Martoja & Martoja-Pierson 1970).

The thickness of the epidermis was measured in 3 sections (4 fields per section), 28µm apart, in each individual. Images were captured using a CoolSNAP-cf camera (Photometrics GmbH, Munich) coupled to a light microscope, and analyzed with Image Pro-Plus 5.0 software (Media Cybernetics, Silver Springs). Thus, for statistical analysis the average value from 12 measurements per individual earthworm were considered (n = 10 individuals per treatment). Epidermal thickness measurements were analysed (with or without $\log_{10}$ transformation, as appropriate) by Two-sample T-test with $p \leq 0.05$ considered the level of significance (Zar 1999).

RESULTS

Chemical analyses of soil and earthworm tissue

Most of the analysed elements in the soils were also present in earthworm's tissues; however, the proportion between concentration in soil and earthworm tissues varied greatly among the analysed chemical elements. For elements such as Ag, Cd, Cs, Hg, Rb, Se and W, the concentrations in soil were below the detection limit but these elements were found in higher concentrations in earthworm tissues. In contrast, elements such as As, Ce, Hf, La, Mg, Ni, Sr, V and Zr, while present in soil they seem that are not readily available to the earthworms. Other elements such as Ba and Pb showed similar concentrations in soil and in the earthworm tissues. Finally, elements such as Al, Ca, Co, Cu, Fe, K, Mn, Mo, Na, Ti and Zn showed to accumulate in earthworm tissues. In fact, more than a 5-fold increase of Zn was observed in earthworm tissues from both sites and a 5-fold increase in Mn and Ti in individuals from Furnas, were observed when compared to the concentrations of such elements in soils (Table III). Statistical concordance determined by the Cohen kappa statistic was 0.562 (n=30, p<.0001), indicating that the concordance between the ranks assigned to each sampling site is moderate and highly significant.

Table III. Concentrations of metals measured by HR-ICP/MS in body tissue of *Pontoscolex corethrurus* (µg element g of dry weight $^{-1}$± SE) and in soils (µg element g of dry weight $^{-1}$) collected from the soils of Furnas and of pineapple greenhouse in Fajã de Baixo (São Miguel island- Azores).

	Furnas		Rank	Fajã		Rank
Chemical Element	Soil	Tissue		Soil	Tissue	
Ag	0.8	2.6 ± 0.2	3	n.d.	0.9 ± 0.6	4
Al	8663	15406 ± 2679	3	6377	3085 ± 1592	1
As	10	6.7 ± 0.4	1	6	3.05 ± 0.4	1
Ba	170	71.9 ± 2.2	1	236	207.9 ± 59	2
Ca	693	1575 ± 1162	3	1615	7734 ± 9972	3
Cd	n.d.	12.7 ± 1.5	4	n.d.	17.7 ± 2.98	4
Ce	228	6.8 ± 4.3	0	119	12.8 ± 4.7	1
Co	n.d.	2.1 ± 0.1	3	9	4.7 ± 0.5	1
Cr	23	6.04 ± 1.03	1	79	13.9 ± 3.3	1
Cs	n.d.	0.3 ± 0.1	4	n.d.	0.2 ± 0.01	4
Cu	8	7.6 ± 0.2	3	41	21.1 ± 3.4	1
Fe	3196	14422 ± 374	3	3217	8377 ± 2968	3
Hf	19.5	9.8 ± 0.3	1	11.5	3.2 ± 2.01	1
Hg	n.d.	8.6 ± 0.3	4	n.d.	10.3 ± 0.5	4
K	3819	13632 ± 428	3	2175	10355 ± 1086	3
La	171	2.9 ± 2.1	0	86.7	5.5 ± 3.9	1
Mg	290	47 ± 53	1	694	251 ± 274	1
Mn	170	894 ± 16	3	139	479 ± 126	3
Mo	3	4.8 ± 0.2	3	2	2.5 ± 0.01	3
Na	3746	2515 ± 328	3	2367	4158 ± 855	3
Ni	7	3.5 ± 0.2	1	34	8.4 ± 2.1	1
Pb	13	10.8 ± 0.7	2	108	76.6 ± 54.3	1
Rb	140	25.1 ± 3.1	4	nd	23.9 ± 0.1	4
Se	n.d.	5.5 ± 0.1	4	5	11.1 ± 1.9	3
Sr	90	25.97 ± 6.5	1	167	79.3 ± 39.5	1
Ti	400	2061 ± 67	3	586	1615 ± 548	3
V	24	11.01 ± 0.4	1	58	16.2 ± 4.6	1
W	n.d.	2.1 ± 0.01	4	n.d.	1.1 ± 0.2	4
Zn	166	868.4 ± 30.9	3	282	1633 ± 173	3
Zr	1066	497.5 ± 23.4	1	452	150.6 ± 105.6	1

n.d. – not detected

Histological and cytological markers

Coelomocytes counts

Although some variation was observed between the two populations of P. corethrurus exposed to volcanogenic or anthropogenic polluted soils, the proportion of different coelomocyte types, eleocytes, granular and non-granular amebocytes, was not significantly different, revealing a similar immune competence. Among these 3 types of coelomocytes, eleocytes are the most abundant (40≈50 %) among the population of circulating coelomocytes, followed by both amebocyte types (Table IV).

Table IV. Proportion of different types of coelomocytes extruded from *Pontoscolex corethrurus* collected from the soils of Furnas and of pineapple greenhouse in Fajã de Baixo (São Miguel island- Azores).

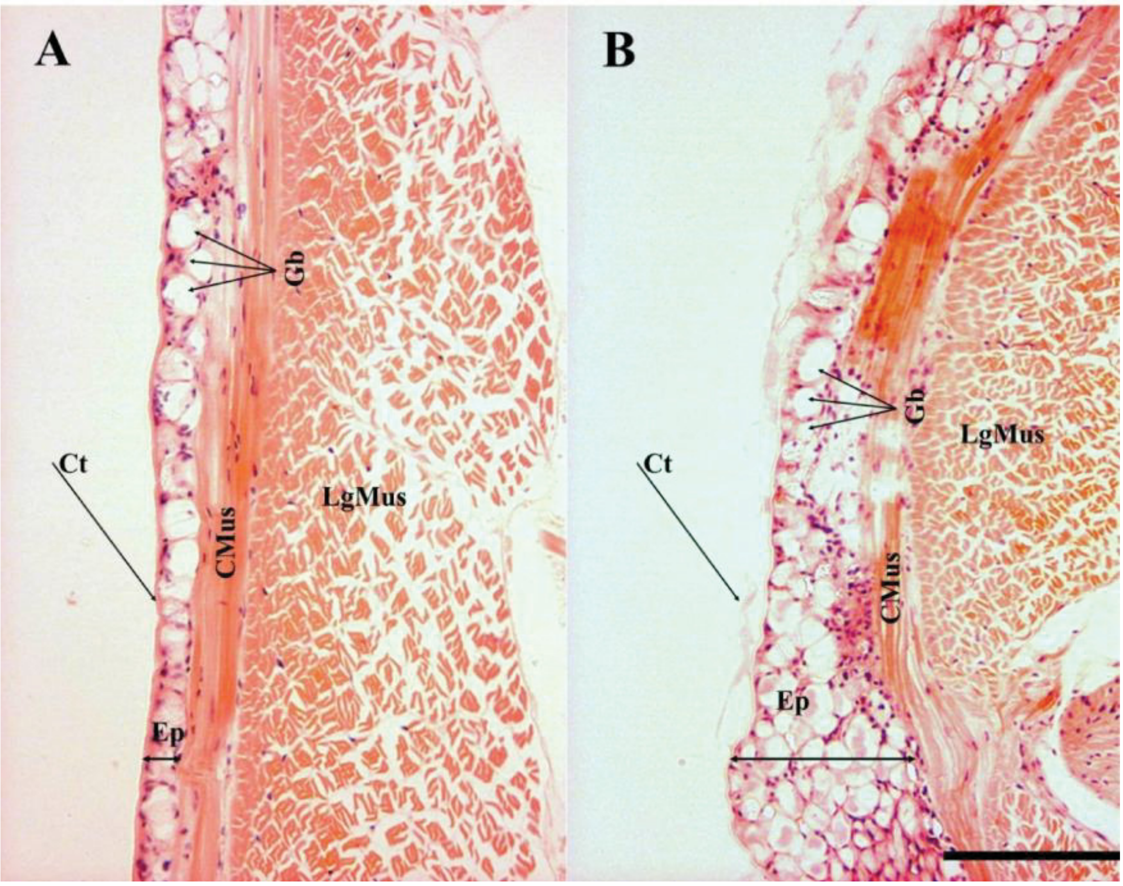
Site	Eleocytes			Granulocytes			Non granulocytes		
Fajã de Baixo	43.3	±	4.7	25.3	±	2.2	31.4	±	3.3
Furnas	52.0	±	4.2	24.1	±	3.0	23.9	±	3.6

Thickness of the epidermis

Representative micrographs of histological HE-stained sections of the epidermis of earthworms from the two populations are given in Figure 1.The epidermis of earthworm's from the geothermal field in Furnas was significantly thinner (t = 3.82, df = 18, $p < 0.001$), with a mean value of 37.24±1.71µm (±SE) when compared to the con-specifics from the pineapple greenhouse, that had an average epidermis height of

51.68±3.38 µm (±SE) (Figure 1). Such reduction was proposed to be an adaptation to the surrounding atmosphere in volcanically-active soils (Cunha *et al.* 2011).

Figure 1. Micrographs of epidermal epithelia of *Pontoscolex corethrurus* collected collected from the soils of Furnas (A) and of pineapple greenhouse in Fajã de Baixo (B), in São Miguel island- Azores. Scale bars = 50 µm. Ct, cuticle;Gb, Goblet cells; Ep, epidermis; CMus, cicular muscle; LgMus, longitudinal muscle.



DISCUSSION

Through the direct measurement of heavy metal concentrations in tissues of *Pontoscolex corethrurus* was possible to assess the soil composition pollution levels in two soils with very different sources of pollution (of volcanogenic or anthropogenic origin). Although, the source of pollutants was completely distinct and, both the mobility and availability of the different chemical elements to the soil biota is considered to be highly dependent of the soil environmental conditions (Somero *et al.* 1977; Barrow 1986; Sandifer & Hopkin 1997; Wieczorek-Olchawa *et al.* 2003; Khan *et al.* 2007), the majority of the analysed chemical elements observed in the soils were also found in *P. corethrurus* tissues collected from both sites. Despite the distinct origins of soil pollutants, the similarities in the chemical contents in both sites can be explained by the fact that pineapple compost substrates are composed by volcanic soil to some extent (70%) and because these soils are frequently enriched by fertilizers. According to Rodrigues et al (2008), fertilizers are the main cause for the high concentrations of some elements, such as Mg, Mn, Zn and Ca found in a soil of a non-volcanically active agricultural field in São Miguel Island. The observed tissue concentrations of some well-known toxic xenobiotics such as Mn and Zn, commonly found in volcanically active environments, are consistent with the results obtained for other organisms exposed to volcanically active environments, like algae (Wallenstein *et al.* 2009), molluscs (Cunha *et al.* 2008), insects (Rodrigues *et al.* 2008) and mammals (Amaral *et al*. 2007) . Also, and not described before, was the presence of high concentrations of Ti with a maximum value of more than 2000 µg per g $^{-1}$ for Furnas earthworms. Some consideration should be taken for Ti and Zn, since for these elements evidence of a potential genotoxic effect was established for concentrations above 1000 µg per g $^{-1}$ using nanoparticle compounds (Hu *et al.* 2010). In addition, the increased concentrations and availability of toxic elements can have an antagonistic effect in the uptake of some essential elements such as Mg and Ca by affinity competition, though any potential interactions are far from being understood (Goyer 1997; Marinussen & Van Der Zee 1997). Interestingly, the presence of some elements in the body tissue of *P. corethrurus* that were not detected in the soil reveals that species can be highly sensible to detect these elements even at very low concentrations. In conclusion, the highly significant moderate statistical concordance between the concentrations of the chemical elements in analysed soils and in the earthworm's tissues shows that the physiological response of *P. corethrurus* to the presence of the selected elements is quite similar in both sites, despite the distinct physicochemical properties of each soil. Such results show that *P. corethrurus* is a good indicator of soil quality, particularly for those under geogenic stress, such as the highly fertile soils of volcanically active regions or agricultural fields with intense manure.

In earthworms coelom immunocytes are responsible for humoral and cellular responses, comparable to those carried out by vertebrate mononuclear phagocytes (Adamowicz & Wojtaszek 2001; Adamowicz 2005). The eleocytes are relatively large cells with a cytoplasm that contains numerous chloragossomes (Plytycz *et al.* 2010). The amebocytes are related to various vital processes, metabolic and regulatory, ensuring proper functioning of the whole organism (Affar *et al.* 1998); they act as multi-function cells mainly involved in cellular immune reactions which include humoral responses (Stein and Cooper, 1983; Ville et al., 1995; Jarosz and Glinski, 1997; Cooper et al., 2001), encapsulation and nodulation (Valembois *et al.* 1992; Wieczorek-Olchawa *et al.* 2003) and phagocytosis (Adamowicz & Wojtaszek 2001; Homa *et al.* 2003). Changes in the numbers of these cells are affected by external environmental factors such heavy metal exposition (Homa *et al.* 2003; Plytycz *et al.* 2011) but also due to internal factors such an annual endogenous rhythm (Kurek & Plytycz 2003). Despite the soil they were collected from, both earthworm populations of *P. corethrurus* showed similar proportions of coelomocytes, indicating that the immune response of their circulating coelomocytes is similar. This result reveals that even under stressful environmental conditions, such the ones present in the geothermal field, the earthworm population is able to maintain the immune response in a level similar to the observed in its conspecifics, living in a more controlled and less stressful environment, such as the pineapple greenhouse. Such observation supports the assumption that P. corethrurus is able to tolerate a wide range of different environmental conditions, being therefore a relevant organism for ecological and ecotoxicological studies due to its survival capacity under stressful environmental conditions (Lavelle *et al.* 1987).

The thickness of the epidermis in *P. corethrurus* was significantly thinner in Furnas earthworms, as it was also observed by Cunha *et al.* (2011). These authors have suggested that such reduction in the thickness of the earthworm's epidermis is an adaptation to the surrounding atmosphere in volcanically-active soils, to increase the efficiency of gas exchanges and compensate for the hypoxia of the surronding atmosphere, which contrasts with the thick epidermis in conspecifics resident in pineapple greenhouses as exactly to what was found for another earthworm species that also inhabits the volcanic environment, an invasive megascoelecid earthworm *Amynthas gracilis* (Cunha et al, 2011). This agrees with the plausible conclusion that the earthworm's responses to the multi-stressor challenges in active volcanic soils are, like the adaptations of arthropods and vertebrates to hypoxic conditions (Fluck *et al.* 2007) or the case of the hydrothermal tubeworm (*Ridgeia piscesae*) (Andersen *et al.* 2006). These changes are probably plastic rather than constitutive, however the molecular possibilities should be investigated in order to reveal and understand the integrated modifications ranging from genetic and biochemical, to cellular and physiological levels of organisation in these volcanic earthworms (Cunha *et al.* 2011).

This study have clearly demonstrated a good correlation between the concentrations of distinct chemical elements in the soil and earthworm tissues for a widely spread pantropical species. Thus we propose that *Pontoscolex corethrurus* should be considered as a relevant bioindicator species for soil quality, since it reflects with accuracy the composition of a given soil, with a valuable independency of the surrounding environmental factors due to its environmental tolerance and plasticity.

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REFERENCES

Adamowicz A. (2005). Morphology and ultrastructure of the earthworm *Dendrobaena veneta* (Lumbricidae) coelomocytes. *Tissue and Cell*, 37, 125-133.

Adamowicz A. & Wojtaszek J. (2001). Morphology and phagocytotic activity of coelomocytes in *Dendrobaena veneta* (Lumbricidae). *Zoologica Poloniae*, 46, 91-104.

Affar E., Dufour M., Poirier G. & Nadeau D. (1998). Isolation, purification and partial characterization of chloragocytes from the earthworm species *Lumbricus terrestris. Molecular and cellular biochemistry*, 185, 123-133.

Aiuppa A., Allard P., D'Alessandro W., Michel A., Parello F., Treuil M. & Valenza M. (2000). Mobility and fluxes of major, minor and trace metals during basalt weathering and groundwater transport at Mt. Etna volcano (Sicily). *Geochim. Cosmochim. Acta*, 64, 1827-1841.

Amaral A. & Rodrigues A (2011). Volcanogenic contaminants: Chronic exposure. *In*: Nriagu, J., ed. Encyclopedia on Environmental Health. pp. 681-689. Elsevier B.V.

Amaral A., Cabral C., Guedes C & Rodrigues A. (2007). Apoptosis, metallothionein, and bioavailable metals in domestic mice (*Mus musculus* L.) from a human-inhabited volcanic area. *Ecotoxicology*, 16: 475-482.

Andersen A., Flores J. & Hourdez S. (2006). Comparative branchial plume biometry between two extreme ecotypes of the hydrothermal vent tubeworm *Ridgeia piscesae. Can. J. Zool.*, 84, 1810-1822.

Bagnato E., Aiuppa A., Parello F., Calabrese S., D'Alessandro W., Mather T., McGonigle A., Pyle D. & Wängberg I. (2007). Degassing of gaseous (elemental and reactive) and particulate mercury from Mount Etna volcano (Southern Italy). *Atmos. Environ.*, 41, 7377-7388.

Barrow N. (1986). Testing a mechanistic model. II. The effects of time and temperature on the reaction of zinc with a soil. *Eur. J. Soil Sci.*, 37, 277-286.

Buch A.C., Brown G.G., Niva C.C., Sautter K.D. & Louren\ccato L.F. (2011). Life cycle of *Pontoscolex corethrurus* (M\"uller, 1857) in tropical artificial soil. *Pedobiologia*, in press.

Cruz J., Coutinho R., Carvalho M., Oskarsson N. & Gislason S. (1999). Chemistry of waters from Furnas volcano, Sao Miguel, Azores: fluxes of volcanic carbon dioxide and leached material. *J. Volcanol. Geotherm. Res.*, 92, 151-167.

Cunha L., Amaral A., Medeiros V., Martins G., Wallenstein F., Couto R., Neto A. & Rodrigues A. (2008). Bioavailable metals and cellular effects in the digestive gland of marine limpets living close to shallow water hydrothermal vents. *Chemosphere*, 71, 1356-1362.

Cunha L., Campos I., Montiel R., Rodrigues A. & Morgan A.J. (2011). Morphometry of the epidermis of an invasive megascoelecid earthworm (*Amynthas gracilis*, Kinberg 1867) inhabiting actively volcanic soils in the Azores archipelago. *Ecotoxicol. Environ. Saf.*, 74, 25-32.

Ferreira T. & Oskarsson N. (1999). Chemistry and isotopic composition of fumarole discharges of Furnas caldera. *J. Volcanol. Geotherm. Res.*, 92, 169-179.

Fluck M., Webster K.A., Graham J., Giomi F., Gerlach F. & Schmitz A. (2007). Coping with cyclic oxygen availability: evolutionary aspects. *Integr. Comp. Biol.*, 47, 524-531.

Goyer R. (1997). Toxic and essential metal interactions. *Annu. Rev. Nutr.*, 17, 37-50.

Hall-Spencer J., Rodolfo-Metalpa R., Martin S., Ransome E., Fine M., Turner S., Rowley S., Tedesco D. & Buia M. (2008). Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature (Lond.)*, 454, 46-47.

Homa J., Niklinska M. & Plytycz B. (2003). Effect of heavy metals on coelomocytes of the earthworm *Allolobophora chlorotica*:: The 7th international symposium on earthworm ecology\textperiodcentered Cardiff\textperiodcentered Wales\textperiodcentered 2002. *Pedobiologia*, 47, 640-645.

Homa J., Olchawa E., Sturzenbaum S.R., John Morgan A. & Plytycz B. (2005). Early-phase immunodetection of metallothionein and heat shock proteins in extruded earthworm coelomocytes after dermal exposure to metal ions. *Environmental Pollution*, 135, 275-280.

Hu C., Li M., Cui Y., Li D., Chen J. & Yang L. (2010). Toxicological effects of TiO2 and ZnO nanoparticles in soil on earthworm *Eisenia fetida. Soil Biology and Biochemistry*, 42, 586-591.

Huynh T.M.D., Diep T.M.H., Pham-Thi A.T. & Rouland-Lefevre C. (2011). Enhanced Lead Phytoextraction of Lantana camara L. by a Biological Agent, Earthworm *Pontoscolex corethrurus. Asian Journal of Biological Sciences*, 4, 375-382.

Khan M., Ahmed S., Salazar A., Gurumendi J., Khan A., Vargas M. & von Catalin B. (2007). Effect of temperature on heavy metal toxicity to earthworm *Lumbricus terrestris* (Annelida: Oligochaeta). *Environ. Toxicol.*, 22.

Kurek A. & Plytycz B. (2003). Annual changes in coelomocytes of four earthworm species:: The 7th international symposium on earthworm ecology, Cardiff, Wales, 2002. *Pedobiologia*, 47, 689-701.

Lavelle P., Barois I., Cruz I., Fragoso C., Hernandez A., Pineda A. & Rangel P. (1987). Adaptive strategies of *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta), a peregrine geophagous earthworm of the humid tropics. *Biology and fertility of soils*, 5, 188-194.

Lavelle P., Lattaud C., Trigo D. & Barois I. (1995). Mutualism and biodiversity in soils. *Plant and Soil*, 170, 23-33.

Liang S.H., Chen S.C., Chen C.Y., Kao C.M., Yang J.I., Shieh B.S., Chen J.H. & Chen C.C. (2011). Cadmium-induced earthworm metallothionein-2 is associated with metal accumulation and counteracts oxidative stress. *Pedobiologia*.

Marinussen M.P.J.C. & Van Der Zee S.E. (1997). Cu accumulation by *Lumbricus rubellus* as affected by total amount of Cu in soil, soil moisture and soil heterogeneity. *Soil Biology and Biochemistry*, 29, 641-647.

Martoja R. & Martoja-Pierson M. (1970). *Técnicas de histología animal*. Toray-Masson, Barcelona.

Morgan J. & Morgan A. (1998). The distribution and intracellular compartmentation of metals in the endogeic earthworm *Aporrectodea caliginosa* sampled from an unpolluted and a metal-contaminated site. *Environ. Pollut.*, 99, 167-175.

Morgan J. & Morgan A. (1999). The accumulation of metals (Cd, Cu, Pb, Zn and Ca) by two ecologically contrasting earthworm species (*Lumbricus rubellus* and *Aporrectodea caliginosa*): implications for ecotoxicological testing. *Appl. Soil Ecol.*, 13, 9-20.

Morgan J.E. & Morgan A.J. (1993). Seasonal changes in the tissue-metal (Cd, Zn and Pb) concentrations in two ecophysiologically dissimilar earthworm species: pollution-monitoring implications. *Environ. Pollut.*, 82, 1-7.

Peijnenburg W. (2002). Bioavailability of metals to soil invertebrates. In: *Bioavailability of metals in terrestrial ecosystems: importance of partitioning for bioavailability to invertebrates, microbes, and plants* (ed. Allen HE). SETAC Pensacola, Florida, pp. 89–112.

Plytycz B., Kielbasa E., Grebosz A., Duchnowski M. & Morgan A.J. (2010). Riboflavin mobilization from eleocyte stores in the earthworm *Dendrodrilus rubidus* inhabiting aerially-contaminated Ni smelter soil. *Chemosphere*, 81, 199-205.

Plytycz B., Klimek M., Homa J., Mazur A.I., Kruk J. & Morgan A.J. (2011). Species-specific sensitivity of earthworm coelomocytes to dermal metal (Cd, Cu, Ni, Pb, Zn) exposures: methodological approach. *Pedobiologia*, in press.

Ribeiro D.C., Martins G., Nogueira R., Cruz J.V. & Brito A.G. (2008). Phosphorus fractionation in volcanic lake sediments (Azores - Portugal). *Chemosphere*, 70, 1256-1263.

Rodrigues A., Cunha L., Amaral A., Medeiros J. & Garcia P. (2008). Bioavailability of heavy metals and their effects on the midgut cells of a phytopaghous insect inhabiting volcanic environments. *Sci. Total Environ.*, 406, 116-122.

Sandifer R. & Hopkin S. (1997). Effects of Temperature on the Relative Toxicities of Cd, Cu, Pb, and Zn *toFolsomia candida* (Collembola). *Ecotoxicol. Environ. Saf.*, 37, 125-130.

Soames J., Macleod R. & Kelly P. (1995). Feulgen hydrolysis profiles and acid-labile DNA in oral squamous cell carcinoma. *European Journal of Cancer. Part B: Oral Oncology*, 31, 222-226.

Somero G., Chow T., Yancey P. & Snyder C. (1977). Lead accumulation rates in tissues of the estuarine teleost fish, *Gillichthys mirabilis*: salinity and temperature effects. *Arch. Environ. Contam. Toxicol.*, 6, 337-348.

Suthar S., Singh S. & Dhawan S. (2008). Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu, Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological category? *Ecol. Eng.*, 32, 99-107.

Valembois P., Lassègues M. & Roch P. (1992). Formation of brown bodies in the coelomic cavity of the earthworm *Eisenia fetida andrei* and attendant changes in shape and adhesive capacity of constitutive cells. *Developmental & Comparative Immunology*, 16, 95-101.

Viveiros F., Ferreira T., Cabral Vieira J., Silva C. & Gaspar J.L. (2008). Environmental influences on soil CO2 degassing at Furnas and Fogo volcanoes (São Miguel Island, Azores archipelago). *J. Volcanol. Geotherm. Res.*, 177, 883-893.

Viveiros F., Ferreira T., Silva C. & Gaspar J. (2009). Meteorological factors controlling soil gases and indoor CO2 concentration: A permanent risk in degassing areas. *Sci. Total Environ.*, 407, 1362-1372.

Wallenstein F.M., Couto R.P., Amaral A.S., Wilkinson M., Neto A.I. & Rodrigues A.S. (2009). Baseline metal concentrations in marine algae from São Miguel (Azores) under different ecological conditions-Urban proximity and shallow water hydrothermal activity. *Journal of Marine Pollution Bulletin*, 58, 424-455.

Wieczorek-Olchawa E., Niklinska M., Miedzobrodzki J. & Plytycz B. (2003). Effects of temperature and soil pollution on the presence of bacteria, coelomocytes and brown bodies in coelomic fluid of *Dendrobaena veneta*: The 7th international symposium on earthworm ecology\textperiodcentered Cardiff, Wales, 2002. *Pedobiologia*, 47, 702-709.

Zar J. (1999). *Biostatistical analysis* 4th edn. Simon & Shuster, New Jersey.

Chapter 4

The crawling of earthworm mitochondrial DNA inside a volcanic crater

The crawling of earthworm mitochondrial DNA inside a volcanic crater

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ABSTRACT

The communities of soil organisms that occupy terrestrial geothermal vents provide an unusual opportunity to study the dynamics of population structure and genetic diversity. The presence and high abundance of *Pontoscolex corethrurus* in the Furnas geothermal field may indicate its relative tolerance to the multifactorial stress challenge posed by the active volcanic environment. Furthermore, the extent of genetic diversity in *P. corethrurus* populations in the Azores archipelago is not known and neither do we presently know the timing and source(s) of such colonization. We analysed 60 individuals from 3 different locations in the island of São Miguel, Azores. Molecular markers included the mitochondrial regions of the cytochrome c oxidase subunit I gene (COI), small ribosomal unit (s- rRNA), the NADH desidrogenase subunit 2 and 3. Analyses of the data using different approaches corroborate the existence of two different genetic lineages living in São Miguel Island. Furnas population showed lower genetic diversity when compared to the populations living within pineapple plantations. The importance of genetic reduction in the population genetic structure of earthworms living under the stress of the volcanic environment is further discussed.

Keywords: *Pontoscolex corethrurus, mitochondrial markers, volcanic soils, genetic erosion, haplotypes, Azores*

INTRODUCTION

The communities of soil organisms that occupy terrestrial geothermal vents provide an unusual opportunity to study the dynamics of population structure and genetic diversity as similar to the communities close to deep hydrothermal vents. In fact geothermal biotopes are reducing environments with certain unique features, mainly characterised by elevated soil, water, and atmospheric elemental composition, together with constant diffuse degassing and high temperatures (Cruz *et al.* 1999; Viveiros *et al.* 2008; Viveiros *et al.* 2009). Volcanic gases in Furnas geothermal field typically comprise water vapour, carbon dioxide, sulphur dioxide, hydrogen sulphide, and hydrogen chloride with lesser amounts of hydrogen fluoride (Ferreira & Oskarsson 1999). Rocks and volatiles of volcanic origin are enriched with metals/metalloids such as Al, As, Cu, Hg, Pb, and Zn (Ferreira & Oskarsson 1999; Aiuppa *et al.* 2000), with the diffusion of acidic volcanic gases through the rocks mobilizing the metals in associated soils and water bodies (Bagnato *et al.* 2007). We have previously collected and identified the native earthworm *Pontoscolex corethrurus* (Müller, 1856), from the geothermal field at Furnas. *P. corethrurus* is a well-known invasive species of most tropical regions and its distribution seems to be only limited by temperature around the globe. Actually, being a geophagous endogeic species (Lavelle *et al.* 1987; Buch *et al.* 2011), *P. corethrurus* shows high plasticity which accordingly with Lavelle et al. (1987) is related to its wide range tolerance to soil physico-chemical characteristics, to moisture and temperature conditions, a very efficient assimilation of low-quality soil organic matter allowing it to survive even in very poor soils (Lavelle *et al.* 1987). The presence and high abundance of this species in the geothermal field may indicate its relative tolerance to the multifactorial stress challenge posed by the active volcanic environment. Furthermore, the extent of genetic diversity in *P. corethrurus* populations in the Azores archipelago is not known. Neither do we presently know the timing and source(s) of the colonization of individual islands in the chain. It is likely that whether the colonization was a singular or multiple event(s), and whether the main immigration agency was 'natural' or anthropogenic, the species has probably experienced genetic bottlenecking (Slatkin 1985, 1987). If this supposition is correct, then it may be predicted that overall genetic diversity within the species in Furnas volcanic crater is low compared with that in potential source populations living outside the crater. Therefore, under such conspicuous environment the demographic instability probably is influencing the genetic diversity within and among discrete populations. This can be due to mutations, genetic drift due to finite population size, and even natural selection supporting adaptations to local environmental conditions which all will lead to the genetic differentiation of the local populations (Slatkin 1985, 1987; Bossart & Pashley Prowell 1998). The primary purpose of determining intra-specific genetic diversity is to provide an insight into the variability available for natural selection in the locally stressed locations (Andre *et al.* 2010a). The general objective of the present investigation was to investigate the genetic diversity and population structure of a native earthworm in volcanic soils, the geophagous *P. corethrurus* by using mitochondrial (mtDNA) markers to genotype *P. corethrurus* from populations with different exposure histories at three environmentally contrasting sites along the island of S. Miguel in the Azores Archipelago. The particular objectives were to determine: (i) the phylogenetic relation between both *P. corethrurus populations*; and (ii) whether or not within-population genetic diversity has been locally reduced.

MATERIALS AND METHODS

Earthworm sources and collection sites

The Azores archipelago comprises nine islands and is located in the North Atlantic Ocean, between 36º45'–39º43'N and 24º45'–31º17'W, at the triple junction of Eurasian, African and North American plates, characterized by a complex tectonic settlement, where seismic and volcanic phenomena are common (Booth *et al.* 1978). São Miguel is the largest island (757 km^2), which presents several active volcanic spots including fumarolic fields, cold and thermal springs and soil diffuse degassing (Viveiros et al., 2008). *P. corethrurus* in the Azores seems to be delimited to the hot soils of the degasing fields in Furnas and inside agricultural greenhouses of pineapple culture around the island. The sampling sites on São Miguel, differing in their contemporary geogenic traits were selected for earthworm capture: (a) Furnas, which displays the most conspicuous degassing and geothermal activity in the entire Azores archipelago, and in pineapple greenhouses in (b) Fajã de Baixo and (c) Vila Franca do Campo. A group of 20 adults (clitellate) P. corethrurus from the geothermal field soils in Furnas (37º 46' 24.6'' N 25º 18' 10.3'' W), another group randomly chosen in two pineapple greenhouse soils (with an average pH of 5.5) in Fajã de Baixo (37º45'12.2N 25º38'21.3''W) and in Vila Franca (37º45'12.5N 25º24'18.3''W) were collected by digging and hand-sorting during summer of 2011 (See Figure 1).. After sampling, the earthworms were immediately transferred to the laboratory, where they were depurated of gut contents by placing them on moistened paper for 36 h.

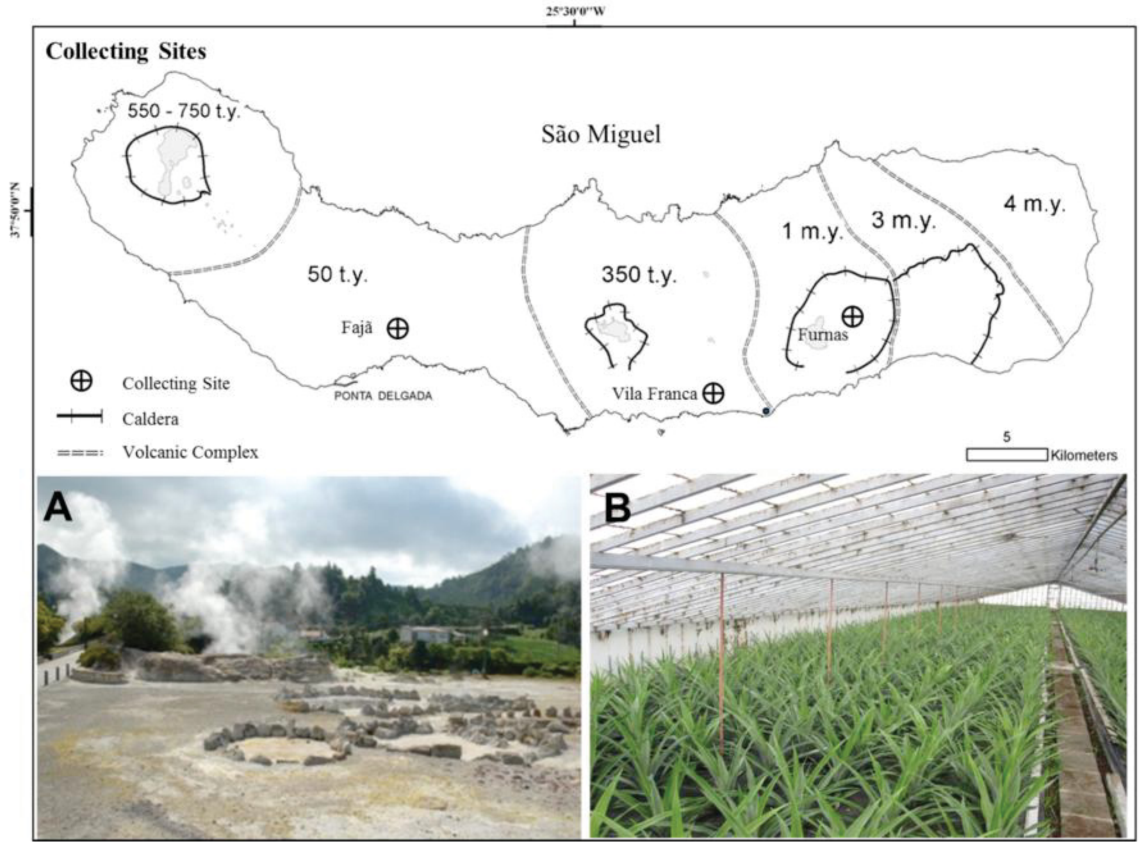

Figure 1. Collecting sites in São Miguel Island. M.y. million years; t.y., thousand years. A, Furnas geothermal field and B, pineapple greenhouse in Fajã de Baixo.

DNA extraction

For DNA extraction 2–3 segments of the caudal end of each individual were used. Any gut content was removed and the remaining tissue was cut into pieces and subjected to a phenol-chloroform protocol (Sambrook & Russell 2001) with the following modifications: the lysis step was accomplished in 2h using 180 µl of ATL Buffer (Qiagen) and 20 µl of Proteinase K (600 mAU/ml, Qiagen) and the DNA pellet was finally dissolved in 50 µl sterile water. DNA concentration was measured using NanoDrop® (ND-1000 spectrophotometer, Thermo Scientific) to dilute extracts with sterile water to 20 ng/µl prior to PCR. All DNA extracts were stored at −20 °C.

Mitochondrial DNA cloning

Due to the paucity of mitochondrial sequence information available for *Pontoscolex corethrurus* it was necessary to retrieve primary sequence information by cloning. A large isolated mitochondrial DNA fragment (4420 bp) including all the NADH dehydrogenase subunits genes as well the adjacent t-RNAs was initially isolated. Briefly, this was achieved by taking earthworm tissue frozen in liquid N_2 and grinding vigorously in a mortar and pestle. One individual was used for the extraction of the mitochondrial fraction by sequential centrifugations using a sucrose density gradient modified from *Wolstenholme et al. (1972) for mitochondria enrichment.* Briefly, to 1 vol of sample were added 5 vol of isolation buffer composed of 0.3 M sucrose, 1 mm disodium ethylenediaminetetraacetate (EDTA), and 0.01 M Tris .HCl (pH 7.4) at maintained4°C with subsequent centrifugation at 480 g for 10 min. The supernatant was removed into a fresh tube and further centrifuged at 9000 g for 12 min. The resulted pellet was incubated with Deoxyribonucleate 5′-oligonucleotido-hydrolase (DNAse I) (Sigma-Aldrich), neutralized with 0.04M EDTA and centrifugated at 9000 g for 5 min. The mitochondrial pellet thus generated was suspended in 0.15 M NaCl, 0.1 M EDTA, and 0.01 M Tris–HCl (pH 8.0) and lysed by adding 0.1 vol of 18% sodium dodecyl sulfate (SDS) and 0.2 vol of 5 M NaCl. After centrifuging, the supernatant was incubated with RNase A, and total DNA was extracted with phenol-chloroform (Sambrook & Russell 2001), ethanol precipitated, and resuspended in nuclease free ultrapure water (Milli-Q). Extracted DNA was purified by column (Quiagen®), sheared by nebulization and end repaired according with pJet 2.1 cloning kit recommendations. Efficient random cloning of sheared DNA (~4.5kb fragments) was accomplished with the commercial vector pJet 2.1 (Fermentas). Plasmid clone was amplified from both ends with standard primers, digested with *Hind*III and *Bgl*II (Bioline®) and the resulted fragments cloned as above and sequenced with standard pJET kit primers on an ABI PRISM® BigDye v3.1 Terminator Sequencing technology (Applied Biosystems, USA) on the ABI PRISM® 3100 DNA sequencer run by the genomics department of LANGEBIO, Mexico. Raw sequence traces were confirmed using Finch TV (Geospiza, http://www.geospiza.com/ finchTV) before being imported into CLC Genomics Workbench v6.6 (CLC Bio, Aarhus, Denmark) for the assembling of the full 4420 bp fragment (See Annex I).

Mitochondrial DNA genotyping

A total of three different fragments including the small ribosomal unit, the Cytochrome oxidase subunit I and the NADH *dehydrogenase* subunit 2 and 3 with some adjacent t-RNAS (Ser, Lys) (see Table I) were used to genotype the mitochondrial DNA of P. corethrurus populations. Primers were designed using Primer Premier v5 (Lalitha 2000).

Table I. The different primer pairs used and the respective fragment size and loci amplified.

Locus covered	Sequence 5' – 3'	Fragment Length	Based on Sequence
Small ribossomal subunit	fwd-CATACCCCAAAAAAGAGAAACC rev-AGGCCTATAGTGTGTTTAAGTG	1243	This study (Annex I)
Cytocrome oxidase subunit I	fwd-GAAGTGTGGGCTGGGATAATCG rev-TGTGGTGGGCTCATACAATGAA	802	JN036370 (PUBMED)
t-RNA-Lys, NAD subunit 3, t-RNA-Ser, NAD subunit 2	fwd-TAGTGCCGGAATAAACGGATAA rev-CAAGGAATGCGCCTGGTTGACT	1697	This study (Annex I)

For each PCR reaction ~40 ng DNA template was amplified using 0.4 pmol/µl forward and reverse primer, 0.2 mM dNTP mix and 1.25U/µl GO Taq DNA polymerase buffered with 1X Mg-free GO Taq Buffer (Promega). PCR amplification buffer was supplemented with $MgCl_2$ to achieve a final concentration of 1.75 mM. The reaction was denatured at 95 C for 3 min and then cycled 35 times at 95 C for 30 s, 30 s at the required primer annealing temperature and 72 °C for 1 min for COI and s-rRNA and 1 min 45 s for the large fragment containing the NAD subunit 2 and NAD subunit 3. This was followed by a 10 min final extension at 72 C. The amplicons were resolved by electrophoresis in 1 X TAE buffer at 90 V for 30 min in a Pharmacia GNA-100 tank.

Nucleic acid bands were then visualised on a UV gel documentation system. Prior to sequencing PCR clean-ups 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 the ABI PRISM® 3100 DNA Sequencer run by the Cardiff University Molecular Biology Support Unit. Raw sequence traces were confirmed using FinchTV (Geospiza,http://www.geospiza.com/finchTV) before being imported into Mega v5.05 (Kumar *et al.* 2008) for alignment and tree construction. Good quality nucleotide sequences (COI:381 bp, s-rRNA:455 bp, ND2: 724 bp, ND3: 889 bp) were aligned from 60 *P. corethrurus* earthworms and sequences from *Lumbricus Terrestris* (NC_001673), *Lumbricus rubellus* (provided by P. Kille) amd *Perionyx excavates* (EF494507) as outgroups.

Phylogenetic Analysis

Relationships between phylogenetic haplotypes were determined by maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods using Mega v5.05 (Kumar *et al.* 2008) and MrBayes respectively (Huelsenbeck & Ronquist 2001). Genes were concatenated using DAMBE (Xia & Xie 2001). JModeltest v0.1.1 (Posada 2008) and the Akaike Information Criterion (AIC) (Akaike 1973) were used to select the optimum model of sequence evolution in order to fit the data of each individual gene. See Table 2 for individual gene base frequencies and best fitted model. In the concatenated matrix unlinked nucleotide substitution models selected by jModelTest were specified for each gene fragment and the nucleotide substitution estimates were allowed to vary independently between each partition (Novo *et al.* 2011). Parameters in MrBayes were set to 10 million generations and 10000 trees were sampled for every 500th generation, using the default random tree option to initiate the analysis. The analysis was performed twice and all sample points prior to the plateau phase were discarded as burn-in. The remaining trees were combined to find the maximum a posteriori probability estimate of phylogeny. Genetic distances were calculated using p-distance in Mega and median-joining networks (Bandelt *et al.* 1999) were drawn using Network v4.6.1.0 (Bandelt *et al.* 1999) and DnaSPsp v5 (Librado & Rozas 2009) after star contraction (Forster *et al.* 2001) for very similar haplotypes. A maximum likelihood (ML) and Maximum parsimony (MP) analysis was performed with Mega v5.05 (Kumar *et al.* 2008) using simultaneous NNI to estimate tree topology. The best-fit evolutionary model selected by jModelTest for the concatenated sequences was specified and the program optimized. For ML clade support was evaluated by 1000 replicates of non-parametric bootstrapping (Felsenstein 1985, 2004).

RESULTS

Mitochondrial DNA haplotypes and phylogenetic trees

The genetic variability values within the analysed *Pontoscolex* population's data set are shown in Table II according to nomenclature used by Novo et al (2011). The mitochondrial ND3 and ND2 were the most variable regions among the populations, showing the higher values for polymorphic sites and number of mutations among the studied genes with values of 189 for ND3 (with 194 mutations), a value of 183 polymorphic sites for ND2 (with 188 mutations). The COI and s-rRNA genes were far less variable, showing values of 26 polymorphic sites in s-rRNA (with 26 mutations) and 29 polymorphic sites in COI (with 30 mutations found). Haplotypes were also lower in Furnas (with the exception of COI) with only 1 main haplotype for the s-rRNA (in a total of 7 haplotypes), 4 haplotypes for ND3 (in a total of 11 haplotypes), 2 haplotypes for ND2 (in a total of 13 haplotypes) and 5 haplotypes for COI (in a total of 7 haplotypes) as well low nucleotide diversity. Interestingly the COI in earthworms of Furnas showed the highest number of point mutation with a value of 23 mutations in a total of 30 mutation sites in the total among all populations. The genes with higher parsimony informative sites were the ND2 and the ND3 which may indicate different substitution rates within these genes. In general the genetic diversity within the population was conspicuously lower in Furnas earthworms when compared to both populations in Fajã de Baixo and Vila Franca. The multiple sequence alignment of the concatenated sequence data used for phylogenetic analyses was 2449 bp in length and descriptive genetics can be viewed in Table III. Using the concatenated fragment with all the genes, the pattern of low genetic diversity in Furnas seems the same although the data is more homogeneous and the total parsimony informative sites reach a total of 450 sites, with a total of 427 polymorphic sites and 438 mutations, when comparing all the populations. Considering the uncorrected p-distances, the population in Furnas showed to be more divergent when compared with the populations at pineapple greenhouses showing a value of 13.5% to Vila Franca and 13.2% to Fajã de Baixo, although the distance between Vila Franca and Fajã de Baixo was only of 1.4% (See Table IV). Interestingly, uncorrected p-distances of individual genes show a wide range of variation, with a value of 19% for ND3; 21% for ND2; and less than 1% for COI and s-rRNA (See Table V), which may indicate that the substitution rate in ND2-3 is very different for those in COI and s-rRNA or that another genomic mechanism is the underlying cause of such genetic distance.

In the concatenated alignment, we found a total of 36 haplotypes (Table VI). From these there were at least 1 major typical haplotype per pineapple greenhouses populations, with frequency values of 20 % for haplotype 9 in Fajã de Baixo, and 30% for haplotype 32 in Vila Franca. In Furnas there are two main haplotypes that share similar relative frequencies and together make 65% of the population, the haplotype 15 present in 35% of the sampled population and haplotype 20 present in 30% of individuals.

Based on the median joining algorithm (Bandelt *et al.* 1999) it was possible to draw a network that clearly aggregates the *Pontoscolex corethrurus* haplotypes in two distinct groups (Figure 2), one group mainly composed by the haplotypes present in Furnas earthworms with also one individual from Fajã de Baixo and another group composed with most of the haplotypes (19/20 individuals) of Fajã de Baixo earthworms and all the haplotypes present in Vila Franca population.

Likelihood ratio tests performed with jModelTest are described in Table VII. The best-fit models of sequence evolution are HKY+G for the concatenated data set, although the COI and the 16s were best fitted with the GTR+G model after the model test run for each gene.

Table II. Diversity values of the gene fragments used for the genotyping of mitochondrial DNA of *Pontoscolex* populations. N: Number of individuals analyzed. NHAP: Number of identified haplotypes; *H*: Haplotypic diversity; π: Nucleotidic diversity; AND: Average number of differences K. Ps: Polymorphic sites and IS: Number of Parsimony Informative Sites are showed in relation to the complete alignment.

	Genotyped Genes															
	s-rRNA (431pb)				**ND3 (878pb)**				**ND2 (717pb)**				**COI (383pb)**			
	Furnas	Fajã de Baixo	Vila Franca	Total	Furnas	Fajã de Baixo	Vila Franca	Total	Furnas	Fajã de Baixo	Vila Franca	Total	Furnas	Fajã de Baixo	Vila Franca	Total
N	20	20	20	60	20	20	20	60	20	20	20	60	20	20	20	60
NHAP	1	4	4	7	4	6	4	11	2	6	8	13	5	4	2	7
H	0	0.432	0.284	0.5	0.363	0.72	0.616	0.79	0.1	0.726	0.742	0.82	0.632	0.537	0.395	0.51
π	0	0.005	0.004	0.01	0.003	0.02	0.001	0.09	0	0.032	0.012	0.11	0.007	0.003	0.001	0
Mutations	0	9	18	26	22	174	4	194	2	170	30	188	23	9	1	30
AND	0	2.17	1.89	2.34	2.38	18.3	0.82	79.4	0.2	22.44	8.22	75.2	2.77	1.24	0.39	1.46
Ps	26				189				183				29			
IS	6				175				167				2			

Table III. Molecular genetics values of concatenated gene arrangement used for the analyses of *Pontoscolex* populations. N: Number of individuals analysed. NHAP: Number of identified haplotypes; *H*: Haplotypic diversity; π: Nucleotidic diversity; AND: Average number of differences K; Nt: Number of transition; Ntr: Number of transversions; Ns: Number of substitutions; Ni: Number of indels. Ps: Polymorphic Sites, and IS: Number of Parsimony Informative Sites are showed in relation to the complete alignment.

	Concatenated Genes (2449 pb)			
	Furnas	**Fajã de Baixo**	**Vila Franca**	**Total**
N	20	20	20	60
NHAP	8	14	14	36
H	0.805	0.953	0.937	0.967
π	0.018	0.002	0.005	0.066
Mutations	47	362	53	438
Nt	217	21	19	257
Ntr	148	26	34	208
Ns	365	47	53	465
Ni	6	4	10	20
AND	5.35	44.15	11.32	158.45
Ps	427			
IS	350			

Table IV. Values for the average p-distance calculated for the concatenated gene fragment of populations of *Pontoscolex corethrurus* from the volcanic geothermal field Furnas, pineapple greenhouses in Fajã de Baixo and Vila Franca and the outgroup composed by *Peryonix excavates*, *Lumbricus terrestris* and *Lumbricus rubellus*. The number of base differences per site from averaging over all sequence pairs between groups are shown. The analysis involved 47 nucleotide sequences (collapse of identical haplotypes). All positions containing gaps and missing data were eliminated. There were a total of 2359 positions in the final dataset.

		Population 2		
		Fajã de Baixo	**Vila Franca**	**Outgroup**
Population 1	**Furnas**	0.132	0.135	0.330
	Fajã de Baixo	-	0.014	0.339
	Vila Franca	-	-	0.337

Table V. Values for the average p-distance for ND3 (A), ND2 (B), COI (C), and s-rRNA (D) gene between populations of *Pontoscolex corethrurus* from the volcanic geothermal field Furnas, pineapple greenhouses in Fajã de Baixo and Vila Franca and the outgroup composed by *Peryonix excavates, Lumbricus terrestris* and *Lumbricus rubellus.* The number of base differences per site from averaging over all sequence pairs between groups is shown. The analysis involved 60 nucleotide sequences (collapse of identical haplotypes). All positions containing gaps and missing data were eliminated.

A		**Population 2**		
		Fajã	**Vila Franca**	**Outgroup**
Population 1	**Furnas**	0.186	0.011	0.379
	Fajã	-	0.194	0.372
	Vila Franca	-	-	0.379
	Outgroup	-	-	-

B		**Population 2**		
		Fajã de Baixo	**Vila Franca**	**Outgroup**
Population 1	**Furnas**	0.206	0.209	0.378
	Fajã de Baixo	-	0.027	0.394
	Vila Franca	-	-	0.391
	Outgroup	-	-	-

C		**Population 2**		
		Fajã de Baixo	**Vila Franca**	**Outgroup**
Population 1	**Furnas**	0.005	0.004	0.219
	Fajã de Baixo		0.002	0.219
	Vila Franca	-	-	0.218
	Outgroup	-	-	-

D		**Population 2**		
		Fajã de Baixo	**Vila Franca**	**Outgroup**
Population 1	**Furnas**	0.008	0.002	0.26
	Fajã de Baixo	-	0.01	0.267
	Vila Franca	-	-	0.261
	Outgroup	-	-	-

Table VI. Descriptive of haplotypes and associated number of individuals in each site found for the concatenated gene analysis. Hap: haplotype. N = 20 for each site.

Haplotype	Individuals	Site	Haplotype	Individuals	Site
Hap_1:	1	Fajã de Baixo	Hap_19:	1	Furnas
Hap_2:	1	Fajã de Baixo	Hap_20:	6	Furnas
Hap_3:	1	Fajã de Baixo	Hap_21:	1	Furnas
Hap_4:	1	Fajã de Baixo	Hap_22:	1	Furnas
Hap_5:	1	Fajã de Baixo	Hap_23:	1	Vila Franca
Hap_6:	1	Fajã de Baixo	Hap_24:	1	Vila Franca
Hap_7:	2	Fajã de Baixo	Hap_25:	1	Vila Franca
Hap_8:	2	Fajã de Baixo	Hap_26:	1	Vila Franca
Hap_9:	4	Fajã de Baixo	Hap_27:	1	Vila Franca
Hap_10:	1	Fajã de Baixo	Hap_28:	1	Vila Franca
Hap_11:	1	Fajã de Baixo	Hap_29:	1	Vila Franca
Hap_12:	1	Fajã de Baixo	Hap_30:	2	Vila Franca
Hap_13:	2	Fajã de Baixo	Hap_31:	1	Vila Franca
Hap_14:	1	Fajã de Baixo	Hap_32:	5	Vila Franca
Hap_15:	7	Furnas	Hap_33:	1	Vila Franca
Hap_16:	1	Furnas	Hap_34:	2	Vila Franca
Hap_17:	1	Furnas	Hap_35:	1	Vila Franca
Hap_18:	2	Furnas	Hap_36:	1	Vila Franca

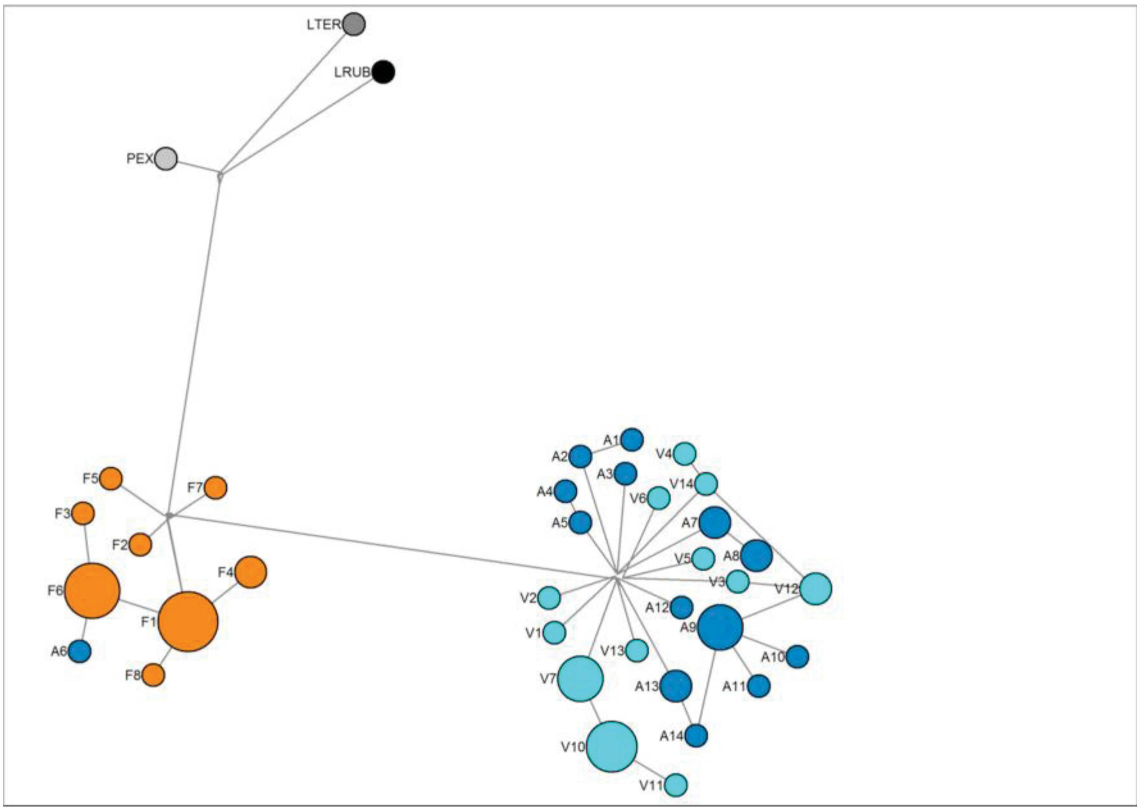

Figure 2. Median-joining (MJ) networks showing phylogenetic relationships between both populations of *Pontoscolex corethrurus* structure in volcanic and non-volcanic soils. The different colour shades refer to the screened *Pontoscolex corethrurus* populations, Furnas haplotypes (orange), Vila Franca pineapple greenhouses haplotypes (light blue), Fajã pineapple greenhouses (blue), *Lumbricus rubellus* (LRUB) and *Lumbricus terrestris* (LTER) (dark green) and *Perionyx excavates* (PEX) (light green). MJ-network based on the concatenated mitochondrial sequences alignment of 63 samples. Exact distances based in the p-distance calculation of the concatenated mitochondrial sequences are given in Annex II.

Table VII. Likelihood ratio tests performed with jModelTest for each gene assessed in the study and also for the concatenated genes.

Loci	Best fitted model	-lnL	fA	fC	fG	fT	Gamma Destribution shape
s-rRNA	GTR+G	1573.0825	0.3785	0.2177	0.1628	0.241	0.872
COI	GTR+G	1617.5566	0.2517	0.2472	0.1979	0.3033	2.025
ND2	HKY+G	3702.6792	0.3162	0.2395	0.1321	0.3122	0.569
ND3	HKY+G	4114.4944	0.325	0.2531	0.1247	0.2973	0.781
Concatenated Genes	HKY+G	11384.9007	0.323	0.2445	0.1362	0.2963	0.59

Intra- and inter-site evolutionary relationships were phylogenetically analysed in a tree based on the Bayesian inference (BI) and are shown in Figure 2. The studied populations of *Pontoscolex corethrurus* clearly reveal two different clades well supported by posterior probabilities. Although attention should be considered to the fact that one individual from Fajã de Baixo is inside cluster mainly composed by Furnas haplotypes. The posterior probability found for the two clusters reveals that Furnas population shows to be a different *Pontoscolex* lineage when compared with most of the individuals from Vila Franca and Fajã de Baixo. As there is no splitting among the haplotypes found in individuals from pineapple greenhouses populations in Fajã de Baixo and Vila Franca reveals that most of the individuals in greenhouses (39/40 individuals) belong to a unique lineage of *Pontoscolex corethrurus* which in fact show a large distance from those haplotypes in Furnas.

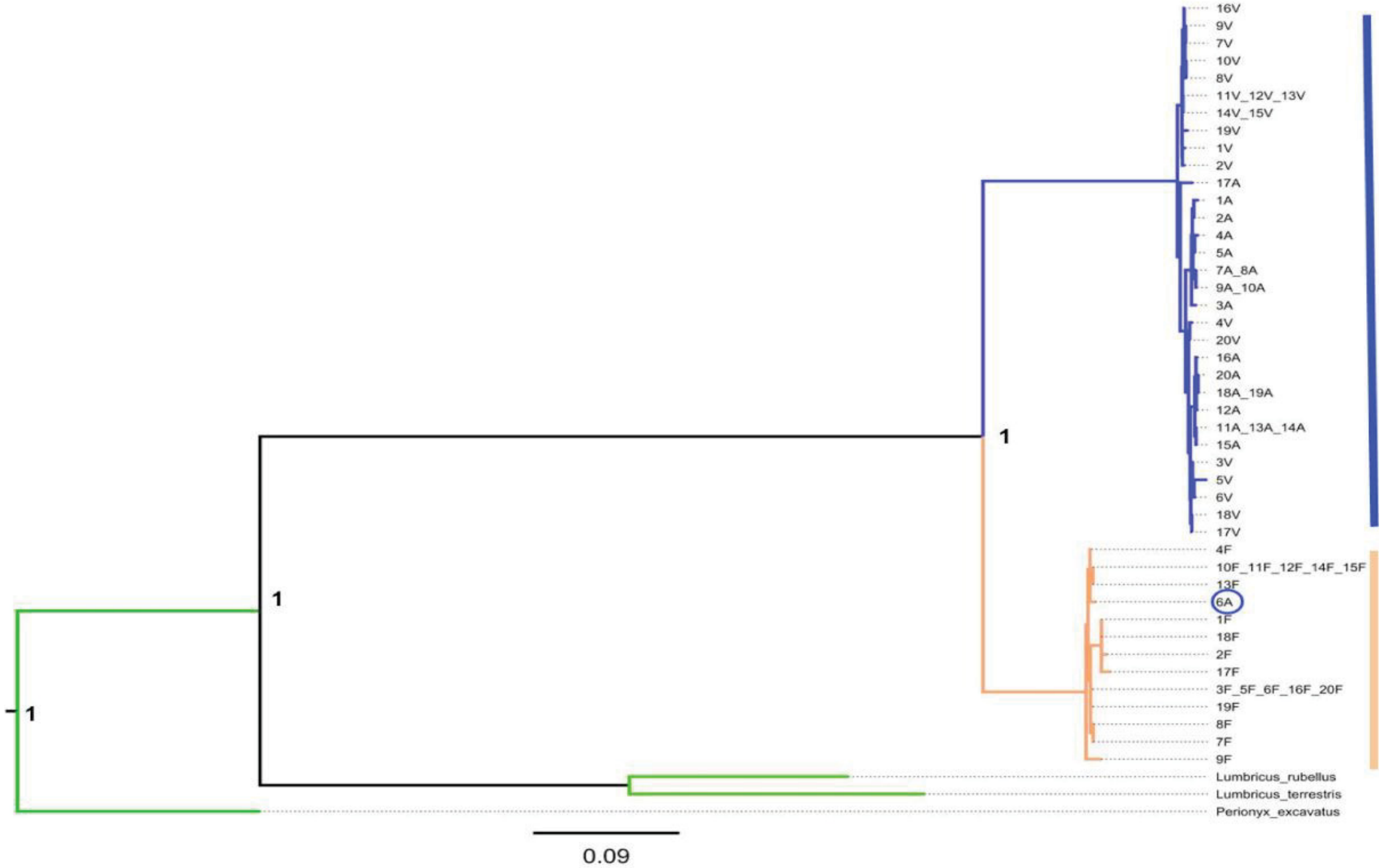

Figure 3. Bayesian phylogenetic hypothesis (BI) based on the four gene fragments amplified for *Pontoscolex* populations. Posterior probability values are shown on the nodes. Two *Pontoscolex* corethrurus lineages are apparent, termed lineage ‘A’ (blue) and ‘F’ (orange), respectively, with a mean inter-lineage mitochondrial DNA sequence divergence of approximately 18%. The outgroup branches were assigned with light green colour. Only one individual from

The maximum likelihood (ML) and maximum parsimony (MP) trees for the aligned data set are shown in Figure 4 and 5. All the individuals are shown and the resulting tree topology was supported by bootstrap analyses. According with trees, haplotypes in Furnas cluster together with one individual from Fajã de Baixo in a cluster clearly separated (bootstrap value = 100) from the group containing most of the haplotypes in Fajã de Baixo and all haplotypes in Vila Franca.

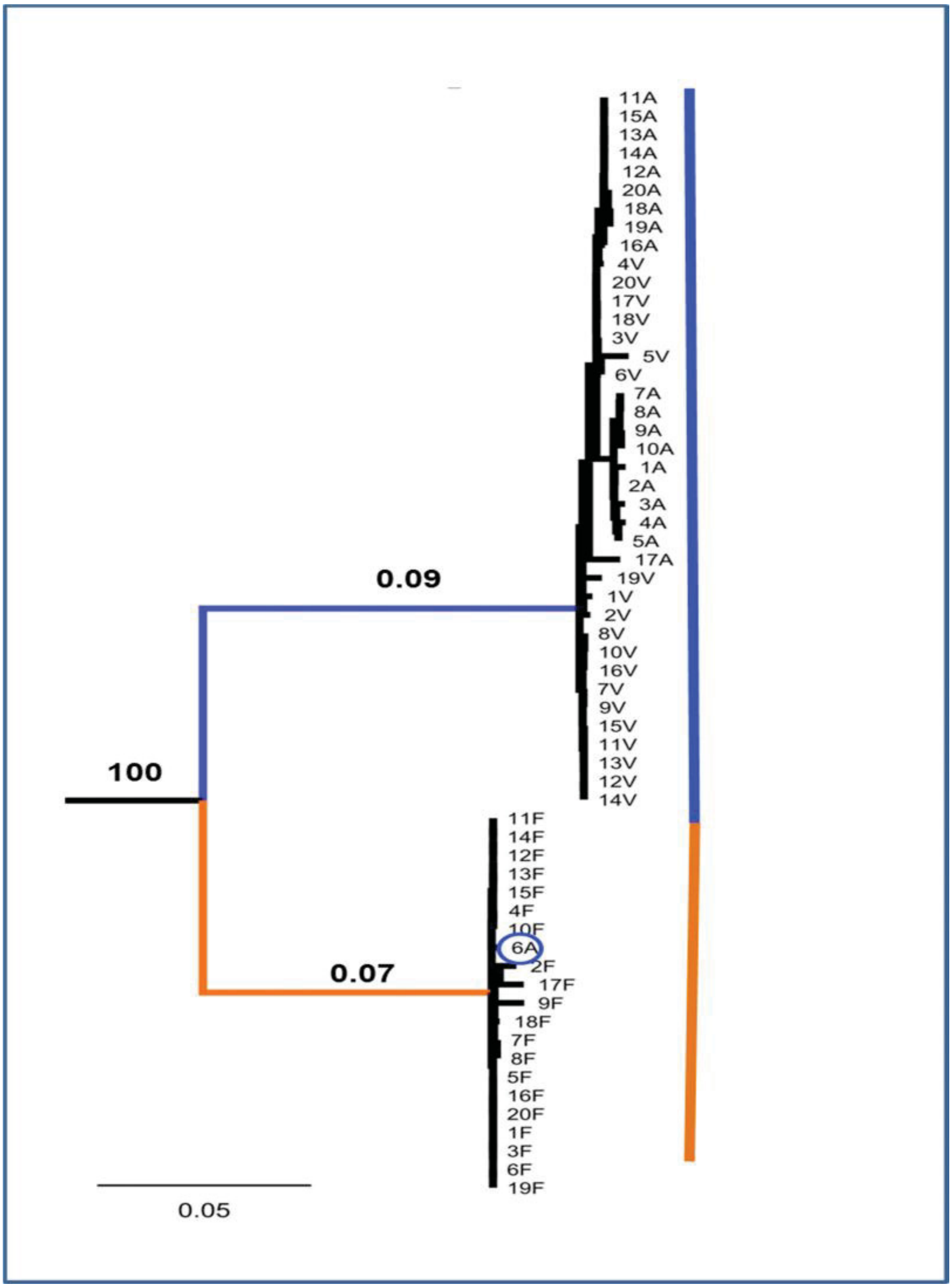

Figure 4. Maximum likelihood tree (ML) based on the four gene fragments amplified for *Pontoscolex corethrurus* populations. The outgroups were deleted from the tree. Two *Pontoscolex corethrurus* lineages are apparent, termed lineage 'A' (blue) and 'F' (orange), respectively, with a mean inter-lineage mitochondrial DNA sequence divergence of approximately 18%. The split between these two lineages was well supported by bootstrap

analyses (bootstrap value = 100). Only one individual from Fajã de Baixo was found in lineage 'F' (blue circle).

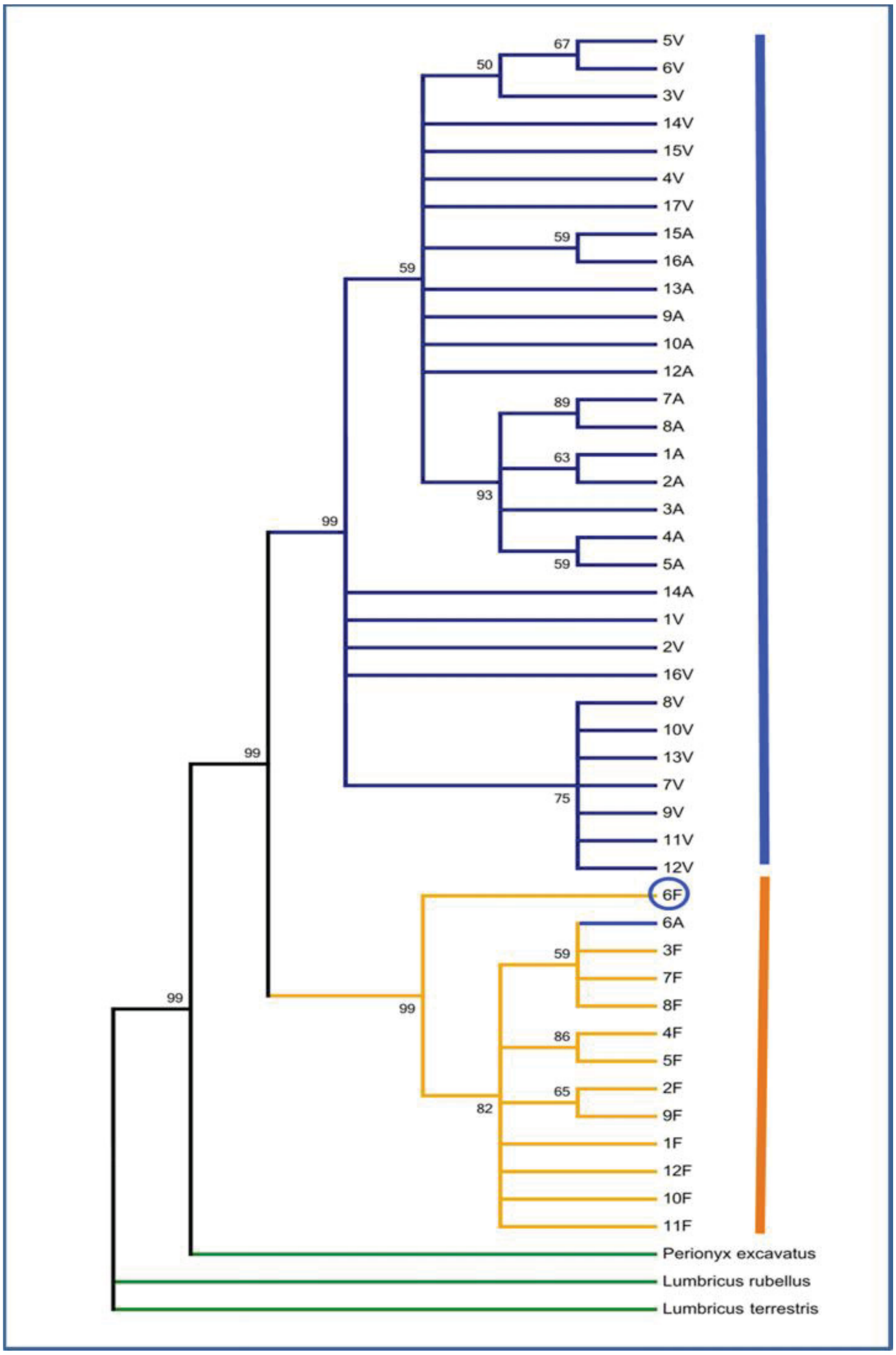

Figure 5. Maximum parsimony (MP) based on the four gene fragments amplified for *Pontoscolex corethrurus*. Bootstrap supports for MP are presented in the branches. Two *Pontoscolex corethrurus* lineages are apparent, termed lineage 'A' (blue) and 'F' (orange),

respectively, with a mean inter-lineage mitochondrial DNA sequence divergence of approximately 18%. The outgroup (*Perionyx excavatus*, *Lumbricus rubellus* and *Lumbricus terrestris*) was assigned with light green colour. The split between these two lineages, A and F, was well supported by bootstrap analyses (bootstrap value = 99). Only one individual from Fajã de Baixo was found in lineage 'F' (blue circle).

All trees share common features. Therefore the *Pontoscoclex* populations sampled in the Fajã de Baixo, Vila Franca and Furnas could be resolved into two distinct genetic lineages. The lineage A composed by 97.5% of the individuals sampled in Fajã de Baixo and Vila Franca and lineage F composed by all the individuals in Furnas and only 1 from Fajã de Baixo, with a mean inter-lineage mitochondrial DNA sequence divergence of 18% according to the Bayesian Inference and the 16% Maximum likelihood tree.

DISCUSSION

All the trees showed that haplotypes of the studied populations described as *Pontoscolex corethrurus* are exclusive to two major clades. Furthermore, there was one individual from Fajã de Baixo that showed to be close to the haplotypes found in Furnas. However, none of the Furnas haplotypes clustered together with any of those haplotypes found in Fajã de Baixo and Vila Franca cluster. Hence, it can be concluded that, based on the phylogenetic analysis, we are dealing with two genetically different phylogenetic lineages. The usual way that new species are described relies largely on the classical taxonomy assessment using a morphologically based classification system (Pfenninger & Schwenk 2007; Vrijenhoek 2009). However, speciation is not always necessarily paired with morphological differentiation (Hebert *et al.* 2004; Ahrens *et al.* 2007) and, actually cryptic or sibling species are typically found in taxa that succeed in complex, patchy, environments and have been discovered by genotyping fauna inhabiting diverse marine, freshwater, and terrestrial habitats (Hebert *et al.* 2004; Hilário *et al.* 2010; Boissin *et al.* 2011; Westram *et al.* 2011; Avrani *et al.* 2012).

In fact, as more studies are developed among the Annelida phylum the more we become aware that cryptic speciation is not an uncommon phenomena (Pfenninger & Schwenk 2007; Hilário *et al.* 2010). In fact, earthworms have been the target of several comprehensive phylogenetic studies using both mitochondrial and nuclear markers revealing high intra-species genetic diversity (Velavan *et al.* 2007; Novo *et al.* 2008) and deeply divergent genetic lineages, possibly in some cases corresponding with cryptic species (Pérez-Losada *et al.* 2009; Andre *et al.* 2010a; Dupont *et al.* 2011; Novo *et al.* 2011). Furthermore, is necessary to consider that the number of *Pontoscolex corethrurus* populations examined was too restricted to draw firm conclusions regarding micro-evolutionary genealogies, but the higher intra-lineage diversity of the earthworms living in pineapple greenhouses is indicative of a relatively stationary population that has undergone multiple introductions or with a very high initial genetic diversity. Moreover, the introduction of pineapple culture in the Azores could be one of the main introductions of *Pontoscolex corethrurus* into the island. However, we must consider that the first and only introduction of pineapple plants was during the first decade of the XIX century with the implementation of pineapple culture by José Bensaude (Stillman & Stillman 1999). It is tempting to relate the low haplotype diversity and apparent reduction in genetic variability in Furnas with natural selective pressures within the volcanic environment, although it is possible to be explained either by genetic drift or inbreeding. The genetic reduction can certainly be part of a small fragmented populations history such as those found inhabiting the 'islands of toxicity' that typify abandoned metal mine sites (Andre *et al.* 2010b). We also cannot detach the fact that the Lineage "F" in Furnas tolerates the unique combination of hyperthermic, anoxic, hypercarbic, and elevated metal-ion stress which constitutes a unique extreme environment far different from those soils inside the pineapple greenhouses. Patchiness, according with Wiens (1976) is organism-defined, and must be considered in terms of the perceptions of the organism rather than those of the investigator. In the case of the lineage "F" from the geothermal field, it is tempting to interpret the comparatively constricted genetic diversity as a hallmark of stress-driven genetic erosion processes related with natural environmental pressures of the geothermalism having acted upon these populations. Also, it must be considered that facultative parthenogenesis is believed to happen in *Pontoscolex corethrurus* (Lavelle *et al.* 1987) and parthenogenesis in the geothermal field inhabitants would not be surprising as this facultative reproductive strategy is highly associated to critical changes in the environment (Glesener & Tilman 1978; Lynch & Gabriel 1983). *Drosophila mercatorum* that has been maintained for several hundred generations has exhibited a gradual increase in parthenogenetic capacity despite the fact that it routinely has been maintained at a small size and occasionally reduced to a single individual (Templeton 1979). In fact, Templeton (1979) goes further and says that genetic revolutions are real phenomena that can quickly alter morphology, development, life history parameters and behaviour in organisms. Indeed, the alterations can be so drastic that a new "species" evolves, complete with pre- and post-mating isolating mechanisms. Therefore, a dioecious and poorly motile species, such as *P. corethrurus* when greatly reduced in abundance by environmental pressures, the probability of encountering a conspecific is reduced and it probably go through parthogenesis in order to assure the structure stability of the population. The species puzzle related to *Pontoscolex corethrurus* revealed in this study has been taken into consideration by Moreno (2004) and, although the existing use of this species in ecological studies (Barois & Lavelle 1986; Barois *et* *al.* 1993; Chapuis-Lardy *et al.* 1998; Chapuis-Lardy *et al.* 2010). Whether or not these high divergent *Pontoscolex corethrurus* lineages warrant the status of (cryptic) species must wait for further genetic and breeding evidence.

CONCLUSION

The apparently accepted fact that the ubiquitous *Pontoscolex corethrurus* is known to be the only peregrine representative from the families Glossoscolecidae (Hendrix) should be reassessed as this can be a group of genetically different species dispersed along the circumtropical region (Moreno 2004). Nevertheless, a physical barrier such as the volcanic crater and the around soil temperature isolating the Furnas population to dispersal can disrupt the possible migration rate between adjacent populations. In fact, genetic evolution models show that the establishment of a long-term physical barrier to dispersal abruptly reduces or stops gene flow between populations and leads to a rapid change in allele frequencies that may be inhibited or accelerated depending on the type of selection at specific loci (Slatkin 1987; Wiens 2004). This often leads to the divergence (by fixation of different mutations) (Slatkin 1985). Certain that the initial number of peregrines, their sources (i.e. migrant vs. introduced haplotype pools), and present rates of gene flow all affect the degree to which demographic unsteadiness affects geographical differentiation. Independent genealogies involving nuclear markers are needed to distinguish the effects of individual processes such as mitochondrial selective bends from demographic processes that leave genome-wide signatures. We also recommend that all the future research on micro or macroevolution, biogeography, ecology, conservation, and ecotoxicology on these taxa should consider their specific taxonomical status and the corresponding implications to the experimental designs. Consequently, we also recommend that researchers should maximize sequence length to increase the probability of sampling regions of high phylogenetic informative genes such the ND2 and ND3 in our study.

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REFERENCES

Ahrens D., Monaghan M.T. & Vogler A.P. (2007). DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). *Molecular Phylogenetics and Evolution*, 44, 436-449.

Aiuppa A., Allard P., D'Alessandro W., Michel A., Parello F., Treuil M. & Valenza M. (2000). Mobility and fluxes of major, minor and trace metals during basalt weathering and groundwater transport at Mt. Etna volcano (Sicily). *Geochim. Cosmochim. Acta*, 64, 1827-1841.

Akaike H. (1973). Information theory and an extension of the maximum likelihood principle. In. Springer Verlag, pp. 267-281.

Andre J., King R., Stürzenbaum S., Kille P., Hodson M.E. & Morgan A. (2010a). Molecular genetic differentiation in earthworms inhabiting a heterogeneous Pb-polluted landscape. *Environmental Pollution*, 158, 883-890.

Andre J., Sturzenbaum S.R., Kille P., Morgan A.J. & Hodson M.E. (2010b). Metal bioaccumulation and cellular fractionation in an epigeic earthworm (*Lumbricus rubellus*): The interactive influences of population exposure histories, site-specific geochemistry and mitochondrial genotype. *Soil Biology and Biochemistry*, 42, 1566-1573.

Avrani S., Ben-Shlomo R. & Inbar M. (2012). Genetic structure of a galling aphid *Slavum wertheimae* and its host tree *Pistacia atlantica* across an Irano-Turanian distribution: from fragmentation to speciation? *Tree Genetics & Genomes*, 1-10.

Bagnato E., Aiuppa A., Parello F., Calabrese S., D'Alessandro W., Mather T., McGonigle A., Pyle D. & Wängberg I. (2007). Degassing of gaseous (elemental and reactive) and particulate mercury from Mount Etna volcano (Southern Italy). *Atmos. Environ.*, 41, 7377-7388.

Bandelt H.J., Forster P. & Röhl A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular biology and evolution*, 16, 37-48.

Barois I. & Lavelle P. (1986). Changes in respiration rate and some physicochemical properties of a tropical soil during transit through *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta). *Soil Biology and Biochemistry*, 18, 539-541.

Barois I., Villemin G., Lavelle P. & Toutain F. (1993). Transformation of the soil structure through *Pontoscolex corethrurus* (Oligochaeta) intestinal tract. *Geoderma*, 56, 57-66.

Boissin E., Stöhr S. & Chenuil A. (2011). Did vicariance and adaptation drive cryptic speciation and evolution of brooding in *Ophioderma longicauda*

(Echinodermata: Ophiuroidea), a common Atlanto-Mediterranean ophiuroid? *Molecular ecology*.

Booth B., Croasdale R. & Walker G. (1978). A quantitative study of five thousand years of volcanism on Sao Miguel, Azores. *Philos. Trans. R. Soc. London, A*, 271-319.

Bossart J. & Pashley Prowell D. (1998). Genetic estimates of population structure and gene flow: limitations, lessons and new directions. *Trends in Ecology & Evolution*, 13, 202-206.

Buch A.C., Brown G.G., Niva C.C., Sautter K.D. & Louren\ccato L.F. (2011). Life cycle of *Pontoscolex corethrurus* (M\"uller, 1857) in tropical artificial soil. *Pedobiologia*, in press.

Chapuis-Lardy L., Brauman A., Bernard L., Pablo A.L., Toucet J., Mano M., Weber L., Brunet D., Razafimbelo T., Chotte J.L. & others (2010). Effect of the endogeic earthworm *Pontoscolex corethrurus* on the microbial structure and activity related to CO2 and N2O fluxes from a tropical soil (Madagascar). *Applied Soil Ecology*, 45, 201-208.

Chapuis-Lardy L., Brossard M., Lavelle P. & Schouller E. (1998). Phosphorus transformations in a ferralsol through ingestion by *Pontoscolex corethrurus*, a geophagous earthworm. *European journal of soil biology*, 34, 61-67.

Cruz J., Coutinho R., Carvalho M., Oskarsson N. & Gislason S. (1999). Chemistry of waters from Furnas volcano, Sao Miguel, Azores: fluxes of volcanic carbon dioxide and leached material. *J. Volcanol. Geotherm. Res.*, 92, 151-167.

Dupont L., Lazrek F., Porco D., King R., Rougerie R., Symondson W., Livet A., Richard B., Deca\.ens T., Butt K. & others (2011). New insight into the genetic structure of the *Allolobophora chlorotica* aggregate in Europe using microsatellite and mitochondrial data. *Pedobiologia*, 54, 217-224.

Felsenstein J. (1985). Phylogenies and the comparative method. *American Naturalist*, 1-15.

Felsenstein J. (2004). Inferring phytogenies. *Sunderland, Massachusetts: Sinauer Associates*.

Ferreira T. & Oskarsson N. (1999). Chemistry and isotopic composition of fumarole discharges of Furnas caldera. *J. Volcanol. Geotherm. Res.*, 92, 169-179.

Forster P., Torroni A., Renfrew C. & Röhl A. (2001). Phylogenetic star contraction applied to Asian and Papuan mtDNA evolution. *Molecular Biology and Evolution*, 18, 1864.

Glesener R.R. & Tilman D. (1978). Sexuality and the components of environmental uncertainty: clues from geographic parthenogenesis in terrestrial animals. *American Naturalist*, 659-673.

Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H. & Hallwachs W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 14812.

Hilário A., Johnson S., Cunha M. & Vrijenhoek R. (2010). High diversity of frenulates (Polychaeta: Siboglinidae) in the Gulf of Cadiz mud volcanoes: A DNA taxonomy analysis. *Deep Sea Research Part I: Oceanographic Research Papers*, 57, 143-150.

Huelsenbeck J.P. & Ronquist F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17, 754-755.

Kumar S., Nei M., Dudley J. & Tamura K. (2008). MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in bioinformatics*, 9, 299-306.

Lalitha S. (2000). Primer premier 5. *Biotech Software & Internet Report: The Computer Software Journal for Scient*, 1, 270-272.

Lavelle P., Barois I., Cruz I., Fragoso C., Hernandez A., Pineda A. & Rangel P. (1987). Adaptive strategies of *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta), a peregrine geophagous earthworm of the humid tropics. *Biology and fertility of soils*, 5, 188-194.

Librado P. & Rozas J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451.

Lynch M. & Gabriel W. (1983). Phenotypic evolution and parthenogenesis. *American Naturalist*, 745-764.

Moreno A.G. (2004). Who is *Pontoscolex corethrurus* (Müller, 1857) (Annelida, Glosscoscolecidae)? . In: *Avances en taxonomía de lombrices de tierra: Advances In Earthworm Taxonomy:(annelida: Oligochaeta)* (eds. Moreno AG & Borges S). Editorial Complutense Madrid, Spain, pp. 63-73.

Novo M., Almodóvar A., Fernández R., Giribet G. & Díaz Cosín D.J. (2011). Understanding the biogeography of a group of earthworms in the Mediterranean basin-the phylogenetic puzzle of Hormogastridae (Clitellata: Oligochaeta). *Molecular Phylogenetics and Evolution*.

Novo M., Velavan T., Almodóvar A., Schulenburg H., DÍAZ-COSÍN D. & Michiels N. (2008). Microsatellite markers for the drought-resistant earthworm *Hormogaster elisae*. *Molecular Ecology Resources*, 8, 901-903.

Pérez-Losada M., Ricoy M., Marshall J.C. & Domínguez J. (2009). Phylogenetic assessment of the earthworm *Aporrectodea caliginosa* species complex (Oligochaeta: Lumbricidae) based on mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, 52, 293-302.

Pfenninger M. & Schwenk K. (2007). Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. *BMC Evolutionary Biology*, 7, 121.

Posada D. (2008). jModelTest: phylogenetic model averaging. *Molecular biology and evolution*, 25, 1253-1256.

Sambrook J. & Russell D.W. (2001). *Molecular cloning: a laboratory manual*. CSHL press.

Slatkin M. (1985). Gene flow in natural populations. *Annual review of ecology and systematics*, 16, 393-430.

Slatkin M. (1987). Gene flow and the geographic structure of natural populations. *Science*, 236, 787.

Stillman Y.K. & Stillman N.A. (1999). *From Iberia to diaspora: studies in Sephardic history and culture*. Brill Academic Pub.

Templeton A.R. (1979). The unit of selection in *Drosophila mercatorum*. II. Genetic revolution and the origin of coadapted genomes in parthenogenetic strains. *Genetics*, 92, 1265.

Velavan T., Schulenburg H. & MICHIELS N.K. (2007). Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris* L.). *Molecular Ecology Notes*, 7, 1060-1062.

Viveiros F., Ferreira T., Cabral Vieira J., Silva C. & Gaspar J.L. (2008). Environmental influences on soil CO2 degassing at Furnas and Fogo volcanoes (São Miguel Island, Azores archipelago). *J. Volcanol. Geotherm. Res.*, 177, 883-893.

Viveiros F., Ferreira T., Silva C. & Gaspar J. (2009). Meteorological factors controlling soil gases and indoor CO2 concentration: A permanent risk in degassing areas. *Sci. Total Environ.*, 407, 1362-1372.

Vrijenhoek R.C. (2009). Cryptic species, phenotypic plasticity, and complex life histories: assessing deep-sea faunal diversity with molecular markers. *Deep Sea Research Part II: Topical Studies in Oceanography*, 56, 1713-1723.

Westram A.M., Jokela J., Baumgartner C. & Keller I. (2011). Spatial Distribution of Cryptic Species Diversity in European Freshwater Amphipods (*Gammarus fossarum*) as Revealed by Pyrosequencing. *PloS one*, 6, e23879.

Wiens J.A. (1976). Population responses to patchy environments. *Annual review of ecology and systematics*, 7, 81-120.

Wiens J.J. (2004). Speciation and ecology revisited: phylogenetic niche conservatism and the origin of species. *Evolution*, 58, 193-197.

Xia X. & Xie Z. (2001). DAMBE: software package for data analysis in molecular biology and evolution. *Journal of Heredity*, 92, 371-373.

Chapter 5

Revealing population structure of a peregrine earthworm inhabiting volcanic soils with AFLPs markers

Revealing population structure of a peregrine earthworm inhabiting volcanic soils with AFLPs markers

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ABSTRACT

The AFLP (amplified fragment length polymorphism) technique was used to analyse and compare the genetic diversity of *Pontoscolex corethrurus* in three sites populations in São Miguel Island,; Furnas, a geothermal field,; Fajã de Baixo; and Vila Franca, with no volcanic activity. Analysis using AFLP markers showed *Pontoscolex corethrurus* to be a genetically heterogeneous complex with direct association with the previous results of mitochondrial divergence. The complete congruence between molecular markers suggests that cryptic speciation is a plausible explanation for the deep mitochondrial divergence in *P. corethrurus* in São Miguel Island. Four pairs of primers generated 425 loci. The average ratio of polymorphic loci among the studied populations was of 84%. Shannon information index was 0.28 with a higher value of 0.3 in Furnas. These results show that the genetic diversity detected with AMOVA was mainly caused by individual differences within a population. In fact, three different ancestral clusters were identified among populations. One cluster showed to be almost exclusive to Furnas individuals showing and confirming the genetic differentiation of this apparently isolated geographic group. The fact that Furnas individuals were mainly associated with one almost exclusive ancestral cluster corroborates the evidence of a different *Pontoscolex corethrurus* lineage inhabiting the geothermal field which confirms previous mitochondrial data. On the one hand it is plausible to consider that the homogenizing effect of selection on genomic diversity would intensify in populations successfully inhabiting intensely stressful environmental conditions, such as actively volcanic soils. In contrast, an intriguing alternative scenario may pertain where chemical contaminants increase genetic diversity by causing genomic mutation which could explain why Furnas population showed the highest number of private bands.

Keywords: *Pontoscolex corethrurus, cryptic speciation, earthworm, volcanic soil, genetic erosion, Azores, AFLP markers*

INTRODUCTION

Sequences derived from the mitochondrial genome (mtDNA) and nuclear introns are the most widely used genetic markers both in species delimitation and historical phylogeography. However, discordance between topologies from nuclear and mitochondrial loci is common because of genes mutating at varying rates and being subjected to different stochastic and population processes (Hebert *et al.* 2004; Mendelson & Simons 2006; Yang & Kenagy 2009; Caitlin & John 2011). In fact, the use of Cytochrome c oxidase I and II (COI and COII), or ribosomal subunits (12s and 16s) is fundamentally based in the assumption of the orthology of characters in order to establish "barcodes" of species (Hebert *et al.* 2003; Hebert *et al.* 2004; Moritz & Cicero 2004). Moreover, this assumption can be violated when paralogous (like nuclear copies of mitochondrial DNA (numts)) sequences are naively treated as orthologs, and therefore incorrect inferences are made (Funk & Omland 2003; Song *et al.* 2008). Nonetheless, deep divergence in mitochondrial DNA sequences (mtDNA) between related individuals is often taken as evidence for the existence of cryptic species (cryptic mtDNA). Variation at level of the species (cryptic) can have important implications for characterising biodiversity and for studies of speciation but also for ecology, conservation, and ecotoxicology approaches (King *et al.* 2008). In contrast, mitochondrial lineages may also be retained through admixture between divergent species or populations (Comas *et al.* 2004; Roy & Buronfosse 2011), or perhaps due to unusual population structures due to natural or human influence (Abiadh *et al.* 2010; Byrne *et al.* 2010) or even due to a more speculative phenomena of possible mitochondrial recombination (White *et al.* 2008; Bovers *et al.* 2009; Galtier *et al.* 2009). Independently of the variation source, we must consider that the possible overlap between mitochondrial genotypes among different populations is likely to be an important phenomena because such mixture provides important sources of genetic novelty for organisms to adapt in changing environments (Tranah *et al.* 2004; Seehausen *et al.* 2008) and could be a key for an highly invasive species as *Pontoscolex corethrurus* (Lavelle *et al.* 1987). In effect, the communities of soil organisms that occupy terrestrial geothermal vents provide an unusual opportunity to study the dynamics of population structure and genetic diversity as similar to the communities close to deep hydrothermal vents. Geothermal biotopes are reducing environments with certain unique features, mainly characterised by elevated soil, water, and atmospheric elemental composition, together with constant diffuse degassing and high temperatures (Cruz *et al.* 1999; Viveiros *et al.* 2008; Viveiros *et al.* 2009). In our previous study, we found that the use of COI or the s-rRNA could not give an informative answer about the phylogenetic relation between an earthworm population living inside a geothermal field in Furnas, Azores when compared to adjacent populations in pineapple cultures, and only when COI and s-rRNA were concatenated with NADH dehydrogenase II and III into a larger fragment the number of informative sites increased abruptly and the genetic relation was clear and highly conspicuous. It is always tempting to relate the low haplotype diversity also found in Furnas and apparent genetic erosion with natural selective pressures within the volcanic environment, although it is possible to be explained either by genetic drift or inbreeding. In fact, population bottlenecks, natural or human-induced, can have significant genetic effects, such as reduced effective population size (Wright 1943; Maruyama & Kimura 1980), loss of heterozygosity (Nei *et al.* 1975), and loss of alleles (Maruyama & Fuerst 1985). Moreover, a physical barrier such as the volcanic crater and the lower soil temperature isolating Furnas population to dispersal can disrupt the possible migration rate between adjacent populations. In fact, genetic evolution models show that the establishment of a long-term physical barrier to dispersal abruptly reduces or stops gene flow between populations and leads to a rapid change in allele frequencies that may be inhibited or accelerated depending on the type of selection at specific loci (Slatkin 1987; Wiens 2004). This often leads to the divergence of the isolated population (by fixation of different mutation (Slatkin 1985). Certain that the initial number of peregrines, their sources (i.e. migrant vs. introduced haplotype pools), and present rates of gene flow all affect the degree to which demographic unsteadiness affects geographical differentiation. Therefore, independent genealogies involving nuclear markers are needed to distinguish the effects of individual processes such as mitochondrial selective bends from demographic processes that leave genome-wide signatures. Individuals of *Pontoscolex corethrurus* in S. Miguel Island fall into two distinct mtDNA clades that show over 18% divergence, with no clear biogeographic explanation as they can appear in a mixed haplotype population inhabiting pineapple greenhouses but in Furnas they all belong to the same cluster. Therefore the deep divergence in the mitochondrial haplotypes is not readily explained through ancestral polymorphism or hybridization. In order to test for cryptic speciation, we complemented these data with nuclear Amplified Fragment Length Polymorphisms (AFLPs) markers, to provide a comparison with nuclear bi-parentally inherited markers (Vos *et al.* 1995). Methods based on variable polymerase chain reaction amplification such as AFLPs can provide a rapid and affordable approach to detect polymorphism in a population on a genomic scale (Campbell *et al.* 2003). Dominant markers such as AFLPs provide an economical way of surveying variation at many loci and therefore will fulfil our aims.

MATERIALS AND METHODS

Earthworm sources and collection sites

The Azores archipelago comprises nine islands and is located in the North Atlantic Ocean, between 36º45'–39º43'N and 24º45'–31º17'W, at the triple junction of Eurasian, African and North American plates, characterized by a complex tectonic settlement, where seismic and volcanic phenomena are common (Booth *et al.* 1978). São Miguel is the largest island (757 km^2), which presents several active volcanic spots including fumarolic fields, cold and thermal springs and soil diffuse degassing (Viveiros et al., 2008). *P. corethrurus* in the Azores seems to be delimited to the hot soils of the degasing fields in Furnas and inside agricultural greenhouses of pineapple culture around the island. The sampling sites on São Miguel, differing in their contemporary geogenic traits were selected for earthworm capture: (a) Furnas, which displays the most conspicuous degassing and geothermal activity in the entire Azores archipelago and in pineapple greenhouses in (b) Fajã de Baixo and (c) Vila Franca do Campo, the only other place around the island that gathers the proper environmental conditions in order to *Pontoscolex corethrurus* establish (apparently its distribution is limited mainly by low temperatures outside the greenhouses as well outside the geothermal field). A group of 21 adults (clitellate) *P. corethrurus* from the geothermal field soils in Furnas (37º 46' 24.6'' N 25º 18' 10.3'' W), another group randomly chosen in two pineapple greenhouse soils (with an average pH of 5.5) in Fajã de Baixo (n=21) (37º45'12.2N 25º38'21.3''W) and in Vila Franca (n=20) (37º45'12.5N 25º24'18.3''W) were collected by digging and hand-sorting during summer of 2011 (See Figure 1). After sampling, the earthworms were immediately transferred to the laboratory, where they were depurated of gut contents by placing them on moistened paper for 36 h.

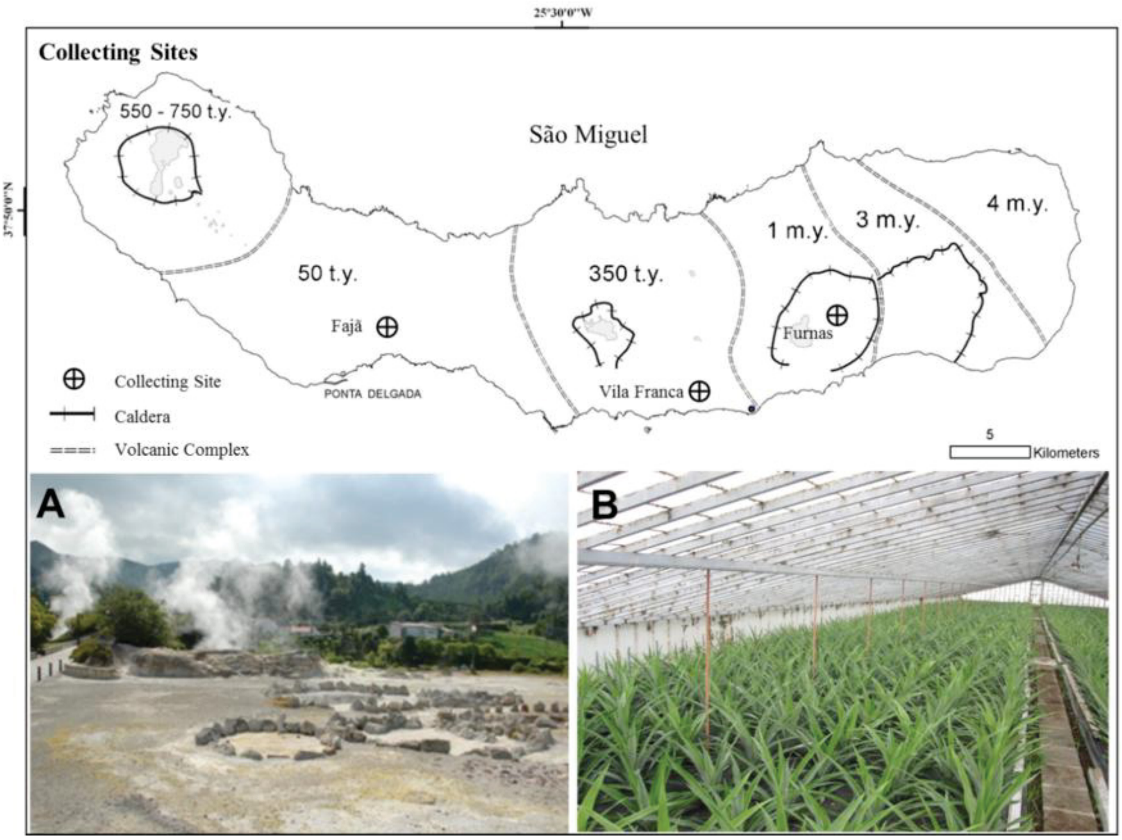

Figure 1. Collecting sites in São Miguel Island. M.y. million years; t.y., thousand years. A, Photograph of Furnas geothermal field and B, pineapple greenhouse in Fajã de Baixo.

DNA extraction

For DNA extraction 2–3 segments of the caudal end of each individual were used. Any gut content was removed and the remaining tissue was cut into pieces and subjected to a phenol-chloroform protocol (Sambrook & Russell 2001) with the following modifications: the lysis step was accomplished in 2h using 180 µl of ATL Buffer (Qiagen) and 20 µl of Proteinase K (600 mAU/ml, Qiagen) and the DNA pellet was finally dissolved in 50 µl sterile water. DNA concentration was measured using NanoDrop® (ND-1000 spectrophotometer, Thermo Scientific) to dilute extracts with sterile water to 20 ng/µl prior to PCR. All DNA extracts were stored at -20 °C.

AFLPs analysis

AFLP analysis was carried out according to Vos *et al.* (1995) with modifications: the enzymes used in the protocol were *Pst*I and *Eco*RI (which are two six base recognition enzymes), with the aim to reduce the overall number of fragments obtained for each primer combination (Hawthorne 2001). Using two six-base (rare) cutters, which generate fewer bands per primer pair than the more commonly used four-base cutters, resulted in clear separation of bands in the fragment analysis output (Mendelson & Simons 2006). A smaller number of bands per primer pair reduces the probability of non-homologous co-migrating bands for all size classes (Hawthorne 2001). Approximately 50 ng of genomic DNA from each specimen was digested with EcoRI (2.5U, New England Biolabs (NEB)) and PstI (2.5U, NEB), Buffer 3 (1x, NEB) adjusted with water to a final volume of 20 µl and incubated for two hours at 37°C. Low-quality DNA samples were excluded and negative controls were run at each step of the genotyping process. Adapters were ligated using 5 pmoles/µl of double-stranded EcoRI adapter (5’- CTCGTAGACTGCGTACC and 5’- AATTGGTACGCAGTC, Sigma), 50 pmoles/µl of double-stranded PstI adapter (5’- CTCGTAGACTGCGTACATGCA and 5’- TGTACGCAGTCTAC, Sigma), ATP (1 mM, Roche), T4 DNA ligase buffer (1x, NEB), and T4 DNA ligase (0.7 U, NEB), adjusted to a final volume of 5 µl with water, then added to the double digested genomic DNA (total volume 25 µl) and incubated at 37 °C for 4 hours. The digestion-ligation template was diluted (1:10) with low TE buffer (2 M Tris-HCl (pH7.5), 0.5 M EDTA (pH 8)). Pre-selective PCRs contained 2.5 µl diluted template DNA, GoTaq mater mix (Promega®), pre-selective EcoRI primer (5`- GACTGCGTACCAATTCA) and PstI primer (5’- GACTGCGTACATGCAGA) (each 2.5 ng/µl, Sigma), adjusted to a total reaction volume of 20 µl with water. Amplification initiated with a denaturing step at 95

°C for 2 min, followed by 30 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s. PCR products (2.5 µl) were run on a 1.5% agarose electrophoresis gel. The pre-selective template was diluted (1:10) with sterile water for use in the selective amplifications. Selective PCR reactions contained 2.5 µl diluted pre-amplified template DNA, GoTaq mater mix (5 µl, Promega®) and EcoRI fluorescent labelled primer (2.5 ng/µl) and PstI (15 ng/µl) primer (Sigma®), adjusted to a total reaction volume of 20 µl with water. Four primer combinations were used each with three overhanging nucleotides at the 3' end (see Table I).

Table I. Selected primer combinations for selective amplification in the AFLP analysis.

Primer pairs	*Pst*I sequence	*Eco*RI sequence
PAAA/E42	5′-GACTGCGTACATGCAGAAA	5′-6Fam-GACTGCGTACCAATTCAGT
PAAT/E42	5′-GACTGCGTACATGCAGAAT	5′-6Fam-GACTGCGTACCAATTCAGT
PAAT/E45	5′-GACTGCGTACATGCAGAAA	5′-6Fam-GACTGCGTACCAATTCATG
PAAC/E45	5′-GACTGCGTACATGCAGAAC	5′-6Fam-GACTGCGTACCAATTCATG

The touchdown thermal cycling programme initiated with a denaturing step at 95 °C for 2 min, followed by 15 cycles of 95 °C for 30 s, 65 °C* for 30 s (*-0.7 °C each cycle), 72 °C for 60 s followed by 25 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s. Amplified products (1 µl) were each added to formamide (10 µl, Applied Biosystems) and GeneScan ROX-500 size standard (0.25 µl, Applied Biosystems). Reactions were run by the Cardiff University Molecular Biology Support Unit and analysed on an Applied Biosystems 3130XL fragment analyser.

AFLP scoring and error rates

Electrophenogram trace files were imported into Genemarker v2.2.0 (Hulce *et al.* 2011). GeneScan 500 size standards (Applied Biosystems®) were applied to the project and manually checked for quality and edited where required. In total 60 individuals were scored, however poor quality profiles (failed amplification) were removed from subsequent analysis. All peaks above 150 rfu (peak height identified as a suitable background noise threshold) and between 50-500bp were scored using Genemarker v2.2.0. A panel was created automatically using all samples. Bin positions were manually checked to identify incorrect bin positioning and low quality or noise peaks (irregular shape or pull-ups). Overlapping bin positions were deleted from the data set to avoid ambiguous scoring due to possible size homoplasy of co-migrating fragments (Vekemans *et al.* 2002). PCR negatives were checked for possible contaminants and any peaks above the background noise threshold were deleted from the respective primer combination. AFLPscore version 1.4b (Whitlock *et al.* 2008) was used to identify thresholds (relating to average locus peak height and relative peak height across all loci) that resulted in acceptable mismatch error rate (< 5%) but maximised the number of loci retained for further analysis (Whitlock *et al.* 2008). Mismatch error rates (Bonin *et al.* 2007), based on 8 repeated genotype profiles (4 in Furnas and 4 in Faja de Baixo) were calculated using the data filtering option, a locus selection threshold of 500 rfu (13% of the total mean normalised peak height across all loci) and a relative phenotype calling threshold of 150 rfu (10% of the total mean normalised peak height across all loci). A binary matrix of retained loci was created in Aflpscore v1.4b for the four primer combinations and a subset were compared to the original electrophenograms to check for computational copying errors. Input files for Arlequin v3.5.1.2 (Excoffier *et al.* 2005) and Structure v2.3.3 (Pritchard *et al.* 2000) were prepared using AFLPdat (Ehrich 2006). Median-joining network (was drawn using Network v4.6.1.0 (Bandelt *et al.* 1999). Microsoft Access, Excel and the Excel macro GenAlEx6.41 (Peakall & Smouse 2006) were used to create a cumulative table of all loci from each individual and transform the data into a binary form. Principal component (PCA) analysis was used to confirm the results and variation of our data. Phylogenetic tree construction, supported by bootstrap analysis (1000 replicates), was performed using the Neighbour-joining algorithm (based upon the distance matrix produced by GenAlEx v6.41 in PHYLIP Phylogeny Inference Package v3.69 (Felsenstein 1993) to estimate tree topology and calculate branch length. Trees were modified for presentation using FigTree v1.3.1 (Rambaut 2009). Additionally, measures of heterozygosity and variation were estimated with GenAlEx v6.41 an Analysis of molecular variance (AMOVA) in Arlequin v3.5.1.2 (Weir & Cockerham 1984; Excoffier *et al.* 2005).

Population structure analysis

We used the presence/absence matrix of AFLP fragments to delineate clusters of individuals on a multi-locus, genotype using a Bayesian clustering algorithm implemented in the program Structure v2.3.3, we set most of parameters to their default values as advised in the user's manual of structure. Specifically, we chose the admixture model and the option of correlated allele frequencies between defined populations, as this configuration is considered best by Falush *et al.* (2007) in cases of subtle population structure. Similarly, we let the degree of admixture alpha be inferred from the data. When alpha is close to zero, most individuals are essentially from one population or another, while alpha > 1 means that most individuals are admixed (Falush *et al.* 2003). We found that a length of the burn-in and MCMC (Markov chain Monte Carlo) of

100000 generations each was adequate. Longer burn-in or MCMC generations did not change significantly the results. The range of possible Ks we tested was from 1 to the true number of populations plus 3 (K from 1 to 6)(Evanno *et al.* 2005). L(K), the modal choice criterion, is calculated in Structure and the true number of populations (K) can be deferred from its maximal value. ΔK, the rate of change in the log probability of data between successive K-values, provides a visual means to easily identify the number of clusters in a sample of individuals (Evanno *et al.* 2005). Each likelihood value was estimated with 15 interactions. The Structure outputs were used to implement the Evanno method (Evanno *et al.* 2005) with the help of Structure Harvest v0.6.92 (Earl & vonHoldt 2011).

RESULTS

The selected primers generated an appropriate amplification pattern with clear and consistent reproducible bands as shown in the reproducibility test, shown in Figure 2, carried out with primer combination nos. 1 and 4 (See Table 1).

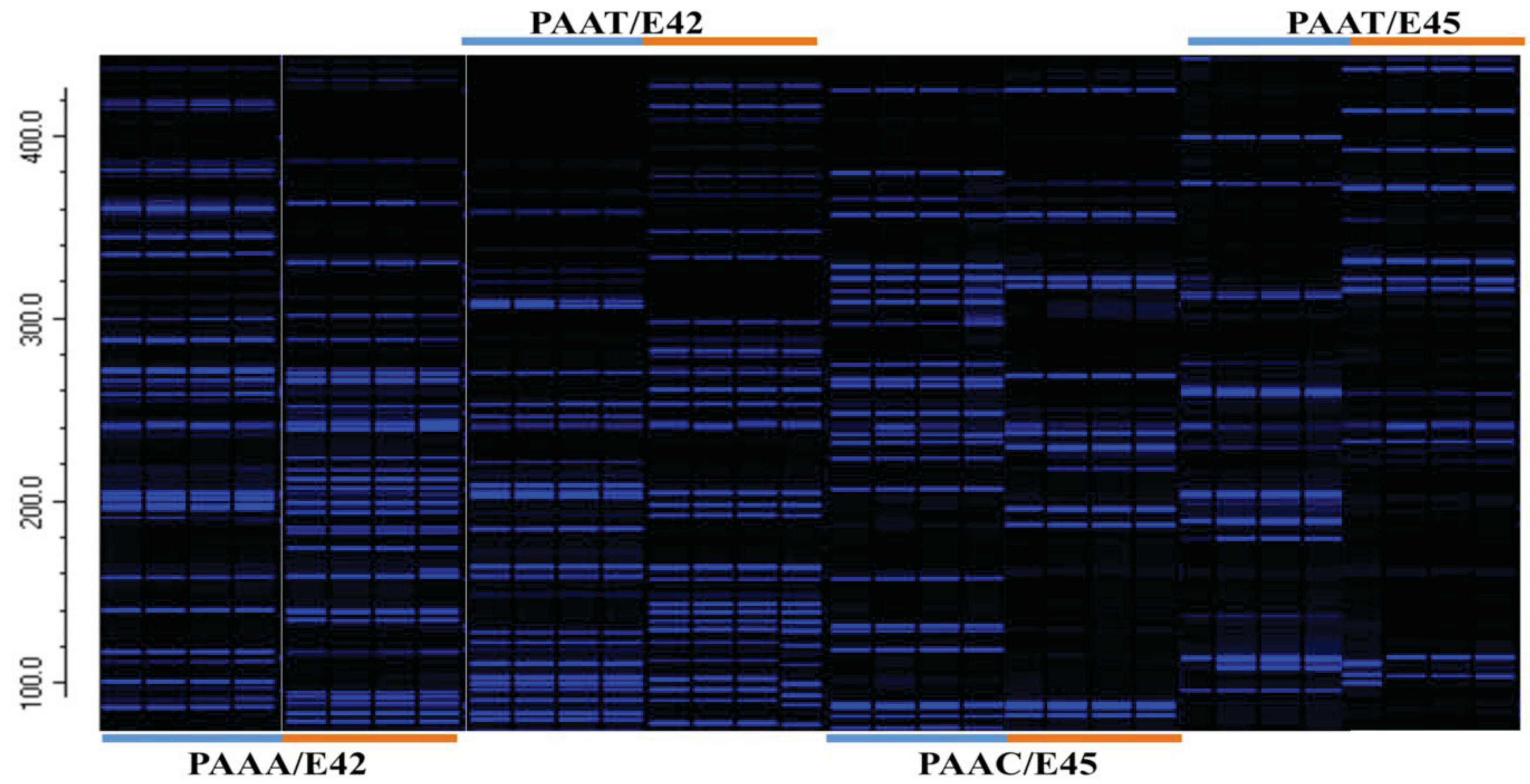

Figure 2. Digitalised electropherogram of AFLP profile of genomic DNA. The figure represents the reproducibility assay performed on Fajã de Baixo (lanes 1, 3, 5, 7 with blue bar in the bottom) and Furnas (lanes 2,4,6,8 with orange bar in the bottom) using primer combination PAAA/E42 in lane 1 and 2, PAAT/E42 in lane 3 and 4, PAAC/E45 in lane 5 and 6 and PAAT/E45 in lane 7 and 8. Scale in left is shown in base pairs (bp).

Population genetics analysis

Of the 425 loci examined, we found an average of 82.4 loci per individual with higher frequency of bands in all populations (Figure 3). Genetic diversity statistics estimated from AFLP data showed that populations are differentiated within and among populations (See Table II). Furnas showed the lowest number of bands (314 bands) when compared to Vila Franca (347 bands) and Fajã de Baixo (404). Private bands were found to be higher in Furnas with a value of 10 unique bands among the analysed individual profiles, Vila Franca did not show any private bands, and Fajã de baixo showed 2 private bands.

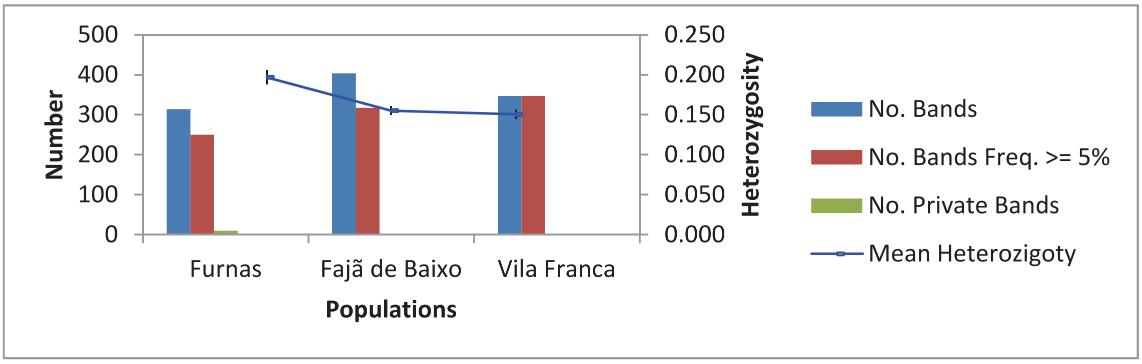

Figure 3. Total band patterns for binary data across the studied populations.

Mean expected heterozygosity was 0.167 (S.E. = 0.004). The higher value for the mean expected heterozygosity was found in Furnas populations with a value of 0.197 (0.155 in Fajã de Baixo and 0.151 in Vila Franca) (Table II). Polymorphic *loci* proportions were higher in Fajã de Baixo with almost 95% of differently dynamic *loci*, Furnas showed the lowest proportion with only 74 % of change in the total of analysed *loci.* The mean percentage of polymorphic loci over all populations was 84%. The mean population diversity using the Shannon information index (I) was 0.28 (S.E. = 0.006). Furnas population was the most diverse (I = 0.305) and lowest diverse population was

Vila Franca (I = 0.26) (Table II). The highest number of effective alleles was in Furnas population (1.325, S.E. = 0.017) and the lowest in Vila Franca (1.204, SE = 0.01), with an average 1.246 (SE = 0.007) over all populations.

Table II. *Pontoscolex corethrurus* population information for Furnas, Fajã de Baixo and Vila Franca. N, number of individuals; Tb, total number of bands; Pb, number of private bands; Plp, percentage of polymorphic *loci*; Na, number of different alleles; Ne, number of effective alleles; I, Shannon's Information Index; He, expected heterozygosity; UHe, Unbiased expected heterozygosity.

Pop.	**N**	**Tb**	**Pb**	**Plp %**	**Na**	**S.E.**	**Ne**	**S.E.**	**I**	**S.E.**	**He**	**S.E.**	**UHe**	**S.E.**
Furnas	21	314	10	74	1.478	0.043	1.325	0.017	0.305	0.013	0.197	0.009	0.202	0.009
Fajã de Baixo	21	404	2	95	1.901	0.021	1.208	0.009	0.274	0.008	0.155	0.005	0.159	0.006
Vila Franc	20	347	0	82	1.633	0.038	1.204	0.010	0.261	0.009	0.151	0.006	0.154	0.006
Mean	21	355	4	84	1.671	0.021	1.246	0.007	0.280	0.006	0.167	0.004	0.172	0.004

Based on the median joining algorithm (Bandelt *et al.* 1999), it was possible to draw a network that aggregates the *Pontoscolex corethrurus* haplotypes in two/three distinct groups (Figure 4), one clear group mainly composed by the haplotypes present in Furnas earthworms with also one individual from Fajã de Baixo and another group composed with most of the haplotypes of Fajã de Baixo earthworms and all the haplotypes present in Vila Franca population and some from Furnas (7 individuals). Apparently the larger group could also be divided into two groups as there is an extension composed by Fajã de Baixo and Vila Franca haplotypes that's opposite to the Furnas cluster.

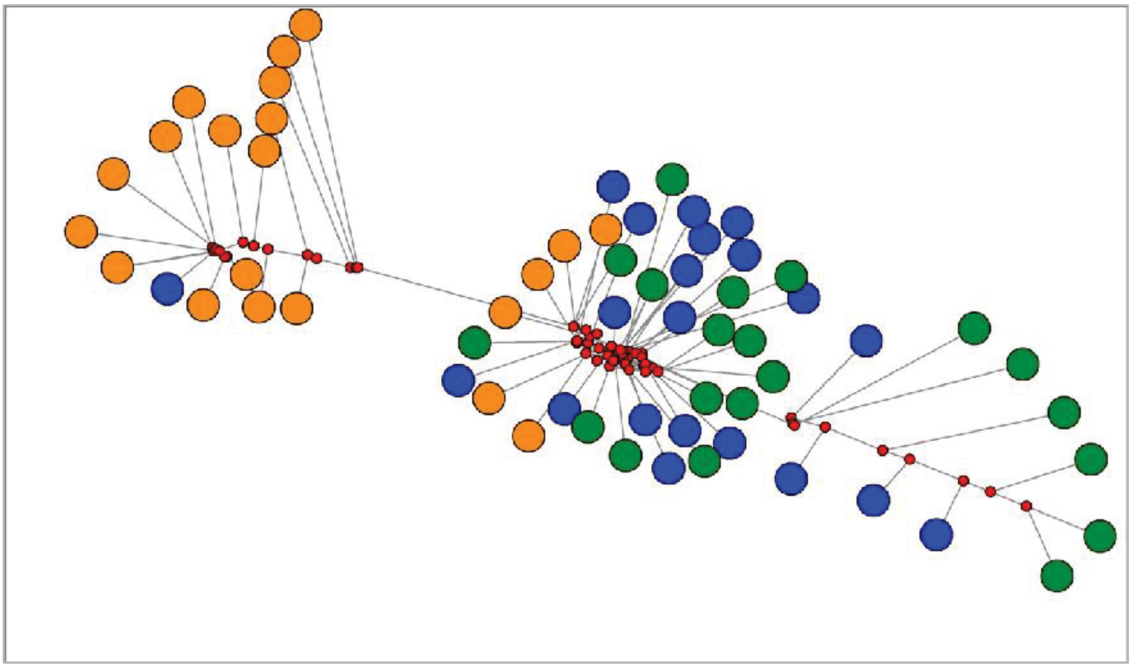

Figure 4. Median-joining (MJ) networks showing phylogenetic relationships between all the individuals from populations of *Pontoscolex corethrurus* in volcanic and non-volcanic soils. The different colour shades refer to the screened *Pontoscolex corethrurus* populations, Furnas haplotypes (orange), Vila Franca pineapple greenhouses haplotypes

(green), Fajã pineapple greenhouses (blue). MJ-network based on the concatenated AFLP profiles of four different primer combinations.

The analysis of molecular variance (AMOVA) for populations showed highly significant differentiation ($P < 0.001$), with 85% of the differentiation attributed to within populations and 15 % attributed to among populations is shown in (Table III).

Table III. Analysis of molecular variance (AMOVA) using AFLP data from three different populations of *Pontoscolex corethrurus* in the geothermal field Furnas, in Fajã de Baixo and Vila Franca. Levels of significance are based on 1000 iterations.

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation %	p
Among Populations	2	485.244	9.14533	14.81	**<0.001**
Within Populations	59	3117.643	52.8414	85.19	**<0.001**
Total	61	3602.887	62.02673		
Fixation Index (F_{ST}) **0.14753 (p<0.001)**					
Population pairwise F_{ST}			**Furnas**	**Fajã de Baixo**	**Vila Franca**
		Furnas	0	…	…
		Fajã de Baixo	0.19272	0	…
		Vila Franca	0	0.21993	0

The Fixation Index (F_{ST}) estimate had a value of 0.15 and highly significant ($P < 0.001$). Additionally, the principal components analysis of molecular variance performed in GenAlex 6.41, based on individual band pattern gave a consistent result, with 87% of the variance in the data explained by differences between populations. The first two axes explained 71.7% (Figure 5). The Neighbour-joining (NJ) algorithm and the genetic distance matrix (Annex III) generated in GenAlEx v6.41 were used to estimate the degree of relationships between the individuals analysed based on common amplified fragments and the constructed tree is in Figure 5.

The NJ tree (Figure 6) shows a trend of three different clusters with different population origin proportions. Nevertheless, most of Furnas individuals (orange) formed a distant cluster from the other two populations.

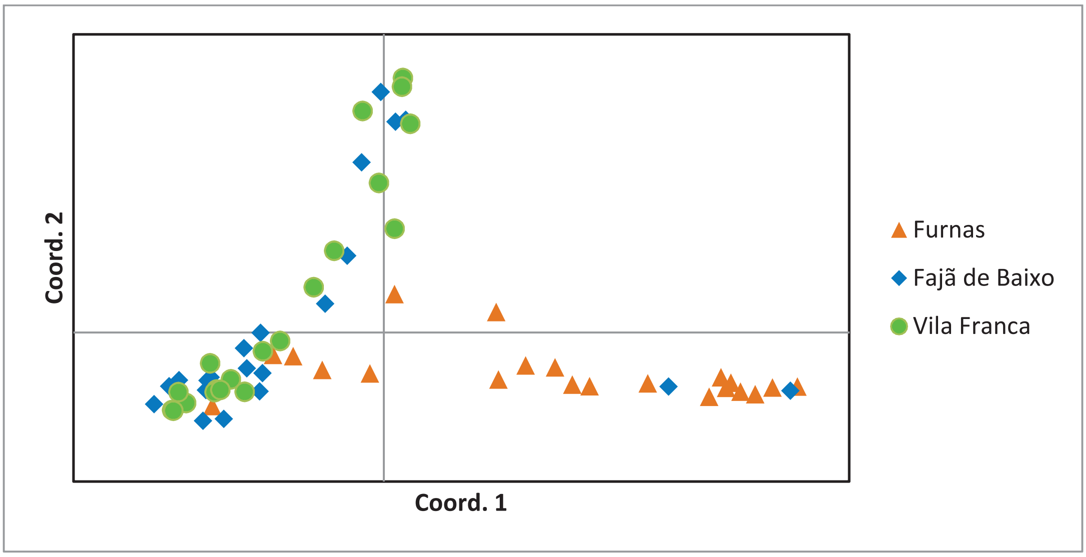

Figure 5. The plot of PCA of 62 individuals using 425 AFLP markers. Populations are represented by orange triangles, blue rhombi and green circles correspond to Furnas, Fajã de Baixo and Vila Franca respectively.

The pattern that is portrayed by the PCA analysis as well in the median-joining network is very similar to the NJ tree. The PCA seems to show three groups that correspond to the three different population's sites, with some Furnas individuals in the biggest cluster. The PCA plot also shows spread shrewdly scattering when compared to Fajã de Baixo and Vila Franca, which both show to be more aggregated together which also agrees with both having higher genetic diversity. The first two coordinates explained 71.7% of the total variation.

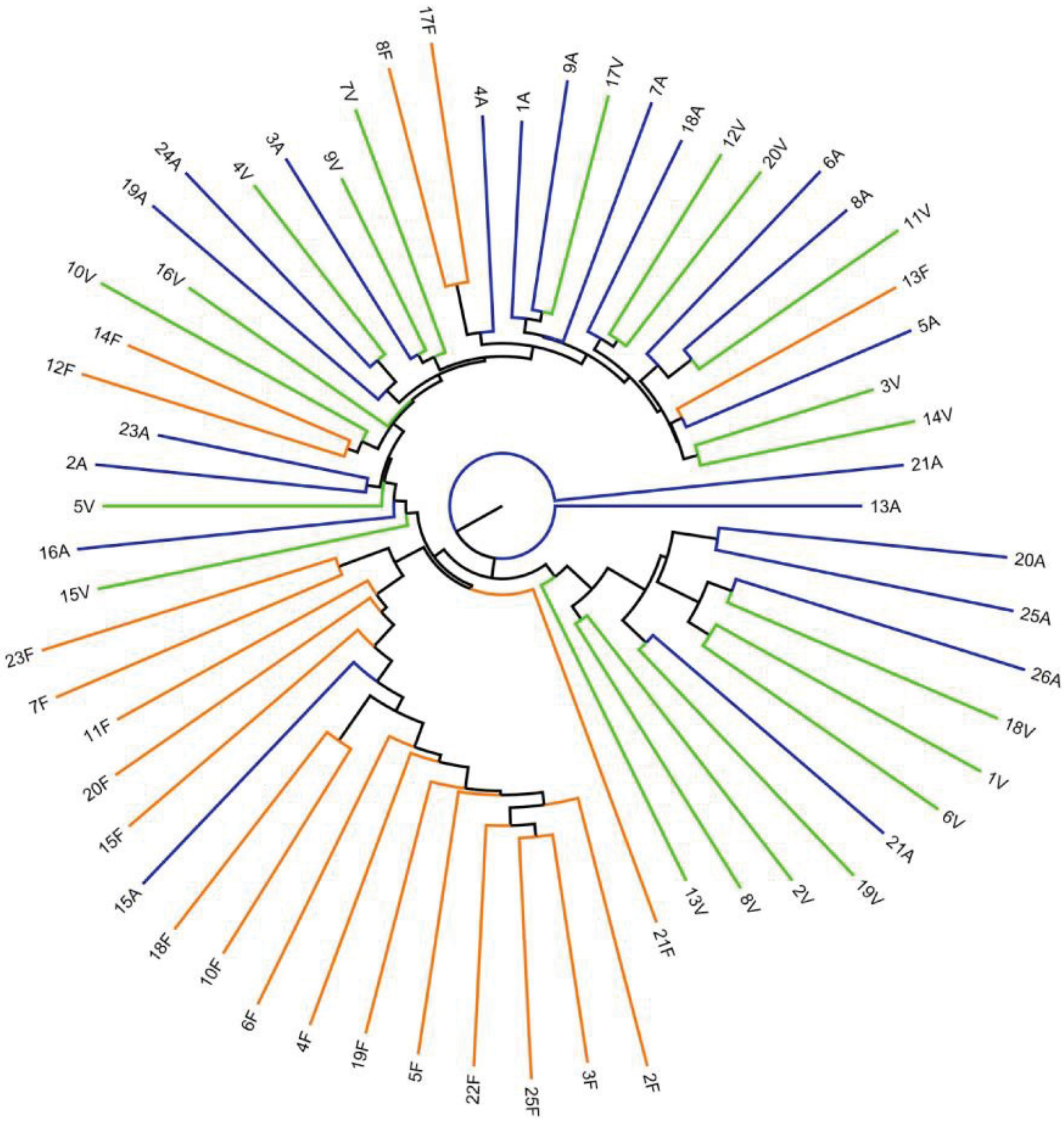

Figure 6. Neighbor-joining tree showing the hypothesized relationships among *Pontoscolex corethrurus* populations based on 425 AFLP characters. The different colour shades refer to the screened *Pontoscolex corethrurus* populations, Furnas haplotypes (orange), Vila Franca pineapple greenhouses haplotypes (green), Fajã pineapple greenhouses (blue).

Population structure analysis

In order to test for congruent differentiation between AFLP markers within the three different populations we ran a structure analysis using all the individuals and the 425 AFLP markers. We compared the mean likelihood values for all runs with the statistic ΔK. The AFLP-based Bayesian population assignment test confirmed that K=3 is the best estimate for these data (Table IV). The number of clusters (K) was determined by comparing the likelihood ratios for K values between 1 to the real number of populations plus 3 (total of 6) (average log probability of data Ln P(D) = −8439.25 ± 7.38, SD) (see Figure 7A and 7B) (Evanno *et al.* 2005). The ancestry proportion of each population in each cluster is shown in Figure 7. The Furnas population was the most distinct with 63% of ancestry in cluster 1. Both Vila Franca and Fajã de Baixo populations were fairly admixed. Fajã de Baixo and Vila Franca populations showed similar ancestry proportions among the cluster 2 and 3, although for both populations the largest proportion of ancestry was in cluster 2 showing values of 70% for Fajã de Baixo population and 68% for Vila Franca population.

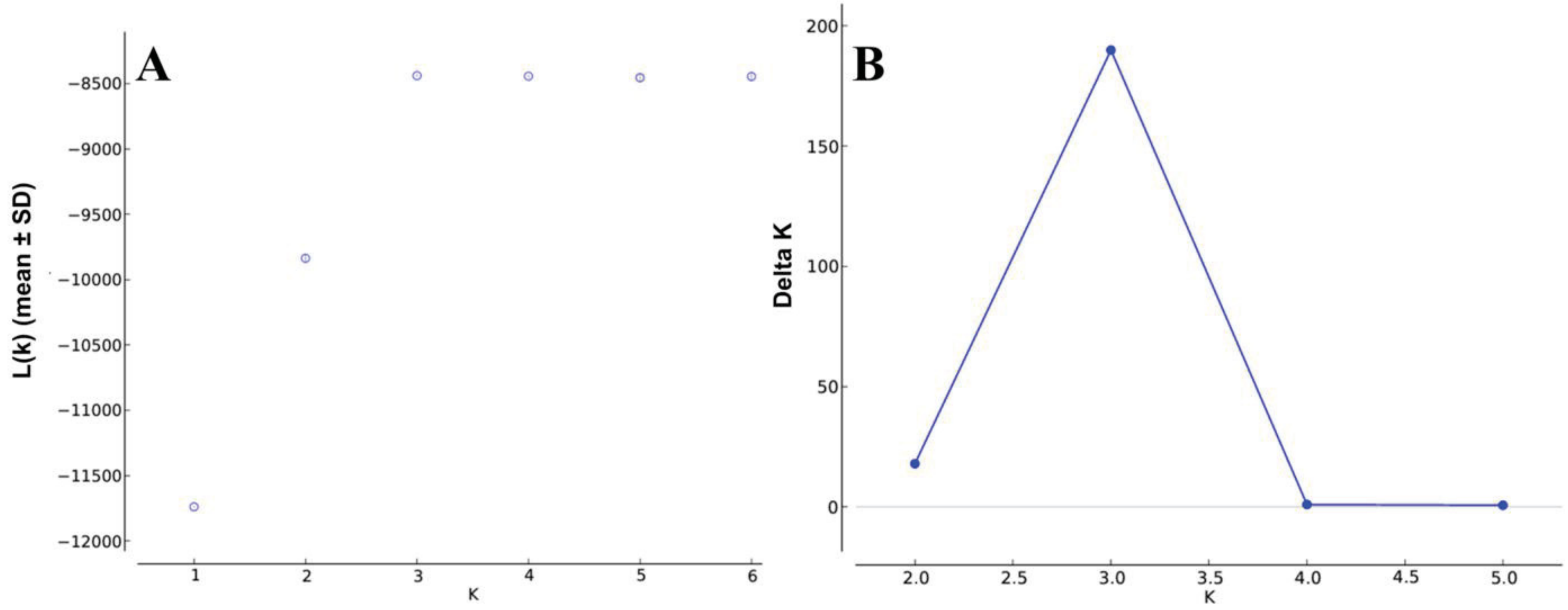

Figure 7. **A,** Log probability of data L(K) as a function of K under exhaustive sampling (mean of 15 replicates). Results are shown for the 425 AFLPsmarkers using all the individuals from Furnas, Fajã and Vila Franca. **B,** Magnitude of ΔK as a function of K (mean of 15 replicates).

Table IV. Result of structure analysis using all the individuals and the 425 AFLP markers from three different populations of *Pontoscolex corethrurus* in the geothermal field Furnas, in Fajã de Baixo and Vila Franca. Levels of significance are based on 1000 iterations.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	15	-11738.6	3.2022	NA	NA	NA
2	15	-9836.64	28.0468	1901.913	504.5267	17.98875
3	**15**	**-8439.25**	**7.3765**	**1397.387**	**1401.12**	**189.9439**
4	15	-8442.99	7.7494	-3.73333	7.92	1.022019
5	15	-8454.64	29.5495	-11.6533	20.74667	0.702099
6	15	-8445.55	29.4257	9.093333	NA	NA

Cluster 3 showed to be the ancestry of 31% of Vila Franca population and 24% of Fajã de Baixo. Only 3% of the ancestry of Furnas individuals belonged to cluster 3 (see also Figure 9). These results were consistent and confirmed the trends verified in the median-joining network (Figure 4), the PCA (Figure 5) as well the NJ tree (Figure 6).

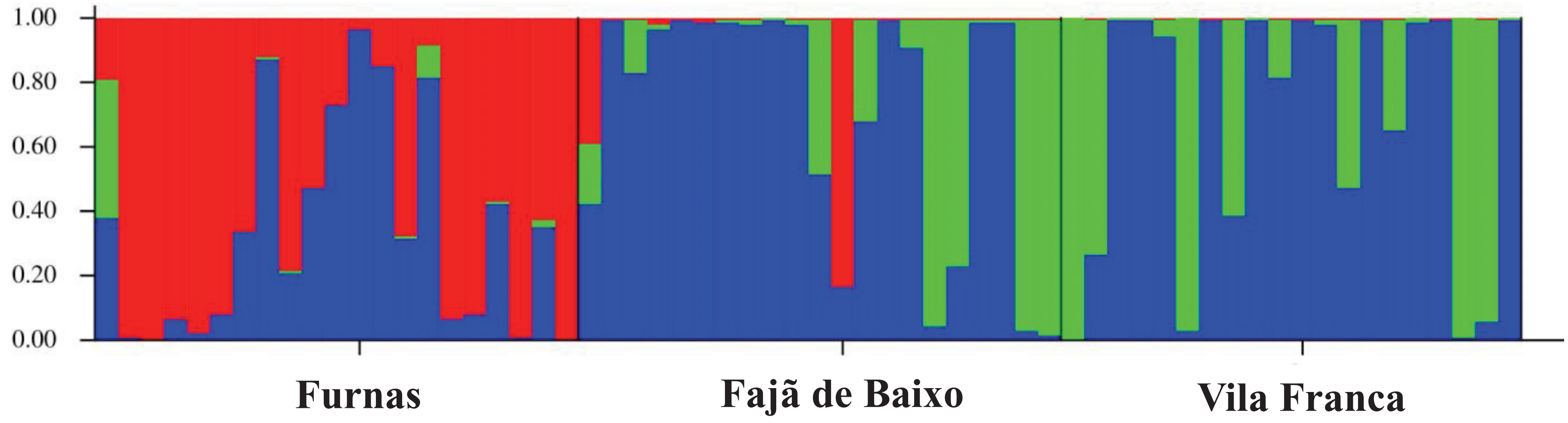

Figure 8. The estimated population structure with a summary plot of the estimates of *Q*. Each individual is represented by a vertical line, which is partitioned into K coloured segments representing the individual's estimated membership fractions in K clusters. The numbers delimited regions correspond to the predefined populations, Furnas, Fajã de Baixo and Vila Franca respectively.

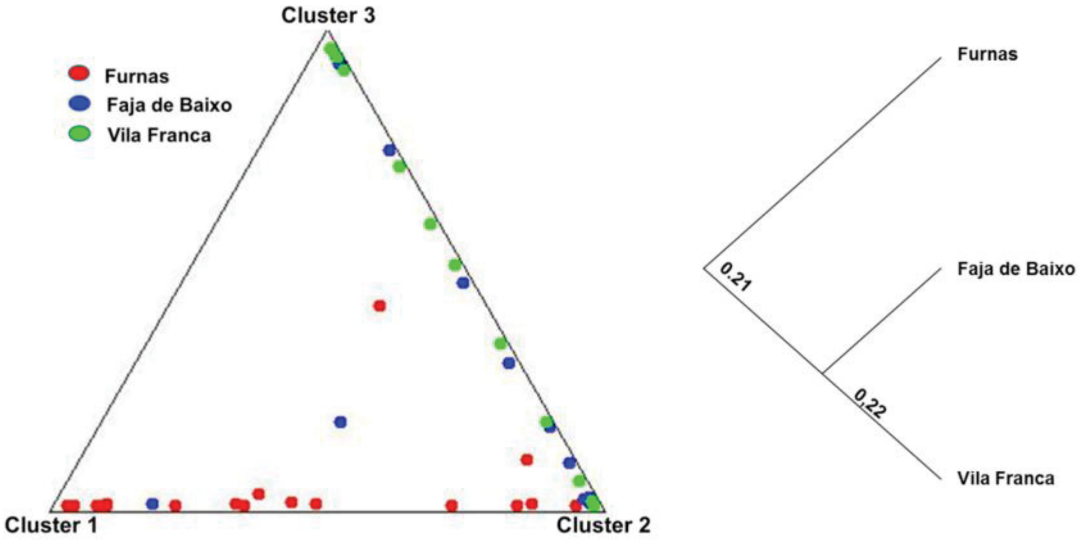

Figure 9. A, Structure analysis of the seven populations when K = 3 clusters are assumed. The Furnas individuals are shown as red spots, blue spots relate to Fajã de Baixo and green spots to the Vila Franca individuals. **B,** Cladogram obtained using allelic frequency divergence among pops (Net nucleotide distance), computed using point estimates obtained in the Structure run. The population allelic frequency divergence against Furnas is over branches.

The results on among-population differentiation suggest different evolutionary histories for Furnas population.

DISCUSSION

The ability to identify and define biological “populations” is crucial for making inferences about the mechanisms that these apparent *Pontoscolex corethrurus* populations were able to colonize and adapt to the different environments in the island of São Miguel which may also be considered for decisions concerning conservation and management. Our previous study showed that *Pontoscocolex corethrurus* populations sampled in the Fajã de Baixo, Vila Franca and Furnas could be resolved into two distinct genetic lineages. The lineage A composed by 97,5% of the individuals sampled in Fajã de Baixo and Vila Franca and lineage F composed by all the individuals in Furnas and only 1 from Fajã de Baixo, with a mean inter-lineage mitochondrial DNA sequence divergence of 18% according with the Bayesian Inference and the 16% Maximum likelihood tree. Interestingly, our present results show in fact the presence of two different clusters but also a minor third cluster mainly shared by populations in Fajã de Baixo and Vila Franca. Moreover, the distinct clusters found are not exclusive of each site population and in fact reveal admixed population sites which are higher than what was revealed by the mitochondrial data. Nevertheless, the usual way that new species are described relies largely on the classical taxonomy assessment using a morphologically based classification system (Pfenninger & Schwenk 2007; Vrijenhoek 2010). However, speciation is not always necessarily paired with morphological differentiation (Hebert *et al.* 2004; Ahrens *et al.* 2007) and, actually cryptic or sibling species are typically found in taxa that succeed in complex, patchy, environments and have been discovered by genotyping fauna inhabiting diverse marine, freshwater, and terrestrial habitats (Hebert *et al.* 2004; Hilário *et al.* 2010; Boissin *et al.* 2011; Westram *et al.* 2011; Avrani *et al.* 2012). As more studies are developed among the Annelida phylum we more become aware that cryptic speciation is not an uncommon phenomenon (Pfenninger & Schwenk 2007; Hilário *et al.* 2010). In fact, earthworms have been the target of several comprehensive phylogenetic studies using both mitochondrial and nuclear markers revealing high intra-species genetic diversity (Velavan *et al.* 2007; Novo *et al.* 2008) and deeply divergent genetic lineages, possibly in some cases corresponding with cryptic species (Pérez-Losada *et al.* 2009; Andre *et al.* 2010; Dupont *et al.* 2011; Novo *et al.* 2011). Furthermore, is necessary to consider that the number of *Pontoscolex corethrurus* populations examined was too restricted to draw firm conclusions regarding micro-evolutionary genealogies, but the higher intra-lineage diversity of the earthworms living in pineapple greenhouses is indicative of a relatively stationary population that has undergone multiple introductions or with a very high initial genetic diversity. Nevertheless, the high number of AFLP markers used in this study may compensate for the low number of individuals used and in fact confirm the mitochondrial relations among the analysed individuals. Furnas individuals were mainly associated with one almost exclusive ancestral cluster agrees with the evidence of a higher proportion of a different *Pontoscolex corethrurus* lineage inhabiting the geothermal field which was confirmed by previous mitochondrial data, although in contrast they showed higher genetic diversity (higher expected heterozigosity). Interestingly, the lower diversity of mtDNA found in Furnas individuals could mean a bias restricting mtDNA diversification or unbalanced sex migration (i.e. different migration rate for males and females). On the one hand it is plausible that the erosive effect of selection on genomic diversity will intensify in populations successfully inhabiting intensely stressful environmental conditions, such as actively volcanic soils. In contrast, an intriguing alternative scenario may pertain where chemical contaminants increase genetic diversity by causing genomic mutations (Hirano & Tamae 2010) which could explain why Furnas show the highest number of private bands. The highest number of private bands in Furnas individuals correlates also with the highest mutations found in mitochondrial data. It is tempting to relate the low haplotype diversity and apparent genetic erosion in Furnas with natural selective pressures within the volcanic environment, although it is possible to be explained either by genetic drift or inbreeding. The genetic erosion can certainly be part of a small fragmented population's history such as those found inhabiting the 'islands of toxicity' that typify abandoned metal mine sites (Andre *et al.* 2010). We also cannot detach the fact that the Lineage "F" in Furnas tolerates the unique combination of hyperthermic, anoxic, hypercarbic, and elevated metal-ion stress which constitutes a unique extreme environment far different from those soils inside the pineapple greenhouses. Patchiness, according with Wiens (1976) is organism-defined, and must be considered in terms of the perceptions of the organism rather than those of the investigator. In the case of the lineage “F” from the geothermal field, it is tempting to interpret the comparatively constricted genetic diversity as a hallmark of stress-driven genetic erosion processes related with natural environmental pressures of the geothermalism having acted upon these populations. Also must be considered that facultative parthenogenesis is believed to happen in *Pontoscolex corethrurus* (Lavelle *et al.* 1987) and parthenogenesis in the geothermal field inhabitants would not be surprising as this facultative reproductive strategy is highly associated to critical changes in the environment (Glesener & Tilman 1978; Lynch & Gabriel 1983) which could also explain the bias introduced by reproduction mode, resulting in more homogeneous mtDNA pool as compared to nuclear variation. Furthermore, there was one individual from Fajã de Baixo that showed to group with the mitochondrial haplotypes found in Furnas showing corroboration with the AFLP data of the exactly same individual (A15). The contrast with mitochondrial data of the previous study was the clustering of some Furnas individuals close to the individuals in Fajã de Baixo, showing to share a common ancestral cluster. Fixation index F_{ST} is a standardizing measure of the degree of genetic differentiation among populations: scores for both measures range from 0 (no differentiation) to 1 (no alleles shared). In this study, the F_{ST} value obtained over all populations was 0.15 and highly significant ($P < 0.001$), suggesting very subtle structuring among the populations. This reveals an interesting fact is that Furnas population is inside a geothermal field and possible gene flow with the adjacent populations is surprising as *Pontoscolex corethrurus* distribution seems to be only limited by the low temperatures in the non-geothermally heated soils. However, we must consider that the introduction of *Pontoscolex corethrurus* could be related to the introduction of pineapple plants was during the first decade of the XIX century with the implementation of pineapple culture by José Bensaude (Stillman & Stillman 1999). Therefore the subtle genetic structure, or better, the absence of a solid population structure among the studied sites may be due to the possible recent introduction (200 years) of *Pontoscolex corethrurus* in the São Miguel Island.

CONCLUSION

Our genetic analysis shows that populations of *Pontoscolex corethrurus* in São Miguel Island are genetically differentiated in three different ancestral genetic clusters, however heterogeneously distributed among the studied sites. It is tempting to relate the high proportion of an almost exclusive ancestral cluster shared by most of the Furnas individuals, which may also reveal some subtle population structure, with the stressful surrounding environment. Nevertheless, surprisingly a physical barrier such as the volcanic crater and the around soil temperature isolating the Furnas population to dispersal seems not enoughpaltry to disrupt the possible migration between adjacent populations and may be responsible for differential sex migration. In addition, the present data corroborates the previous finding of an exclusive Furnas lineage. Our results support the assertion that mtDNA evidence alone should be optimized with nuclear markers in order to delimiting species boundaries. We also recommend that all the future research on micro or macroevolution, biogeography, ecology, conservation, and ecotoxicology on these taxa should consider their specific taxonomical status and the corresponding implications to the experimental designs.

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REFERENCES

Abiadh A., Lamine-Cheniti T., Capanna E., Colangelo P. & others (2010). Molecular phylogenetics of the genus< i> *Gerbillus* </i>(Rodentia, Gerbillinae): Implications for systematics, taxonomy and chromosomal evolution. *Molecular Phylogenetics and Evolution*, 56, 513-518.

Ahrens D., Monaghan M.T. & Vogler A.P. (2007). DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). *Molecular Phylogenetics and Evolution*, 44, 436-449.

Andre J., King R., Stürzenbaum S., Kille P., Hodson M.E. & Morgan A. (2010). Molecular genetic differentiation in earthworms inhabiting a heterogeneous Pb-polluted landscape. *Environmental Pollution*, 158, 883-890.

Avrani S., Ben-Shlomo R. & Inbar M. (2012). Genetic structure of a galling aphid *Slavum wertheimae* and its host tree *Pistacia atlantica* across an Irano-Turanian distribution: from fragmentation to speciation? *Tree Genetics & Genomes*, 1-10.

Bandelt H.J., Forster P. & Röhl A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, 16, 37-48.

Boissin E., Stöhr S. & Chenuil A. (2011). Did vicariance and adaptation drive cryptic speciation and evolution of brooding in *Ophioderma longicauda* (Echinodermata: Ophiuroidea), a common Atlanto-Mediterranean ophiuroid? *Molecular ecology*.

Bonin A., Ehrich D. & Manel S. (2007). Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. *Molecular Ecology*, 16, 3737-3758.

Booth B., Croasdale R. & Walker G. (1978). A quantitative study of five thousand years of volcanism on Sao Miguel, Azores. *Philos. Trans. R. Soc. London, A*, 271-319.

Bovers M., Hagen F., Kuramae E.E. & Boekhout T. (2009). Promiscuous mitochondria in *Cryptococcus gattii*. *FEMS yeast research*, 9, 489-503.

Byrne M., Rowe F. & Uthicke S. (2010). Molecular taxonomy, phylogeny and evolution in the family Stichopodidae (Aspidochirotida: Holothuroidea) based on COI and 16S mitochondrial DNA. *Molecular Phylogenetics and Evolution*, 56, 1068-1081.

Caitlin F.R.M. & John W. (2011). What are the consequences of combining nuclear and mitochondrial data for phylogenetic analysis? Lessons from *Plethodon* salamanders and 13 other vertebrate clades. *BMC Evolutionary Biology*, 11.

Campbell D., Duchesne P. & Bernatchez L. (2003). AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. *Molecular Ecology*, 12, 1979-1991.

Comas D., Plaza S., Wells R.S., Yuldaseva N., Lao O., Calafell F. & Bertranpetit J. (2004). Admixture, migrations, and dispersals in Central Asia: evidence from maternal DNA lineages. *European journal of human genetics*, 12, 495-504.

Cruz J., Coutinho R., Carvalho M., Oskarsson N. & Gislason S. (1999). Chemistry of waters from Furnas volcano, Sao Miguel, Azores: fluxes of volcanic carbon dioxide and leached material. *J. Volcanol. Geotherm. Res.*, 92, 151-167.

Dupont L., Lazrek F., Porco D., King R., Rougerie R., Symondson W., Livet A., Richard B., Deca\.ens T., Butt K. & others (2011). New insight into the genetic structure of the *Allolobophora chlorotica* aggregate in Europe using microsatellite and mitochondrial data. *Pedobiologia*, 54, 217-224.

Earl D.A. & vonHoldt B.M. (2011). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, 1-3.

Ehrich D. (2006). AFLPdat: a collection of R functions for convenient handling of AFLP data. *Mol. Ecol. Notes*, 6, 603-604.

Evanno G., Regnaut S. & Goudet J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular ecology*, 14, 2611-2620.

Excoffier L., Laval G. & Schneider S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary bioinformatics online*, 1, 47.

Falush D., Stephens M. & Pritchard J.K. (2003). Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics*, 164, 1567.

Falush D., Stephens M. & Pritchard J.K. (2007). Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes*, 7, 574-578.

Felsenstein J. (1993). *PHYLIP (phylogeny inference package), version 3.5 c*. Joseph Felsenstein.

Funk D.J. & Omland K.E. (2003). Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, 397-423.

Galtier N., Nabholz B., Glemin S. & Hurst G. (2009). Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology*, 18, 4541-4550.

Glesener R.R. & Tilman D. (1978). Sexuality and the components of environmental uncertainty: clues from geographic parthenogenesis in terrestrial animals. *American Naturalist*, 659-673.

Hawthorne D.J. (2001). AFLP-based genetic linkage map of the Colorado potato beetle Leptinotarsa decemlineata: sex chromosomes and a pyrethroid-resistance candidate gene. *Genetics*, 158, 695.

Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H. & Hallwachs W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 14812.

Hebert P.D.N., Ratnasingham S. & de Waard J.R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 270, S96.

Hilário A., Johnson S., Cunha M. & Vrijenhoek R. (2010). High diversity of frenulates (Polychaeta: Siboglinidae) in the Gulf of Cadiz mud volcanoes: A DNA taxonomy analysis. *Deep Sea Research Part I: Oceanographic Research Papers*, 57, 143-150.

Hirano T. & Tamae K. (2010). Heavy metal-induced oxidative DNA damage in earthworms: a review. *Applied and Environmental Soil Science*, 2010.

Hulce D., Li X., Snyder-Leiby T. & Liu C.S.J. (2011). GeneMarker\textregistered® Genotyping Software: Tools to Increase the Statistical Power of DNA Fragment Analysis. *Journal of Biomolecular Techniques: JBT*, 22, S35.

King R.A., Tibble A.M.Y.L. & Symondson W.O.C. (2008). Opening a can of worms: unprecedented sympatric cryptic diversity within British lumbricid earthworms. *Molecular Ecology*, 17, 4684-4698.

Lavelle P., Barois I., Cruz I., Fragoso C., Hernandez A., Pineda A. & Rangel P. (1987). Adaptive strategies of *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta), a peregrine geophagous earthworm of the humid tropics. *Biology and fertility of soils*, 5, 188-194.

Lynch M. & Gabriel W. (1983). Phenotypic evolution and parthenogenesis. *American Naturalist*, 745-764.

Maruyama T. & Fuerst P.A. (1985). Population bottlenecks and nonequilibrium models in population genetics. II. Number of alleles in a small population that was formed by a recent bottleneck. *Genetics*, 111, 675.

Maruyama T. & Kimura M. (1980). Genetic variability and effective population size when local extinction and recolonization of subpopulations are frequent. *Proceedings of the National Academy of Sciences*, 77, 6710.

Mendelson T.C. & Simons J.N. (2006). AFLPs resolve cytonuclear discordance and increase resolution among barcheek darters (Percidae: Etheostoma: Catonotus). *Molecular phylogenetics and evolution*, 41, 445-453.

Moritz C. & Cicero C. (2004). DNA barcoding: promise and pitfalls. *PLoS Biology*, 2, e354.

Nei M., Maruyama T. & Chakraborty R. (1975). The bottleneck effect and genetic variability in populations. *Evolution*, 1-10.

Novo M., Almodóvar A., Fernández R., Giribet G. & Díaz Cosín D.J. (2011). Understanding the biogeography of a group of earthworms in the Mediterranean basin-the phylogenetic puzzle of Hormogastridae (Clitellata: Oligochaeta). *Molecular Phylogenetics and Evolution*.

Novo M., Velavan T., Almodóvar A., Schulenburg H., DÍAZ-COSÍN D. & Michiels N. (2008). Microsatellite markers for the drought-resistant earthworm *Hormogaster elisae*. *Molecular Ecology Resources*, 8, 901-903.

Peakall R. & Smouse P.E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288-295.

Pérez-Losada M., Ricoy M., Marshall J.C. & Domínguez J. (2009). Phylogenetic assessment of the earthworm *Aporrectodea caliginosa* species complex (Oligochaeta: Lumbricidae) based on mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, 52, 293-302.

Pfenninger M. & Schwenk K. (2007). Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. *BMC Evolutionary Biology*, 7, 121.

Pritchard J.K., Stephens M. & Donnelly P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155, 945.

Rambaut A. (2009). FigTree v1. 2.2. *Computer program and documentation distributed by the author, website http://tree. bio. ed. ac. uk/software*.

Roy L. & Buronfosse T. (2011). Using Mitochondrial and Nuclear Sequence Data for Disentangling Population Structure in Complex Pest Species: A Case Study with *Dermanyssus gallinae*. *PloS one*, 6, e22305.

Sambrook J. & Russell D.W. (2001). *Molecular cloning: a laboratory manual*. CSHL press.

Seehausen O., Takimoto G., Roy D. & Jokela J. (2008). Speciation reversal and biodiversity dynamics with hybridization in changing environments. *Molecular Ecology*, 17, 30-44.

Slatkin M. (1985). Gene flow in natural populations. *Annual review of ecology and systematics*, 16, 393-430.

Slatkin M. (1987). Gene flow and the geographic structure of natural populations. *Science*, 236, 787.

Song H., Buhay J.E., Whiting M.F. & Crandall K.A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences*, 105, 13486.

Stillman Y.K. & Stillman N.A. (1999). *From Iberia to diaspora: studies in Sephardic history and culture*. Brill Academic Pub.

Tranah G., Campton D. & May B. (2004). Genetic evidence for hybridization of pallid and shovelnose sturgeon. *Journal of Heredity*, 95, 474.

Vekemans X., Beauwens T., Lemaire M. & Roldán-Ruiz I. (2002). Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. *Mol. Ecol.*, 11, 139-151.

Velavan T., Schulenburg H. & MICHIELS N.K. (2007). Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris* L.). *Molecular Ecology Notes*, 7, 1060-1062.

Viveiros F., Ferreira T., Cabral Vieira J., Silva C. & Gaspar J.L. (2008). Environmental influences on soil CO2 degassing at Furnas and Fogo volcanoes (São Miguel Island, Azores archipelago). *J. Volcanol. Geotherm. Res.*, 177, 883-893.

Viveiros F., Ferreira T., Silva C. & Gaspar J. (2009). Meteorological factors controlling soil gases and indoor CO2 concentration: A permanent risk in degassing areas. *Sci. Total Environ.*, 407, 1362-1372.

Vos P., Hogers R., Bleeker M., Reijans M., Lee T., Hornes M., Friters A., Pot J., Paleman J., Kuiper M. & others (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic acids research*, 23, 4407.

VRIJENHOEK R.C. (2010). Genetic diversity and connectivity of deep-sea hydrothermal vent metapopulations. *Molecular ecology*, 19, 4391-4411.

Weir B. & Cockerham C.C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution*, 1358-1370.

Westram A.M., Jokela J., Baumgartner C. & Keller I. (2011). Spatial Distribution of Cryptic Species Diversity in European Freshwater Amphipods (*Gammarus fossarum*) as Revealed by Pyrosequencing. *PloS one*, 6, e23879.

White D.J., Wolff J.N., Pierson M. & Gemmell N.J. (2008). Revealing the hidden complexities of mtDNA inheritance. *Molecular Ecology*, 17, 4925-4942.

Whitlock R., Hipperson H., Mannarelli M., Butlin R. & Burke T. (2008). An objective, rapid and reproducible method for scoring AFLP peak-height data that minimizes genotyping error. *Molecular Ecology Resources*, 8, 725-735.

Wiens J.A. (1976). Population responses to patchy environments. *Annual review of ecology and systematics*, 7, 81-120.

Wiens J.J. (2004). Speciation and ecology revisited: phylogenetic niche conservatism and the origin of species. *Evolution*, 58, 193-197.

Wright S. (1943). Isolation by distance. *Genetics*, 28, 114.

Yang D.S. & Kenagy G. (2009). Nuclear and mitochondrial DNA reveal contrasting evolutionary processes in populations of deer mice (*Peromyscus maniculatus*). *Molecular Ecology*, 18, 5115-5125.

Chapter 6

Living in the Edge at Extreme Environments of Volcanic Origin: Azorean Earthworms and its Resident/Symbiotic Microbial Populations

"Living beings defy neat definition. They fight, they feed, they dance, they mate, they die. At the base of the creativity of all large familiar forms of life, symbiosis generates novelty"

Lynn Margulis

Living in the Edge at Extreme Environments of Volcanic Origin: Azorean Earthworms and its Resident/Symbiotic Microbial Populations

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ABSTRACT

The extant volcanic activity in certain terrestrial locations in São Miguel, one of the nine islands comprising the Azores archipelago, provide good "field-laboratories" to study life in extreme environments of volcanic origin. Microorganisms are believed to play an essential role in earthworm's physiological adaptation process, especially on what concerns resident microbial flora that may enhance nutrients absorption as well as facilitate the hosts digestion. In this study we used a genomic approach to compare the bacterial community profile associated with Pontoscolex corethrurus populations from two geographical distinct regions, one living within a terrestrial fumarolic field in Azores and the other, a population resident in pastures of Veracruz, Mexico. For each studied population a bacterial genomic library was created and clones were successfully sequenced and analysed. The several microbial taxa were identified by blast search on the NCBI database. Most of the bacterial diversity found was represented by rare species with few dominant ones. Bacteria belonging to the subclass *α* (alpha) proteobacteria (Proteobacteria Classe) dominated the genomic libraries. The Azorean earthworm population harboured a higher richness of associated microbial species and therefore displayed a higher diversity when compared with Mexico earthworms. Some bacteria were found in both earthworm populations as resident microbial flora (e.g. Nitrobacter, Serratia, Bradyrhizobium, and Methylobacterium) while others seem to be restrict to one of the studied populations. The Azorean *P. corethrurus* has some conspicuous genera such Anaeromyxobacter and Desulfovibrio that may result of adaptations to the environment in which the host lives. This is also the first report of Verminephrobacter phylotypes within the Pontoscolex genus. These bacteria were firstly described as earthworm strict symbionts for other earthworm genera such as Lumbricus, Aporrectodea, Octolasion, and Eisenia. Further study will be needed in order to better understand how the resident microbial flora metabolism can enhance the host fitness and adaptability.

Keywords: Azores, bacterial populations, drilosphere*, Pontoscolex corethrurus*

INTRODUCTION

The extant volcanic activity in certain terrestrial locations on São Miguel, one of the nine islands comprising the Azores archipelago, provide good “field-laboratories” for investigating aspects of the capacity of soil-dwelling macroinvertebrates to inhabit natural habitats continuously receiving potentially toxic chemical inputs in a milieu conducing bioavailability. Geothermal biotopes are highly reducing environments with certain unique features, mainly characterised by elevated soil, water, and atmospheric elemental composition, together with constant diffuse degassing and high temperatures (Cruz *et al.* 1999; Viveiros *et al.* 2008; Viveiros *et al.* 2009). Although, the well documented studies about adaptations of microbial life to a vast environmental molecular constraints together with the knowledge about deep-sea and shallow waters hydrothermal vents, very little is known for eukaryotic and multi cellular organisms exposed to geothermal habitats and even less about the relationships between prokaryotes and eukaryotes within these environments, (Reysenbach *et al.* 1999; Le Bris & Gaill 2007; Cunha *et al.* 2008).

Earthworms are detritivorous macroinvertebrates conferred with the status of ‘ecological engineers and it is known that from the exact moment that soil is in contact with the earthworm body, either superficially or internally, physicochemical and microbiological changes take place (Brown & Doube 2004). These changes are mainly revealed by the modification of the distribution of nutrients and their availability to other organisms (Milleret *et al.* 2009), whilst the earthworm behaviour and the biogenic structures that they build profoundly affect soil physical properties such as as aeration, water-holding capacity or aggregate (Brown 1995; McLean & Parkinson 1997; Jouquet *et al.* 2006). Earthworms also play a significant role in decomposition of organic material and thus in nutrient cycling (Edwards & Fletcher 1988; Cai *et al.* 2002). Such ability can be very valuable for soil fertility establishment in young volcanic soils (Yamaguchi & Haruki 2003).

Pontoscolex corethrurus is a well-known invasive species of many regions around the globe. Its extraordinarily plasticity, accordingly with Lavelle et al (1987) is related to its wide range tolerance to soil physico-chemical characteristics, to moisture and temperature conditions, an outstanding ability to colonize due to its demographic profile and a very efficient assimilation of low-quality soil organic matter. Earthworms are known to feed mainly in organic matter in soil, however its diet also include different types of microorganism like protozoans, actinomycetes, bacteria and even fungi (Furlong *et al.* 2002; Byzov *et al.* 2007; Monroy *et al.* 2008). Nevertheless, this relationship between microorganisms and annelids is not limited to predatory processes but recently discoveries have proved the existence of several ecological relationships like mutualism, symbiosis and even obligated symbiosis, clearly revealed by the polychaetes inhabiting deep-sea hydrothermal vents (Haddad *et al.* 1995; Campbell *et al.* 2003; Hourdez & Lallier 2007) and terrestrial earthworms (Davidson & Stahl 2006, 2008; Lund *et al.* 2010a). Microorganisms are believed to play an essential role in earthworm's adaptation process, especially the ones passing through the guts and thereby facilitating digestion among other features (Knapp *et al.* 2009). The mutualistic relationship, according to Martin et al. (1987) is mainly supported by the fact that earthworms enhance and activate microbial activities by providing in their gut a mucus consisting of energetic and easily metabolizable compounds and favorable physico-chemical conditions: neutral pH, high moisture and temperature conditions (Barois & Lavelle 1986; Zhang *et al.* 1993; Horn *et al.* 2003). Actually, these animals seem to have poor enzymatic abilities and they appear to rely upon the microbial flora in order to degrade soil organic matter (Barois & Lavelle 1986; Zhang *et al.* 1993).

Such microbial population own an immense diversity (Drake & Horn 2007; Brito-Vega & Espinosa-Victoria 2009; Knapp *et al.* 2009) however, it is poorly known mostly due to the non-cultivatable traits of an outsized quantity of microorganisms (Knapp *et al.* 2009). The main objective of the present study was to compare predominant bacterial populations present in two distinct populations of *P. Corethrurus,* one living inside a hydrothermal field in Azores and the other, a population resident in pastures in Veracruz, Mexico. By comparing populations with different life histories we try to elucidate how *P. corethrurus* can live under such adverse conditions.

MATERIALS AND METHODS

Groups of adult (clitellate) *Pontoscolex corethrurus* were collected by digging and hand-sorting during the summer of 2009 in two collecting sites differing at exposure environment and geographical position. One from Azores islands, Portugal (37º 46' N 25º 18' W) Furnas, São Miguel, which displays the most conspicuous degassing and geothermal activity in the entire Azores archipelago, and other from Mexico (b) Botanical Garden in Xalapa, Veracruz (19°30'N, 96°57'W) which does exhibit any thermal and degassing phenomena. After sampling, individuals were immediately transferred to the laboratory, washed thoroughly with distilled water and depurated of gut contents by placing them on moistened paper for 36 h. The samples were stored at -80°C until processed. The earthworm tissue was frozen in liquid N_2 and ground vigorously in a mortar and pestle. Each group was used to extract the microbial fraction by sequential centrifugations using a sucrose density gradient modified from *Wolstenholme et al. (1972) for mitochondria enrichment which we verified that can be efficiently used for general microbial enrichment due to their similar density (See Figure 1 for the schematic representation of the experimental strategy)*. Briefly, to 1 vol of sample were added 5 vol of isolation buffer composed by 0..3 M sucrose, 1 mm disodium ethylenediaminetetraacetate (EDTA), and 0.01 M Tris .HCl (pH 7.4) at 4°C and centrifuged at 480 g for 10 min. The supernatant was further centrifuged at 9000 g for 12 min. The resulted pellet was incubated with Deoxyribonucleate 5′-oligonucleotido-hydrolase (DNAse I) (Sigma-Aldrich), neutralized with EDTA and centrifugated at 9000 g for 5 min. The resulted microbial pellet was suspended in 0.15 M NaCl, 0.1 M EDTA, and 0.01 M Tris–HCl (pH 8.0) and lysed by adding 0.1 vol of 18% sodium dodecyl sulfate (SDS) and 0.2 vol of 5 M NaCl. After centrifuging, the supernatant was incubated with RNase A, and total DNA was extracted with phenol-chloroform, ethanol precipitated, and ressuspended in ultrapure water (Milli-Q). Extracted DNA was purified, sheared, end repaired. Efficient random cloning of sheared DNA (~4.5kb fragments) was accomplished with the commercial vector pJet 2.1 (Fermentas). Plasmid clone was amplified from both ends with standard primers, digested with HindIII and BgIII (Bioline®) and the resulted fragments cloned as above and sequenced with standard pJET kit primers on an ABI PRISM® BigDye v3.1 Terminator Sequencing technology (Applied Biosystems, USA) on the ABI PRISM® 3100 DNA sequencer run by the genomics department of LANGEBIO, Mexico. Raw sequence traces were confirmed using Finch TV (Geospiza, http://www.geospiza.com/finchTV) before being imported into CLC Genomics Workbench v6.6 (CLC Bio, Aarhus, Denmark) for the to test sequence similarity searched against Genbank using BLASTN (2.2.22+) (Altschul *et al.* 1990; Altschul *et al.* 1997). Relative abundances were calculated using the maximum number of clones of each group. Biodiversity indexes were calculated using the relative abundances of clones in each library.

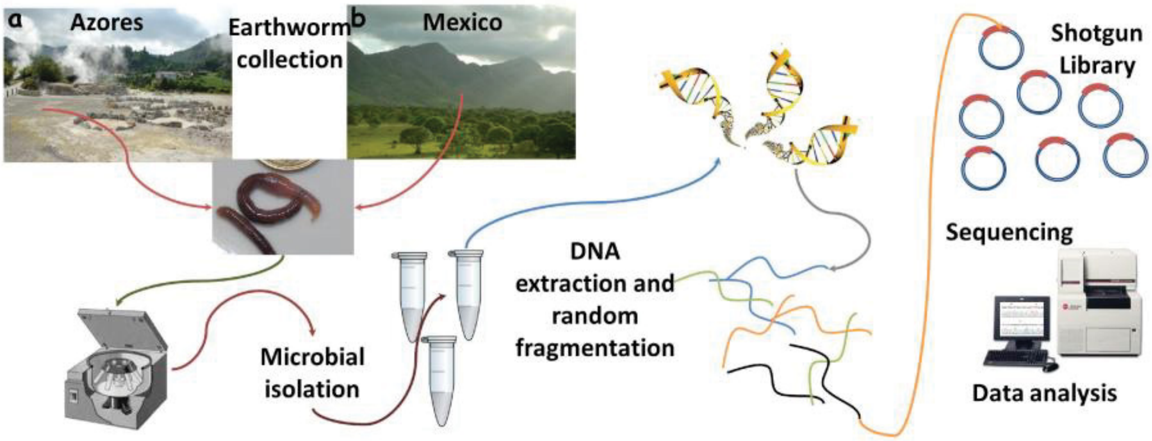

Figure 1. Schematic design of the methodological strategy.

RESULTS

There were few numerically dominant species as well of intermediated abundance but a large number of rare species. In both libraries the microbial abundance was dominated by the α-subclass of the class *Proteobacteria* (Figures 2A and 2B).

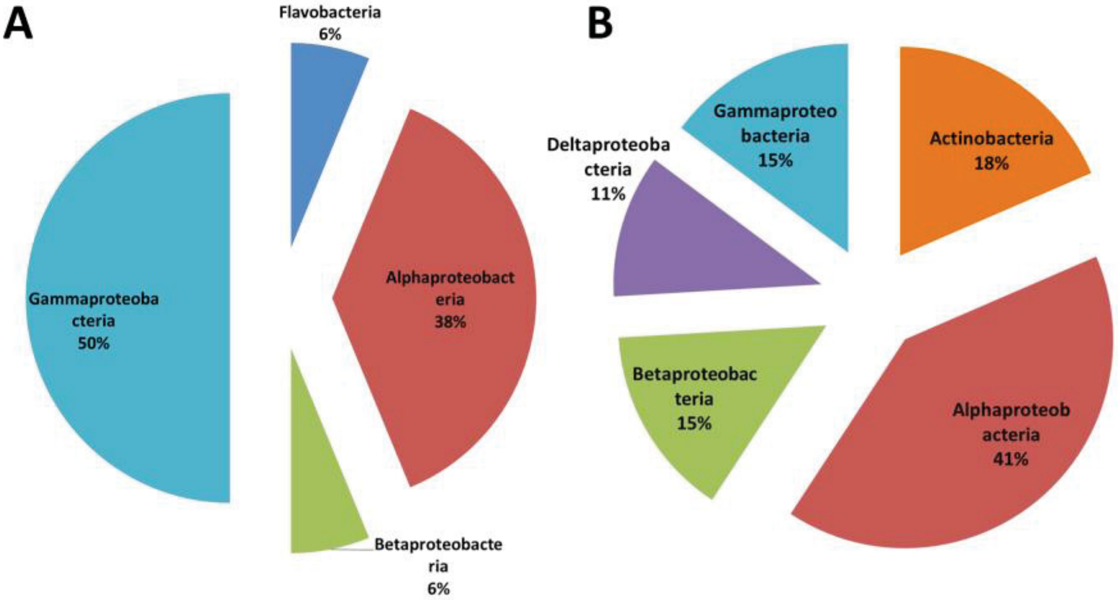

Figure 2. Proportions of the different bacterial subclasses found in *Pontoscolex corethrurus* from México (A) and from the volcanic geothermal field in Azores (B).

Twenty species corresponding to sixteen genus and five phylotypes were found in Mexican clone library. Thirty seven species corresponding to thirty one genus and 9 different microbial types were found in Azorean clone library. The relative abundance of coincident phylotype varied slightly different in both genomic libraries. Tables with the relative abundance (Ab. %) of phylotypes found in the clone library of *P. corethrurus* from Botanical Garden in Xalapa, Mexico (Mx) and Furnas, Azores (Az) are described in full in the annex IV (the names of the most closely related known bacterial species are indicated together the identity used as similarity index). Azorean population was found to have higher species richness and diversity than those from Mexico. The predominant genus in Mexican samples were found to be *Bradyrhizobium, Methylobacterium, Nitrobacter, Paracoccus, Pectobacterium, Rhizobium, Rhodopseudomonas, Burkholderia, Pseudomonas, Stenotrophomonas, Blattabacterium, Dickeya, Edwardsiella, Escherichia and Serratia.* In Azorean populations dominant microbial groups belonged to genus *Agrobacterium, Azorhizobium, Bradyrhizobium, Caulobacter, Mesorhizobium, Methylobacterium, Methylocella, Nitrobacter, Rhizobium, Rhodobacter, Rhodopseudomonas, Acidovorax, Bordetella, Burkholderia, Verminephrobacter, Anaeromyxobacter, Desulfovibrio, Haliangium, Acinetobacter, Pseudomonas, Xanthomonas, Arthrobacter, Clavibacter, Conexibacter, Corynebacterium, Eggerthella, Serratia, Cyanothece and Gloeobacter.* In the Mexican library, no phylotypes affiliated with the gama subclass of class *Proteobacteria,* high GC gram positive bacteria, cyanobacteria, microsporidians and ascomycetes were found, whereas in the second library they constituted together 12% of the clones (Figure 3). In contrast, Azorean library showed no phylotypes, affiliated with the CFB group bacteria, a group closely related with symbiotic bacteria inhabitants of cockroach’s digestive tract (Clark & Kambhampati 2003), which in Mexican library composed 3% of total number of clones (Figure 3).

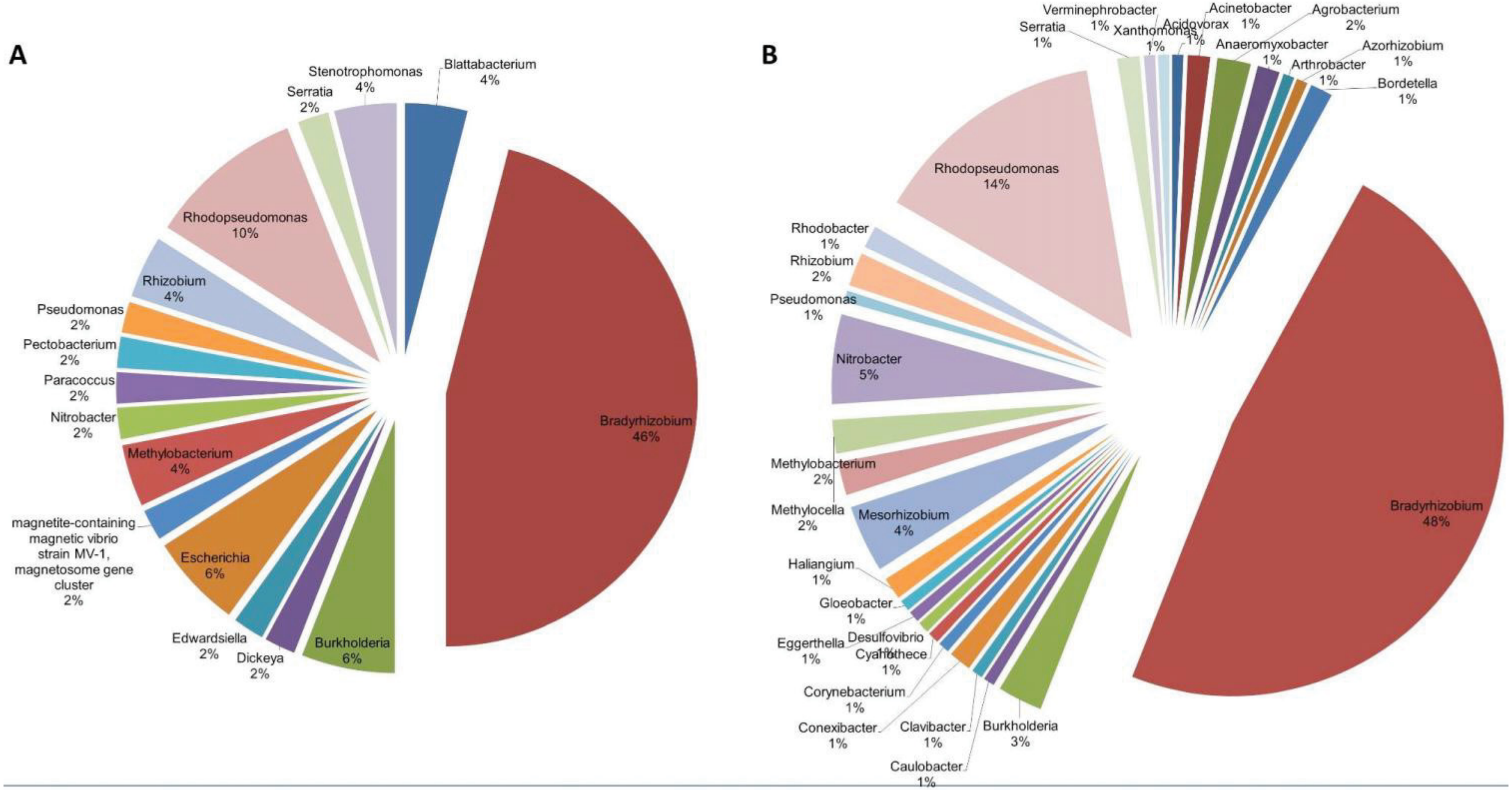

Figure 3. Proportions of the different bacterial groups found in *Pontoscolex corethrurus* from México (A) and from the volcanic geothermal field in Azores (B).

The highest relative abundance belonged to Bradyryzobium-like group which was found in 39% of the Mexican clones and 47% of the clones in the Azorean library. Phylotypes with no similarity to any sequence in the database were denominated as unknown clones, of which Mexican library showed 15% against 3% in the Azorean library.

DISCUSSION

The clones library presented in this paper are, to our knowledge, the first approach in order to reveal the predominant microbial population resident in two important components of the drilosphere (Bouché 1975; Lavelle 1988): the internal micro-environment of the earthworm gut and the earthworm surface in contact with soil. The earthworm body, both internally and externally, is the site of metabolic processes such as external and internal mucus production, respiration, gut passage and nitrogenous excretion (Brown *et al.* 2000). The altered composition of metabolically active groups in the different earthworm's origins could lead to profound changes in the biochemical capacity of the earthworm associated microbiota with the inhabited environment (Horn *et al.* 2003; Ihssen *et al.* 2003), such alterations would not be obvious using culture studies mostly due to the non-cultivatable traits of an outsized quantity of microorganisms (Knapp *et al.* 2009). The altered species diversity could have important adaptation implications for the Azorean *P.corethrurus* to the high and variable temperature, hypoxia and acidic conditions of Furnas soils. Molecular analyses are useful alternatives and can provide ecological information on extremely problematic organisms (Knapp *et al.* 2009). Therefore by using a random sampling of DNA fragments we can delineate future approaches based on oligonucleotide probe design and cellular access. Information from as many sources as possible should be considered when studying microbial community structure associated with earthworms.

Molecular techniques concerning the analysis of DNA fragments like the 16s and 23s gene sequences and also using free-alignment methods have greatly advanced our understanding of phylogenetic diversity of the microbial universe. Nevertheless, each technique has its own problems and solutions.

The active volcanic environment at Furnas poses a multifactorial stress that challenges the local soil-dwelling organisms, including the earthworm P. corethrurus. The microbial species diversity associated with these organisms result in an enhancement for the Azorean *P.corethrurus* that enables these animals to thrive in such high and variable temperature, hypoxia and acidic conditions in Furnas soils. The high numbers obtained for nitrate-dissimilating and nitrifying bacteria in the earthworm flora suggest that the earthworms may be influencing the nutrient cycling in these soils (Ihssen *et al.* 2003) and in the case of the Azorean population this action may be crucial for the nutrient transformation to more plant-available forms (Edwards & Fletcher 1988). Furnas´s earthworms populations not only harbour several nitrogen-fixation microbes like their close relatives from Mexico but possess also several microorganisms linked with metal detoxification, C1 compounds reduction and exopolymers producers. Verminephrobacter was detected only on the Azorean population. This seems to be the result of an ancient symbiosis that enhances the host’s fitness under stress situations by increasing the success of hatched cocoons and increasing maturation rates (Lund *et al.* 2010a; Lund *et al.* 2010b; Bakke *et al.* 2011).

The higher richness found within the Furnas´s population is characteristic of a population subject to highly variable ecosystem as one would expect from a geothermal soil area.

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REFERENCES

Altschul S., Gish W., Miller W., Myers E. & Lipman D. (1990). Basic local alignment search tool. *Journal of molecular biology*, 215, 403-410.

Altschul S., Madden T., Schaffer A., Zhang J., Zhang Z., Miller W. & Lipman D. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research*, 25, 3389.

Bakke B.B., Wilson R. & Rudi K. (2011). Prevalence and dispersal of a facultative bacterial symbiont associated with an endemic metazoan host. *Pedobiologia*, 54, 153-157.

Barois I. & Lavelle P. (1986). 26.Changes in respiration rate and some physicochemical properties of a tropical soil during transit through *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta). *Soil Biology and Biochemistry*, 18, 539-541.

Bouché M. (1975). Action de la faune sur les états de la matière organique dans les écosystèmes. *Biodégradation et Humification. Pierron, Sarrugemines*, 157–168.

Brito-Vega H. & Espinosa-Victoria D. (2009). Bacterial Diversity in the Digestive Tract of Earthworms(Oligochaeta). *Journal of Biological Sciences*, 9, 192-199.

Brown G. (1995). How do earthworms affect microfloral and faunal community diversity? *Plant and Soil*, 170, 209-231.

Brown G., Barois I. & Lavelle P. (2000). Regulation of soil organic matter dynamics and microbial activityin the drilosphere and the role of interactionswith other edaphic functional domains *European Journal of Soil Biology*, 36, 177-198.

Brown G. & Doube B. (2004). Functional interactions between earthworms, microorganisms, organic matter, and plants. *Earthworm ecology. 2nd edn. CRC Press, Boca Raton, FL*, 213–239.

Byzov B., Khomyakov N., Kharin S. & Kurakov A. (2007). Fate of soil bacteria and fungi in the gut of earthworms. *European Journal of Soil Biology*, 43, S149-S156.

Cai H., Zarda B., Mattison G., Schönholzer F. & Hahn D. (2002). Fate of Protozoa transiting the digestive tract of the earthworm *Lumbricus terrestris* L. *Pedobiologia*, 46, 161-175.

Campbell B., Stein J. & Cary S. (2003). Evidence of chemolithoautotrophy in the bacterial community associated with *Alvinella pompejana*, a hydrothermal vent polychaete. *Applied and environmental microbiology*, 69, 5070.

Clark J. & Kambhampati S. (2003). Phylogenetic analysis of Blattabacterium, endosymbiotic bacteria from the wood roach, Cryptocercus (Blattodea: Cryptocercidae), including a description of three new species* 1. *Molecular Phylogenetics and Evolution*, 26, 82-88.

Cruz J., Coutinho R., Carvalho M., Oskarsson N. & Gislason S. (1999). Chemistry of waters from Furnas volcano, Sao Miguel, Azores: fluxes of volcanic carbon dioxide and leached material. *J. Volcanol. Geotherm. Res.*, 92, 151-167.

Cunha L., Amaral A., Medeiros V., Martins G., Wallenstein F., Couto R., Neto A. & Rodrigues A. (2008). Bioavailable metals and cellular effects in the digestive gland of marine limpets living close to shallow water hydrothermal vents. *Chemosphere*, 71, 1356-1362.

Davidson S. & Stahl D. (2006). Transmission of nephridial bacteria of the earthworm *Eisenia fetida*. *Applied and Environmental Microbiology*, 72, 769.

Davidson S. & Stahl D. (2008). Selective recruitment of bacteria during embryogenesis of an earthworm. *The ISME Journal*, 2, 510-518.

Drake H. & Horn M. (2007). As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes.

Edwards C. & Fletcher K. (1988). Interactions between earthworms and microorganisms in organic-matter breakdown. *Agriculture, Ecosystems & Environment*, 24, 235-247.

Furlong M., Singleton D., Coleman D. & Whitman W. (2002). Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. *Applied and environmental microbiology*, 68, 1265.

Haddad A., Camacho F., Durand P. & Cary S. (1995). Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete *Alvinella pompejana*. *Applied and environmental microbiology*, 61, 1679.

Horn M., Schramm A. & Drake H. (2003). 18.The earthworm gut: an ideal habitat for ingested N2O-producing microorganisms. *Applied and environmental microbiology*, 69, 1662.

Hourdez S. & Lallier F. (2007). Adaptations to hypoxia in hydrothermal-vent and cold-seep invertebrates. *Rev. Environ. Sci. Biotechnol.*, 6, 143-159.

Ihssen J., Horn M., Matthies C., Gossner A., Schramm A. & Drake H. (2003). N2O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. *Applied and Environmental Microbiology*, 69, 1655.

Jouquet P., Dauber J., Lagerlöf J., Lavelle P. & Lepage M. (2006). Soil invertebrates as ecosystem engineers: intended and accidental effects on soil and feedback loops. *Applied Soil Ecology*, 32, 153-164.

Knapp B., Podmirseg S., Seeber J., Meyer E. & Insam H. (2009). Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. *Soil Biology and Biochemistry*.

Lavelle P. (1988). Earthworm activities and the soil system. *Biology and Fertility of Soils*, 6, 237-251.

Lavelle P., Barois I., Cruz I., Fragoso C., Hernandez A., Pineda A. & Rangel P. (1987). Adaptive strategies of *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta), a peregrine geophagous earthworm of the humid tropics. *Biology and fertility of soils*, 5, 188-194.

Le Bris N. & Gaill F. (2007). How does the annelid *Alvinella pompejana* deal with an extreme hydrothermal environment? *Reviews in environmental science and biotechnology*, 6, 197-221.

Lund M., Holmstrup M., Lomstein B., Damgaard C. & Schramm A. (2010a). Beneficial effect of *Verminephrobacter nephridial* symbionts on the fitness of the earthworm *Aporrectodea tuberculata*. *Applied and Environmental Microbiology*.

Lund M.B., Davidson S.K., Holmstrup M., James S., Kjeldsen K.U., Stahl D.A. & Schramm A. (2010b). Diversity and host specificity of the Verminephrobacter–earthworm symbiosis. *Environmental microbiology*, 12, 2142-2151.

Margulis L. (1999). *Symbiotic planet: a new look at evolution*. Basic Books (AZ).

Martin A., Cortez J., Barois I. & Lavelle P. (1987). The production of intestinal mucus by earthworms: A key process in their interactions with the soil microflora. *Rev. Ecol. Biol. Sol.*, 24, 549-558.

McLean M. & Parkinson D. (1997). Changes in structure, organic matter and microbial activity in pine forest soil following the introduction of *Dendrobaena octaedra* (Oligochaeta, Lumbricidae). *Soil Biology and Biochemistry*, 29, 537-540.

Milleret R., Le Bayon R. & Gobat J. (2009). Root, mycorrhiza and earthworm interactions: their effects on soil structuring processes, plant and soil nutrient concentration and plant biomass. *Plant and Soil*, 316, 1-12.

Monroy F., Aira M. & Domínguez J. (2008). Changes in density of nematodes, protozoa and total coliforms after transit through the gut of four epigeic earthworms (Oligochaeta). *Applied Soil Ecology*, 39, 127-132.

Reysenbach A., Seitzinger S., Kirshtein J. & McLaughlin E. (1999). Molecular constraints on a high-temperature evolution of early life. *Biological Bulletin*, 196, 367-372.

Viveiros F., Ferreira T., Cabral Vieira J., Silva C. & Gaspar J.L. (2008). Environmental influences on soil CO2 degassing at Furnas and Fogo volcanoes (São Miguel Island, Azores archipelago). *J. Volcanol. Geotherm. Res.*, 177, 883-893.

Viveiros F., Ferreira T., Silva C. & Gaspar J. (2009). Meteorological factors controlling soil gases and indoor CO2 concentration: A permanent risk in degassing areas. *Sci. Total Environ.*, 407, 1362-1372.

Wolstenholme D., Kirschner R. & Gross N. (1972). Heat denaturation studies of rat liver mitochondrial DNA: A denaturation map and changes in molecular configurations. *Journal of Cell Biology*, 53, 393.

Yamaguchi T. & Haruki M. (2003). Developing soil from nutrient-poor volcanic ash using earthworms and leaf litter. *Journal of the Japanese Society of Revegetation Technology*, 28, 417-425.

Zhang B., Rouland C., Lattaud C. & Lavelle P. (1993). 32.Activity and origin of digestive enzymes in gut of the tropical earthworm *Pontoscolex corethrurus*. *European Journal of Soil Biology*, 29, 7-11.

Chapter 7

Final considerations and future studies

Final considerations and future studies

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FINAL CONSIDERATIONS

During the different chapters of this document was possible to reveal how a soil dwelling organism such earthworms are challenged by the multifactorial stress posed by the "extreme" volcanic soil environment. The differentiation found from individuals to the geographically defined populations reveals how the conspicuous volcanic environment could act as furnace for evolution. Survival in active volcanic soils was the result of very specific cellular adjustments in the epidermis structure, closely linked with biochemical and also metabolic alterations of the function of this vital organ. It is tempting to relate the acquiring of tolerance as a direct consequence of the exposure to multiple stressors, including elevated metal availability, hypoxia, hypercapnia and increased temperature. Interestingly, these findings are identical for both earthworms' species that live in the geothermal field, although belonging to distinct ecological groups. The carried genetic characterisation should be perceived as an essential prelude to future environmental diagnostics and functional genomics. In fact, recent advances in "omics" technologies render tractable the challenges alluded to above, such that they represent an unparalleled opportunity in environmental diagnostics to describe the genetic background of a species that has evolved on a remote island of relatively well established geological history. The conspicuous genetic mitochondrial differentiation of Furnas earthworms affects the questions about the timing and source(s) of the colonization. How many lineages conquered the Island and how/when such event occurred? Are the exclusive genetic nuclear markers found in Furnas earthworms a result of evolution within the Island, and if so can we relate them to historical events? These earthworms have an amazing story to tell and just waiting to be discovered.

We also must consider that population isolation and bottlenecks caused by human and volcanic activity on São Miguel in relatively recent times may be a possible cause (Van Riel *et al.* 2003). However, in contrast to the mtDNA data the nuclear marker in Furnas revealed a subtle but higher heterosygosity than in the other analysed groups. As this species is known to be parthenogenetic (Lavelle *et al.* 1987) such unexpected heterosygosity could be explained as the bias introduced by reproduction mode, resulting in more homogeneous mtDNA pool as compared to nuclear variation. Previous results about the genetic distances of a land snail *Leptaxis azorica*, endemic on the Azores suggested the presence of four distinct lineages which are well-matched with colonization proceeding from the eastern, older islands (Santa Maria and São Miguel) to the west (Flores and Corvo). On São Miguel, genetic and morphometric differentiation is concordant with the separate colonisation of two islands that gave rise to the current island 50 000 years ago. The maximum time available for differentiation in isolation (0.55 million years) suggests a high rate of allozyme change between the two lineages on São Miguel. This is more prominent in the eastern region where populations are also characterised by reduced genetic variation (loss of alleles and heterozygosity) compared to populations elsewhere (Van Riel *et al.* 2003). Therefore it should be deemed to analyse some *P. corethrurus* conspecific populations in Africa and South/Central America. The primary purpose will be determining the global intra-specific genetic diversity in order to provide an insight into the variability available for natural selection in the locally stressed locations.

FUTURE STUDIES

"It is important to foster and catalyse research activities on life processes in extreme environments, not only because they are applicable to some of the main scientific

questions such as understanding the basics of life processes, the origin of life or the potential existence of extraterrestrial life, but also because the economic potential behind these activities is important. For all these reasons, 'investigating life in extreme environments' represents a major challenge for earth's inhabitants at the beginning of the 21st century."

European Science Foundation (ESF) (2007)

Revealing how some organisms deal with extreme conditions in their environment is becoming very useful to understand the origin and evolution of life, but without doubt, the major impetus driving extremophile research is the biotechnological potential associated with them (De Champdoré *et al.* 2007). Indeed, the application of extremophiles in industrial processes has opened a new era in biotechnology. Each group has unique features that can be exploited to provide biomolecules and mechanisms with a wide variety of applications (Kim & Peeples 2006; Rath *et al.* 2009). Although little is known about eukaryotic life in extreme environments in comparison to prokaryotic extremophiles, recent advances have provided new insights into the metabolic adaptations of eukaryotes living under extreme conditions (Bris & Gaill 2007; Mary *et al.* 2010).

Under a recently approved research project by the Portuguese National Government we are now able to start the sequencing of the full genome of *P. corethrurus* which will open new "gates" of study and therefore make possible the assessment of several mechanistic aspects of the influence of such environment but also the genomics associated with the population genetics and its phylogenetic relationships which will enable tools that will make possible to infer the correct answers assumed within the present document. The value added aspect of this research will be technology development and transfer through the acquisition of a rich body of DNA sequences that can readily be used for subsequent functional studies, using customized functional transcriptomics technology. Therefore, ***some*** attention will also be given to the fact that tolerance to the elevated metal ion concentrations within the volcanic soils may be dependent on the elevation of metallo-chaperones, metallothionein or phylochelatins, together with generic stress response pathways. The latter breakthrough would be unprecedented and well received by the research community because the expanded resource will undoubtedly also prove invaluable in diverse comparative ecophysiology, phylogenetic, and phylogeography applications. Due to our recent finding, ideally the approach should consider that transcriptomic assays should be performed on a single genotyped 'strain' of adult *P. corethrurus*. Within this context a body of evidence is accumulating indicating significant differences in the responses to certain inorganic and organic environmental toxicants of cryptic (sibling) species belonging to a number of aquatic taxa (Linke-Gamenick *et al.* 2000a; Linke-Gamenick *et al.* 2000b; Palmqvist & Forbes 2008). Very recently a number of lumbricid earthworm 'species' have been shown to possess exceptionally high genetic diversity, with two or more lineages in certain instances probably warranting the status of cryptic or sibling species (Andre *et al.* 2010). These findings are critically important because they raise the spectre that the different genotypes display differential responses or susceptibilities to environmental contaminants and it should be taken in consideration in our further studies in *Pontoscolex corethrurus.*

REFERENCES

Amils R., Ellis-Evans C. & Hinghofer-Szalkay H. (2007). *Life in extreme environments*. Springer Verlag.

Andre J., King R., Stürzenbaum S., Kille P., Hodson M.E. & Morgan A. (2010). Molecular genetic differentiation in earthworms inhabiting a heterogeneous Pb-polluted landscape. *Environmental Pollution*, 158, 883-890.

Bris N. & Gaill F. (2007). How does the annelid *Alvinella pompejana* deal with an extreme hydrothermal environment? *Life in Extreme Environments*, 315-339.

De Champdoré M., Staiano M., Rossi M. & D'Auria S. (2007). Proteins from extremophiles as stable tools for advanced biotechnological applications of high social interest. *Journal of The Royal Society Interface*, 4, 183-191.

Kim J.W. & Peeples T.L. (2006). Screening extremophiles for bioconversion potentials. *Biotechnology progress*, 22, 1720-1724.

Lavelle P., Barois I., Cruz I., Fragoso C., Hernandez A., Pineda A. & Rangel P. (1987). Adaptive strategies of *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta), a peregrine geophagous earthworm of the humid tropics. *Biol. Fertil. Soils*, 5, 188-194.

Linke-Gamenick I., Forbes V.E. & Méndez N. (2000a). Effects of chronic fluoranthene exposure on sibling species of *Capitella* with different development modes.

Linke-Gamenick I., Vismann B. & Forbes V.E. (2000b). Effects of fluoranthene and ambient oxygen levels on survival and metabolism in three sibling species of *Capitella* (Polychaeta).

Mary J., Rogniaux H., Rees J.F. & Zal F. (2010). Response of *Alvinella pompejana* to variable oxygen stress: a proteomic approach. *Proteomics*, 10, 2250-2258.

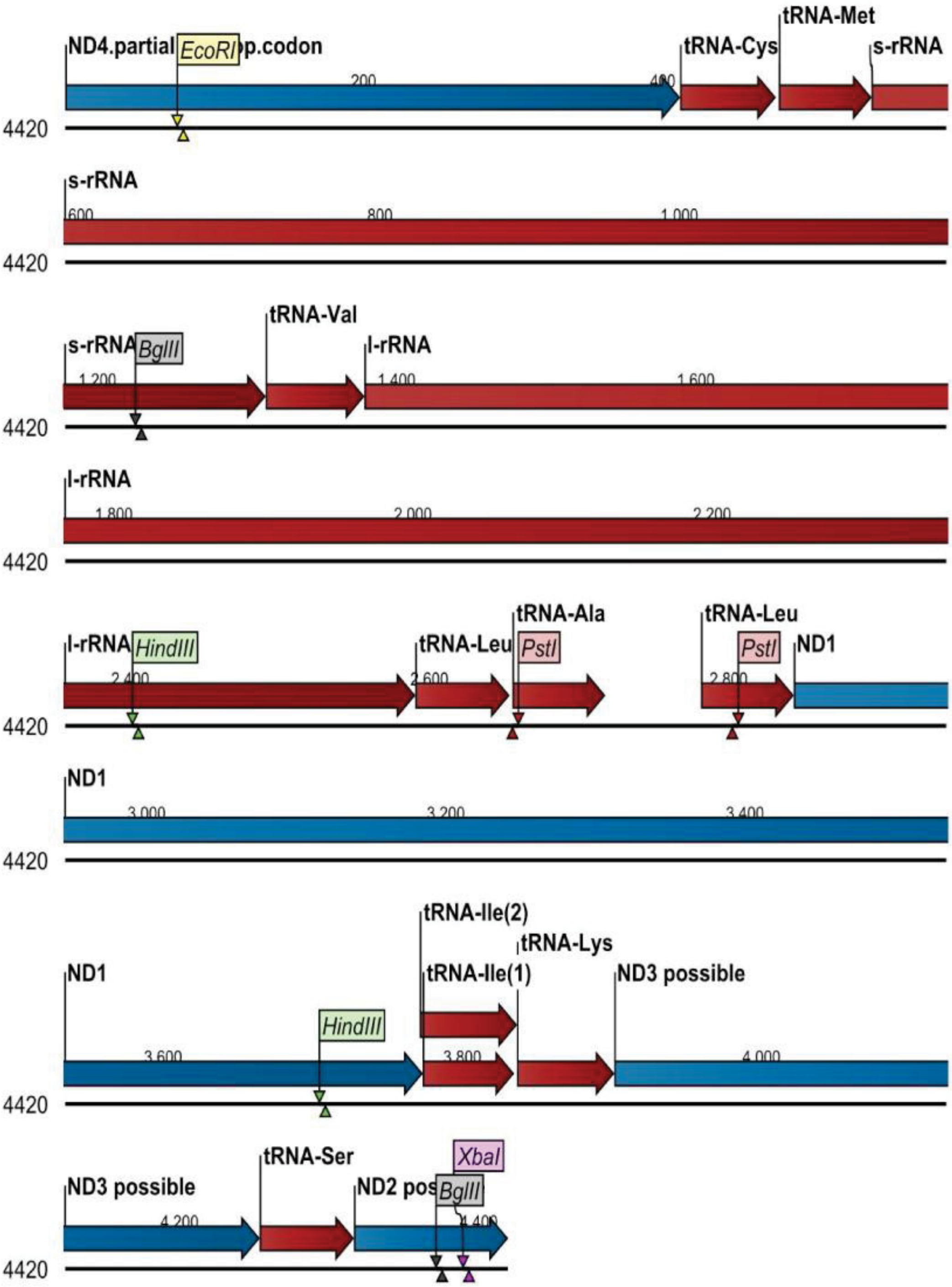
Palmqvist A. & Forbes V.E. (2008). Demographic Effects of the polycyclic aromatic hydrocarbon fluoranthene on two sibling species of the polychaete *Capitella capitata*. *Demographic toxicity: methods in ecological risk assessment*, 200.

Rath C.C., Mohanta H. & Dash S. (2009). Extremophiles as Novel Cell Factories. *Biotechnology Applications*, 282.

Van Riel P., Jordaens K., Verhagen R., Martins A.M.F. & Backeljau T. (2003). Genetic differentiation reflects geological history in the Azorean land snail, *Leptaxis azorica*. *Heredity*, 91, 239-247.

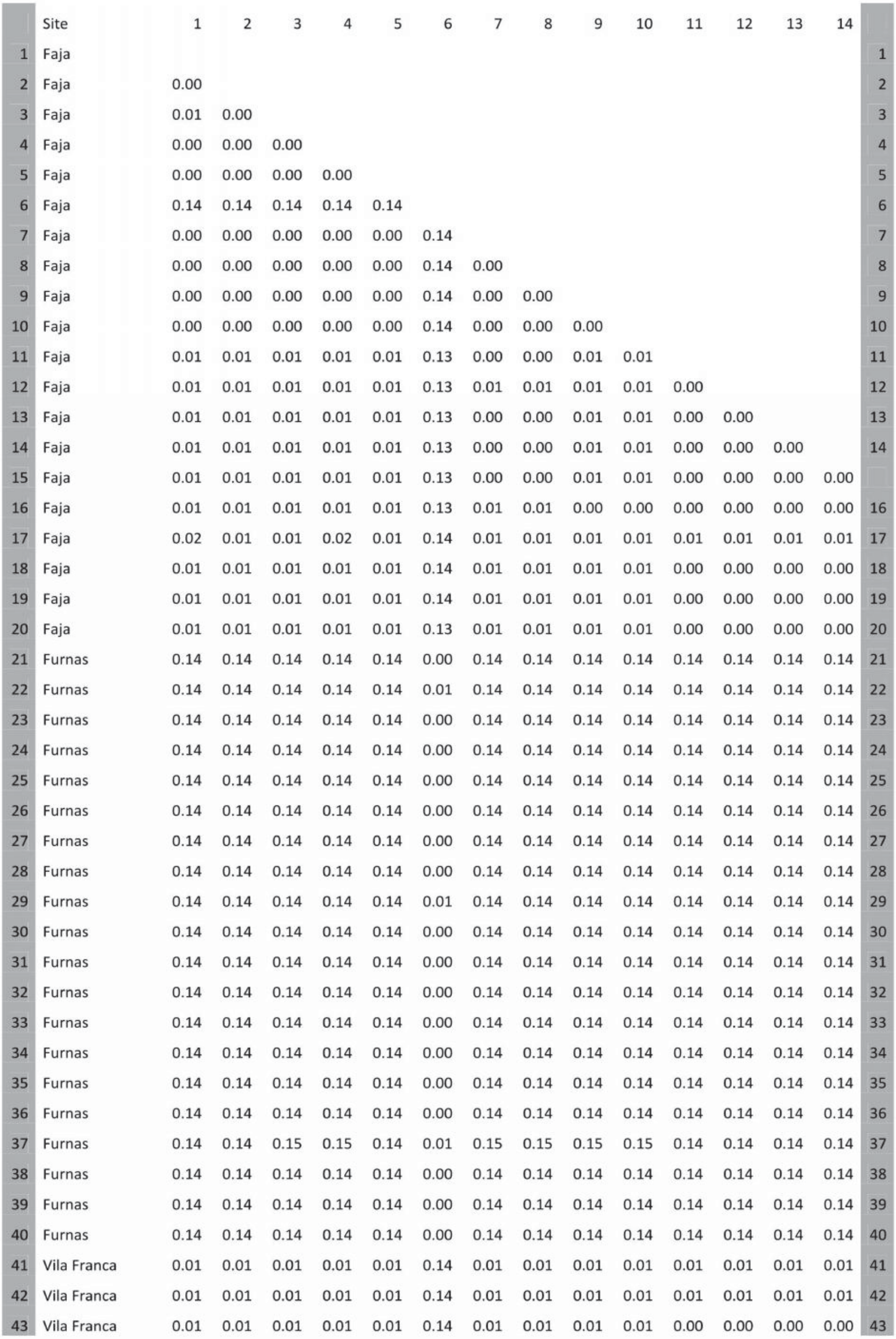
Annex I

Figure of the cloned mitochondrial fragment used for the most part of the mitochondrial genotyping of different populations of *Pontoscolex corethrurus* in the study.


ND4.partial
EcoRI
p.codon
200
400
tRNA-Cys
tRNA-Met
s-rRNA
4420
s-rRNA
600
800
1 000
4420
s-rRNA
BglII
1 200
tRNA-Val
l-rRNA
1 400
1 600
4420
l-rRNA
1 800
2 000
2 200
4420
l-rRNA
HindIII
2 400
tRNA-Leu
2 600
tRNA-Ala
PstI
tRNA-Leu
PstI
2 800
ND1
4420
ND1
3 000
3 200
3 400
4420
ND1
3 600
HindIII
tRNA-Ile(2)
tRNA-Lys
tRNA-Ile(1)
3 800
ND3 possible
4 000
4420
ND3 possible
4 200
tRNA-Ser
ND2 pos
XbaI
BglII
4 400
4420

Annex II

Table with all the individual values for the average p-distance calculated for the concatenated gene fragment of populations of *Pontoscolex corethrurus* from the volcanic geothermal field Furnas, pineapple greenhouses in Faja de Baixo and Vila Franca and the outgroup composed by *Peryonix excavates*, *Lumbricus terrestris* and *Lumbricus Rubellus*. The number of base differences per site from averaging over all sequence pairs between groups are shown. The analysis involved 63 nucleotide sequences (collapse of identical haplotypes). All positions containing gaps and missing data were eliminated. There were a total of 2359 positions in the final dataset.



	Site	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1	Faja															1
2	Faja	0.00														2
3	Faja	0.01	0.00													3
4	Faja	0.00	0.00	0.00												4
5	Faja	0.00	0.00	0.00	0.00											5
6	Faja	0.14	0.14	0.14	0.14	0.14										6
7	Faja	0.00	0.00	0.00	0.00	0.00	0.14									7
8	Faja	0.00	0.00	0.00	0.00	0.00	0.14	0.00								8
9	Faja	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00							9
10	Faja	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00						10
11	Faja	0.01	0.01	0.01	0.01	0.01	0.13	0.00	0.00	0.01	0.01					11
12	Faja	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.00				12
13	Faja	0.01	0.01	0.01	0.01	0.01	0.13	0.00	0.00	0.01	0.01	0.00	0.00			13
14	Faja	0.01	0.01	0.01	0.01	0.01	0.13	0.00	0.00	0.01	0.01	0.00	0.00	0.00		14
15	Faja	0.01	0.01	0.01	0.01	0.01	0.13	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	
16	Faja	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	16
17	Faja	0.02	0.01	0.01	0.02	0.01	0.14	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	17
18	Faja	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	18
19	Faja	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	19
20	Faja	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	20
21	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	21
22	Furnas	0.14	0.14	0.14	0.14	0.14	0.01	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	22
23	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	23
24	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	24
25	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	25
26	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	26
27	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	27
28	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	28
29	Furnas	0.14	0.14	0.14	0.14	0.14	0.01	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	29
30	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	30
31	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	31
32	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	32
33	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	33
34	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	34
35	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	35
36	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	36
37	Furnas	0.14	0.14	0.15	0.15	0.14	0.01	0.15	0.15	0.15	0.15	0.14	0.14	0.14	0.14	37
38	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	38
39	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	39
40	Furnas	0.14	0.14	0.14	0.14	0.14	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	40
41	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	41
42	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	42
43	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	43

44	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	44
45	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	45
46	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	46
47	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	47
48	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	48
49	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	49
50	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	50
51	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	51
52	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	52
53	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	53
54	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	54
55	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	55
56	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.13	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	56
57	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	57
58	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	58
59	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	59
60	Vila Franca	0.01	0.00	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	60
61	P.excavatus	0.33	0.33	0.33	0.33	0.33	0.32	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	61
62	L.rubellus	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	62
63	L.terrestris	0.36	0.36	0.36	0.36	0.36	0.34	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	63

		15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
16	Faja	0.00																16
17	Faja	0.01	0.01															17
18	Faja	0.00	0.00	0.01														18
19	Faja	0.00	0.00	0.01	0.00													19
20	Faja	0.00	0.00	0.01	0.00	0.00												20
21	Furnas	0.14	0.14	0.14	0.14	0.14	0.14											21
22	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00										22
23	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00									23
24	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.01	0.00								24
25	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00							25
26	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00						26
27	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.01	0.00	0.00	0.00	0.00					27
28	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.01	0.00	0.00	0.00	0.00	0.00				28
29	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01			29
30	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01		30
31	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	
32	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	32
33	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	33
34	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	34
35	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	35
36	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	36
37	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	37
38	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	38

39	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	39
40	Furnas	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	40
41	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	41
42	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	42
43	Vila Franca	0.00	0.00	0.01	0.00	0.00	0.00	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	43
44	Vila Franca	0.00	0.00	0.01	0.00	0.00	0.00	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	44
45	Vila Franca	0.01	0.01	0.02	0.01	0.01	0.01	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	45
46	Vila Franca	0.00	0.00	0.01	0.00	0.00	0.00	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	46
47	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	47
48	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	48
49	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	49
50	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	50
51	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	51
52	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	52
53	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	53
54	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	54
55	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	55
56	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	56
57	Vila Franca	0.00	0.00	0.01	0.00	0.00	0.00	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	57
58	Vila Franca	0.00	0.00	0.01	0.00	0.00	0.00	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	58
59	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	59
60	Vila Franca	0.00	0.00	0.01	0.00	0.00	0.00	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	60
61	P.excavatus	0.33	0.33	0.33	0.33	0.33	0.33	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	61
62	L.rubellus	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	62
63	L.terrestris	0.36	0.36	0.36	0.36	0.36	0.36	0.34	0.35	0.34	0.34	0.34	0.34	0.34	0.34	0.35	0.34	63

		31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	
32	Furnas	0.00															32
33	Furnas	0.00	0.00														33
34	Furnas	0.00	0.00	0.00													34
35	Furnas	0.00	0.00	0.00	0.00												35
36	Furnas	0.00	0.00	0.00	0.00	0.00											36
37	Furnas	0.01	0.01	0.01	0.01	0.01	0.01										37
38	Furnas	0.00	0.00	0.00	0.00	0.00	0.00	0.01									38
39	Furnas	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00								39
40	Furnas	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00							40
41	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13						41
42	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.14	0.13	0.13	0.00					42
43	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.01	0.01				43
44	Vila Franca	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.01	0.01	0.00			44
45	Vila Franca	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.01	0.01	0.01	0.01		45
46	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.14	0.13	0.13	0.01	0.01	0.00	0.00	0.01	46
47	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	47
48	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	48
49	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	49

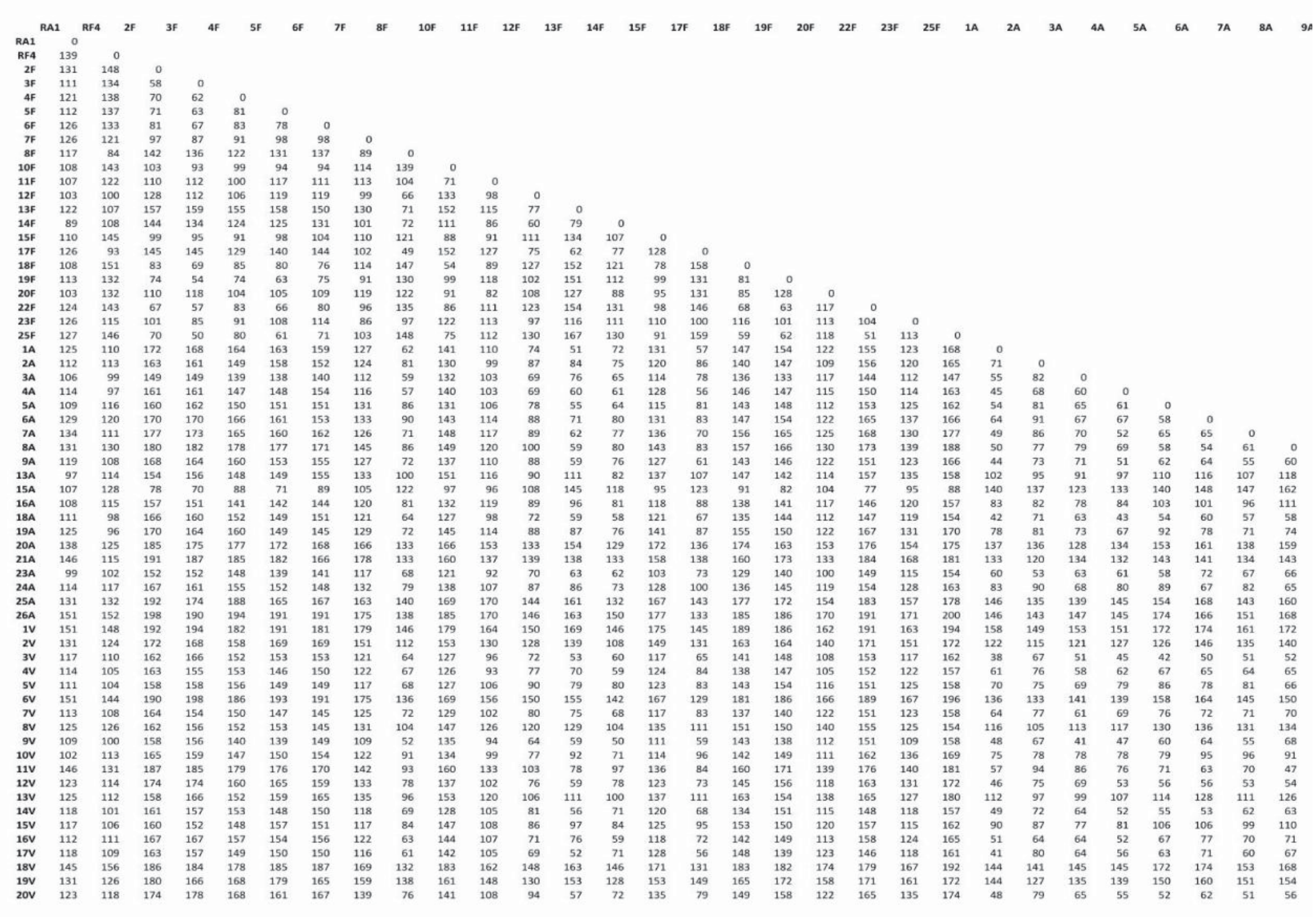
50	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	50
51	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	51
52	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	52
53	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	53
54	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	54
55	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	55
56	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.00	0.00	0.01	0.01	0.01	56
57	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.01	0.01	0.00	0.00	0.01	57
58	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.01	0.01	0.00	0.00	0.01	58
59	Vila Franca	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.01	0.01	0.01	0.01	0.01	59
60	Vila Franca	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.14	0.13	0.13	0.01	0.01	0.00	0.00	0.01	60
61	P.excavatus	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.33	0.33	0.33	0.33	0.33	61
62	L.rubellus	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	62
63	L.terrestris	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.35	0.36	0.35	0.36	0.36	63

		46	47	48	49	50	51	52	53	54	55	56	57	58	59	
47	Vila Franca	0.01														47
48	Vila Franca	0.01	0.00													48
49	Vila Franca	0.01	0.00	0.00												49
50	Vila Franca	0.01	0.00	0.00	0.00											50
51	Vila Franca	0.01	0.00	0.00	0.00	0.00										51
52	Vila Franca	0.01	0.00	0.00	0.00	0.00	0.00									52
53	Vila Franca	0.01	0.00	0.00	0.00	0.00	0.00	0.00								53
54	Vila Franca	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00							54
55	Vila Franca	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00						55
56	Vila Franca	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					56
57	Vila Franca	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01				57
58	Vila Franca	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00			58
59	Vila Franca	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		59
60	Vila Franca	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	60
61	P.excavatus	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	61
62	L.rubellus	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	62
63	L.terrestris	0.36	0.35	0.35	0.36	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.36	63

		60	61	62	
61	P.excavatus	0.33			61
62	L.rubellus	0.33	0.29		62
63	L.terrestris	0.35	0.29	0.20	63

Annex III

Table with all the individual values for the total genetic distance calculated based in 425 AFLP markers of *Pontoscolex corethrurus* from the volcanic geothermal field Furnas, pineapple greenhouses in Faja de Baixo and Vila Franca.



	RA1	RF4	2F	3F	4F	5F	6F	7F	8F	10F	11F	12F	13F	14F	15F	17F	18F	19F	20F	22F	23F	25F	1A	2A	3A	4A	5A	6A	7A	8A	9A
RA1	0																														
RF4	139	0																													
2F	131	148	0																												
3F	111	134	58	0																											
4F	121	138	70	62	0																										
5F	112	137	71	63	81	0																									
6F	126	133	81	67	83	78	0																								
7F	126	121	97	87	91	98	98	0																							
8F	117	84	142	136	122	131	137	89	0																						
10F	108	143	103	93	99	94	94	114	139	0																					
11F	107	122	110	112	100	117	111	113	104	71	0																				
12F	103	100	128	112	106	119	119	99	66	133	98	0																			
13F	122	107	157	159	155	158	150	130	71	152	115	77	0																		
14F	89	108	144	134	124	125	131	101	72	111	86	60	79	0																	
15F	110	145	99	95	91	98	104	110	121	88	91	111	134	107	0																
17F	126	93	145	145	129	140	144	102	49	152	127	75	62	77	128	0															
18F	108	151	83	69	85	80	76	114	147	54	89	127	152	121	78	158	0														
19F	113	132	74	54	74	63	75	91	130	99	118	102	151	112	99	131	81	0													
20F	103	132	110	118	104	105	109	119	122	91	82	108	127	88	95	131	85	128	0												
22F	124	143	67	57	83	66	80	96	135	86	111	123	154	131	98	146	68	63	117	0											
23F	126	115	101	85	91	108	114	86	97	122	113	97	116	111	110	100	116	101	113	104	0										
25F	127	146	70	50	80	61	71	103	148	75	112	130	167	130	91	159	59	62	118	51	113	0									
1A	125	110	172	168	164	163	159	127	62	141	110	74	51	72	131	57	147	154	122	155	123	168	0								
2A	112	113	163	161	149	158	152	124	81	130	99	87	84	75	120	86	140	147	109	156	120	165	71	0							
3A	106	99	149	149	139	138	140	112	59	132	103	69	76	65	114	78	136	133	117	144	112	147	55	82	0						
4A	114	97	161	161	147	148	154	116	57	140	103	69	60	61	128	56	146	147	115	150	114	163	45	68	60	0					
5A	109	116	160	162	150	151	151	131	86	131	106	78	55	64	115	81	143	148	112	153	125	162	54	81	65	61	0				
6A	129	120	170	170	166	161	153	133	90	143	114	88	71	80	131	83	147	154	122	165	137	166	64	91	67	67	58	0			
7A	134	111	177	173	165	160	162	126	71	148	117	89	62	77	136	70	156	165	125	168	130	177	49	86	70	52	65	65	0		
8A	131	130	180	182	178	177	171	145	86	149	120	100	59	80	143	83	157	166	130	173	139	188	50	77	79	69	58	54	61	0	
9A	119	108	168	164	160	153	155	127	72	137	110	88	59	76	127	61	143	146	122	151	123	166	44	73	71	51	62	64	55	60	0
13A	97	114	154	156	148	149	155	133	100	151	116	90	111	82	137	107	147	142	114	157	135	158	102	95	91	97	110	116	107	118	
15A	107	128	78	70	88	71	89	105	122	97	96	108	145	118	95	123	91	82	104	77	95	88	140	137	123	133	140	148	147	162	
16A	108	115	157	151	141	142	144	120	81	132	119	89	96	81	118	88	138	141	117	146	120	157	83	82	78	84	103	101	96	111	
18A	111	98	166	160	152	149	151	121	64	127	98	72	59	58	121	67	135	144	112	147	119	154	42	71	63	43	54	60	57	58	
19A	125	96	170	164	160	149	145	129	72	145	114	88	87	76	141	87	155	150	122	167	131	170	78	81	73	67	92	78	71	74	
20A	138	125	185	175	177	172	168	166	133	166	153	133	154	129	172	136	174	163	153	176	154	175	137	136	128	134	153	161	138	159	
21A	146	115	191	187	185	182	166	178	133	160	137	139	138	133	158	138	160	173	133	184	168	181	133	120	134	132	143	141	134	143	
23A	99	102	152	152	148	139	141	117	68	121	92	70	63	62	103	73	129	140	100	149	115	154	60	53	63	61	58	72	67	66	
24A	114	117	167	161	155	152	148	132	79	138	107	87	86	73	128	100	136	145	119	154	128	163	83	90	68	80	89	67	82	65	
25A	131	132	192	174	188	165	167	163	140	169	170	144	161	132	167	143	177	172	154	183	157	178	146	135	139	145	154	168	143	160	
26A	151	152	198	190	194	191	191	175	138	185	170	146	163	150	177	133	185	186	170	191	171	200	146	143	147	145	174	166	151	168	
1V	151	148	192	194	182	191	181	179	146	179	164	150	169	146	175	145	189	186	162	191	163	194	158	149	153	151	172	174	161	172	
2V	131	124	172	168	158	169	169	151	112	153	130	128	139	108	149	131	163	164	140	171	151	172	122	115	121	127	126	146	135	140	
3V	117	110	162	166	152	153	153	121	64	127	96	72	53	60	117	65	141	148	108	153	117	162	38	67	51	45	42	50	51	52	
4V	114	105	163	155	153	146	150	122	67	126	93	77	70	59	124	84	138	147	105	152	122	157	61	76	58	62	67	65	64	65	
5V	111	104	158	158	156	149	149	117	68	127	106	90	79	80	123	83	143	154	116	151	125	158	70	75	69	79	86	78	81	66	
6V	151	144	190	198	186	193	191	175	136	169	156	150	155	142	167	129	181	186	166	189	167	196	136	133	141	139	158	164	145	150	
7V	113	108	164	154	150	147	145	125	72	129	102	80	75	68	117	83	137	140	122	151	123	158	64	77	61	69	76	72	71	70	
8V	125	126	162	156	152	153	145	131	104	147	126	120	129	104	135	111	151	150	140	155	125	154	116	105	113	117	130	136	131	134	
9V	109	100	158	156	140	139	149	109	52	135	94	64	59	50	111	59	143	138	112	151	109	158	48	67	41	47	60	64	55	68	
10V	102	113	165	159	147	150	154	122	91	134	99	77	92	71	114	96	142	149	111	162	136	169	75	78	78	78	79	95	96	91	
11V	146	131	187	185	179	176	170	142	93	160	133	103	78	97	136	84	160	171	139	176	140	181	57	94	86	76	71	63	70	47	
12V	123	114	174	174	160	165	159	133	78	137	102	76	59	78	123	73	145	156	118	163	131	172	46	75	69	53	56	56	53	54	
13V	125	112	158	166	152	159	165	135	96	153	120	106	111	100	137	111	163	154	138	165	127	180	112	97	99	107	114	128	111	126	
14V	118	101	161	157	153	148	150	118	69	128	105	81	56	71	120	68	134	151	115	148	118	157	49	72	64	52	55	53	62	63	
15V	117	106	160	152	148	157	151	117	84	147	108	86	97	84	125	95	153	150	120	157	115	162	90	87	77	81	106	106	99	110	
16V	112	111	167	167	157	154	156	122	63	144	107	71	76	59	118	72	142	149	113	158	124	165	51	64	64	52	67	77	70	71	
17V	118	109	163	157	149	150	150	116	61	142	105	69	52	71	128	56	148	139	123	146	118	161	41	80	64	56	63	71	60	67	
18V	145	156	186	184	178	185	187	169	132	183	162	148	163	146	171	131	183	182	174	179	167	192	144	141	145	145	172	174	153	168	
19V	131	126	180	166	168	179	165	159	138	161	148	130	153	128	153	149	165	172	158	171	161	172	144	127	135	139	150	160	151	154	
20V	123	118	174	178	168	161	167	139	76	141	108	94	57	72	135	79	149	158	122	165	135	174	48	79	65	55	52	62	51	56	

13A	15A	16A	18A	19A	20A	21A	23A	24A	25A	26A	1V	2V	3V	4V	5V	6V	7V	8V	9V	10V	11V	12V	13V	14V	15V	16V	17V	18V	19V	20V	
																															RA1
																															RF4
																															2F
																															3F
																															4F
																															5F
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																															6A
																															7A
																															8A
																															9A
0																															13A
138	0																														15A
101	133	0																													16A
100	138	95	0																												18A
102	144	99	70	0																											19A
109	161	134	131	131	0																										20A
115	169	142	131	135	114	0																									21A
90	132	69	60	70	135	119	0																								23A
109	139	100	73	73	144	144	79	0																							24A
114	166	135	142	140	79	115	136	151	0																						25A
128	184	137	150	146	129	93	142	179	118	0																					26A
126	180	129	152	148	105	121	148	169	108	84	0																				1V
124	160	115	118	130	101	131	114	137	110	132	134	0																			2V
100	138	81	44	80	143	131	48	75	144	154	156	126	0																		3V
99	133	90	55	71	128	126	55	52	137	155	155	121	51	0																	4V
102	134	81	78	74	143	131	54	85	142	142	148	118	74	73	0																5V
124	184	129	144	152	103	135	138	165	122	112	78	128	138	147	142	0															6V
106	134	87	62	76	143	137	60	71	152	158	162	134	46	61	74	152	0														7V
118	146	113	112	120	119	141	102	117	118	138	132	94	122	117	118	126	122	0													8V
90	128	75	48	72	137	133	52	67	146	146	154	126	32	53	68	136	44	112	0												9V
103	141	102	71	93	132	132	71	88	135	161	157	111	77	78	89	149	81	109	77	0											10V
127	155	116	65	87	162	150	79	72	167	177	179	153	63	68	87	165	77	133	77	92	0										11V
102	146	91	46	74	143	125	64	73	146	160	156	136	48	61	84	146	68	128	56	79	51	0									12V
100	150	95	108	106	101	141	94	109	118	162	134	112	104	105	116	120	106	110	96	95	133	110	0								13V
105	137	84	45	85	144	136	57	80	145	151	151	121	37	58	73	141	63	119	55	84	64	53	109	0							14V
104	132	97	84	100	117	127	86	99	128	142	124	120	90	85	86	130	94	108	74	95	111	96	104	95	0						15V
99	139	78	49	73	138	134	57	74	141	141	149	117	53	60	63	137	65	111	49	70	72	63	105	60	83	0					16V
99	127	90	55	83	138	136	61	86	149	151	155	131	49	66	75	149	75	113	53	82	68	53	107	58	87	66	0				17V
134	174	115	152	150	103	127	146	171	120	80	92	106	154	155	142	86	160	118	146	153	175	154	136	153	138	141	151	0			18V
122	170	137	136	152	105	85	132	145	102	98	108	102	134	135	142	116	138	120	134	131	159	142	128	135	114	139	145	104	0		19V
106	148	101	40	80	153	139	64	77	158	166	170	138	44	69	86	158	70	122	54	91	67	42	116	47	108	67	61	168	152	0	20V

Annex IV

Tables with the relative abundance (Ab. %) of phylotypes in the clone library from the total earthworm body from: **A,** Microbial phylotypes found in the clone library of *P. corethrurus* from Botanical Garden in Xalapa, Mexico (Mx); **B,** Microbial phylotypes found in the clone library of *P. corethrurus* from Furnas, Azores (Az). The names of the most closely related known bacterial species are indicated together the identity used as similarity index.

A: BG	Microbial Type	Genera	Closest Species	Ab. %	Identity
	α-proteobacteria	Bradyrhizobium	*Bradyrhizobium japonicum*	31	86.8
			Bradyrhizobium sp.	8	75.9
		Methylobacterium	*Methylobacterium extorquens*	2	35.2
			Methylobacterium populi	2	75.5
		Nitrobacter	*Nitrobacter hamburgensis*	2	69.2
		Paracoccus	*Paracoccus denitrificans*	2	72.1
		Pectobacterium	*Pectobacterium wasabiae*	2	79.7
		Rhizobium	*Rhizobium etli*	2	32.4
			Rhizobium leguminosarum	2	61.8
		Rhodopseudomonas	*Rhodopseudomonas palustris*	8	67.6
		Vibrio strain MV-1	*Magnetite-containing magnetic Vibrio*	2	64.5
	β-proteobacteria	Burkholderia	*Burkholderia multivorans*	3	69.5
			Burkholderia xenovorans	2	78.8
	γ-proteobacteria	Pseudomonas	*Pseudomonas fluorescens*	2	92.5
		Stenotrophomonas	*Stenotrophomonas maltophilia*	3	94.5
	CFB group bacteria	Blattabacterium	*Blattabacterium sp.*	3	34.9
	enterobacteria	Dickeya	*Dickeya dadantii*	2	9.4
		Edwardsiella	*Edwardsiella tarda*	2	78.1
		Escherichia	*Escherichia coli*	5	99.9
		Serratia	*Serratia proteamaculans*	2	87.8
	Unknown			15	0

B: AZ	Microbial Type	Genera	Closest Species	Fr %	Identity
	α-proteobacteria	Agrobacterium	*Agrobacterium radiobacter*	2	67.5
		Azorhizobium	*Azorhizobium caulinodans*	1	32.1
		Bradyrhizobium	*Bradyrhizobium japonicum*	32	87.3
			Bradyrhizobium sp.	15	89
		Caulobacter	*Caulobacter crescentus*	1	13.7
		Mesorhizobium	*Mesorhizobium loti*	4	79.8
		Methylobacterium	*Methylobacterium sp.*	1	4.7
			Methylobacterium chloromethanicum	1	39.5
			Methylobacterium extorquens	1	83.4
			Methylobacterium radiotolerans	1	30.7
		Methylocella	*Methylocella silvestris*	1	81.1
		Nitrobacter	*Nitrobacter hamburgensis*	5	89
		Rhizobium	*Rhizobium sp.*	2	76.4
		Rhodobacter	*Rhodobacter sphaeroides*	1	74.1
		Rhodopseudomonas	*Rhodopseudomonas palustris*	13	91.8
	β-proteobacteria	Acidovorax	*Acidovorax sp.*	1	68.1
		Bordetella	*Bordetella sp.*	1	58.4
		Burkholderia	*Burkholderia pseudomallei*	1	75.4
			Burkholderia sp.	1	52.3
			Burkholderia thailandensis	1	37.1
		Verminephrobacter	*Verminephrobacter eiseniae*	1	71
	δ-proteobacteria	Anaeromyxobacter	*Anaeromyxobacter dehalogenans*	1	14.5
		Desulfovibrio	*Desulfovibrio magneticus*	1	4.3
		Haliangium	*Haliangium ochraceum*	1	93.6
	γ-proteobacteria	Acinetobacter	*Acinetobacter baumannii*	1	75.2
		Pseudomonas	*Pseudomonas sp.*	1	90.1
		Xanthomonas	*Xanthomonas albilineans*	1	71
	high GC Gram+	Arthrobacter	*Arthrobacter sp.*	1	7.5
		Clavibacter	*Clavibacter michiganensis*	1	22.1
		Conexibacter	*Conexibacter woesei*	1	24.4
		Corynebacterium	*Corynebacterium glutamicum*	1	22.6
		Eggerthella	*Eggerthella lenta*	1	9.6
	enterobacteria	Serratia	*Serratia proteamaculans*	1	86.3
	cyanobacteria	Cyanothece	*Cyanothece sp.*	1	60.3
		Gloeobacter	*Gloeobacter violaceus*	1	68.3
	microsporidians	Enterocytozoon	*Enterocytozoon bieneusi*	1	72.5
	ascomycetes	Candida	*Candida glabrata*	1	50.9
	Unknown			3	0