Evaluating evolutionary pressures and phylogenetic signal in earthworms: a case study – the number of typhlosole lamellae in Hormogastridae (Annelida, Oligochaeta)

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Rarely have phylogenetic comparative methods been used to study the correlation between phenotypic traits and environmental variables in invertebrates. With the widespread convergence and conservativeness of the morphological characters used in earthworms, these comparative methods could be useful to improve our understanding of their evolution and systematics. One of the most prominent morphological characters in the family Hormogastridae, endemic to Mediterranean areas, is their multilamellar typhlosole, traditionally thought to be an adaptation to soils poor in nutrients. We tested the correlation of body size and soil characteristics with the number of typhlosole lamellae through a phylogenetic generalized least squares (PGLS) analysis. An ultrametric phylogenetic hypothesis was built with a 2580-bp DNA sequence from 90 populations, used in combination with three morphological and 11 soil variables. The best-supported model, based on the Akaike information criterion, was obtained by optimizing the parameters lambda (λ), kappa (κ), and delta (δ). The phylogenetic signal was strong for the number of typhlosole lamellae and average body weight, and was lower for soil variables. Increasing body weight appeared to be the main evolutionary pressure behind the increase in the number of typhlosole lamellae, with soil texture and soil richness having a weaker but significant effect. Information on the evolutionary rate of the number of typhlosole lamellae suggested that the early evolution of this character could have strongly shaped its variability, as is found in an adaptive radiation. This work highlights the importance of implementing the phylogenetic comparative method to test evolutionary hypotheses in invertebrate taxa.

INTRODUCTION

Comparative methods have been used extensively on some animal groups, such as vertebrates, to study the correlation between phenotypic traits and environmental variables (Schondube, Herrera-M & del Rio, 2001; Díaz, 2002; Medina *et al.*, 2012; Prevosti *et al.*, 2012; Boyles *et al.*, 2013; Edwards *et al.*, 2013; Riek & Geiser, 2014). Because of the non-independence of species with shared evolutionary history, phylogenetic comparative methods are needed to take their relationships into account (Felsenstein,

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1985). Although this methodology is an effective approach to explaining morphological variation in the light of evolution, it has been rarely used on invertebrates (e.g. Jervis, Boggs & Ferns, 2007; Goncalves-Souza, Diniz-Filho & Romero, 2014).

Evaluating the taxonomic informativeness and phylogenetic signal of morphological characters is a necessity for earthworms, as most of the characters available tend to overlap between close and distantly related species because of rampant convergence (Pop, Wink & Pop, 2003; Briones, Moran & Posada, 2009; Pérez-Losada *et al.*, 2009). In addition, morphological evolution in earthworms appears to be heavily constrained by their homogeneous habitat (Novo *et al.*, 2012), by means of stabilizing selection (Bickford *et al.*, 2007).

The digestive system of earthworms (Annelida, Oligochaeta) is one of the most prominent elements of their body, to the point that earthworms have been described as a 'tube within a tube'. One of the specialized structures of their digestive tract is the typhlosole: one or more longitudinal folds of the dorsal intestinal wall, the function of which is proposed to increase the functional surface of the intestine for greater efficiency, and not just for greater absorption (e.g. Edwards & Bohlen, 1996), but also for digestion and storage. The morphology and degree of development of the typhlosole show a great variability among earthworm families and species. For example, in Lumbricidae it can be simple and cylindrical, plicate, lyre-shaped, T-shaped, anchor-shaped, and bifid or even trifid in cross section (for a detailed review, see Csuzdi & Zicsi, 2003).

The family Hormogastridae is characterized by their multilamellar typhlosole, and the number of longitudinal lamellae is variable across the family, ranging from three to twenty-three (Fig. 1), but there is no variation within populations (and well-established species). This makes it useful as a taxonomic character (Rota, 1993), and suggests that the number of typhlosole lamellae may play an important role in the biology and evolution of hormogastrid earthworms.

Currently, the only proposed hypothesis to explain the evolution of this character is that an increased number of lamellae would be expected in nutrient-poor soils, as it would facilitate a more efficient digestion and absorption of the scarce nutrients. Perel (1977) and James & Cunningham (1989) associated the greater convolution of the intestinal surface with lower quality diets. As endogeic earthworms, hormogastrids have an exclusively geophagous diet (but for a suggestion of partially anecic species, see Bouché, 1972). Therefore, soil characteristics could influence the development of the typhlosole and its number of lamellae. For example, a more convoluted, cumbersome typhlosole could hinder the flow of coarse-textured soils through the digestive tract; however, many other evolutionary pressures could explain the observed variability in this character.

The members of the family Hormogastridae (including Ailoscolecidae; James & Davidson, 2012; Novo *et al.*, 2016) show a wide range of body sizes (Fig. 1), from 0.5 to 100 g and from 3 to 90 cm in length, approximately, for clitellated specimens (Cobolli Sbordoni *et al.*, 1992; Omodeo & Rota, 2008). The basal position of the smallest species (i.e. *Hemigastrodrilus monicae* Bouché, 1970 and *Ailoscolex lacteospumosus* Bouché, 1969) in their phylogeny (Novo *et al.*, 2016) suggests an evolutionary trend towards increasing body size. With the inverse relationship between body size and the ratio of surface area to volume (Haldane, 1926), the proportional inner absorption surface of an earthworm's intestine is expected to decrease in larger species. It can be hypothesized that the development of a typhlosole composed of a higher number of lamellae would be advantageous for these species.

In this study, we obtained morphological and soil-variable data from previously available and newly discovered populations of hormogastrid earthworms, in order to perform a comparative analysis. We reconstructed a phylogenetic hypothesis based on molecular sequences and used it together with the variables in a phylogenetic generalized least squares (PGLS) analysis. This analysis is particularly suited to the study of continuous variables (Grafen, 1989; Pagel, 1999; Rohlf, 2001). PGLS takes into account the statistical non-independence of the measured variables among related species by incorporating phylogenetic information into the error structure of the model with an explicit model of evolution. For that, Pagel's λ (Pagel, 1999) is estimated using maximum likelihood: it ranges from 0 (indicating independence from phylogeny) to 1 (indicating that traits covary proportionally to the degree of shared evolutionary history, following the Brownian motion model). Along with λ, kappa (κ) and delta (δ) can be used as branch-length scaling parameters to improve the fit of the data. They represent the rate of evolution of characters over time, with values equal to 1 equating direct gradualism (change is linear to branch length/time). κ or δ values over 1 mean a greater contribution to change from longer branches/paths, whereas the opposite is true for values under 1 (Pagel, 1999).

In this context, our objective was to find which variables better explained the variation in the number of typhlosole lamellae, as well as to explore the
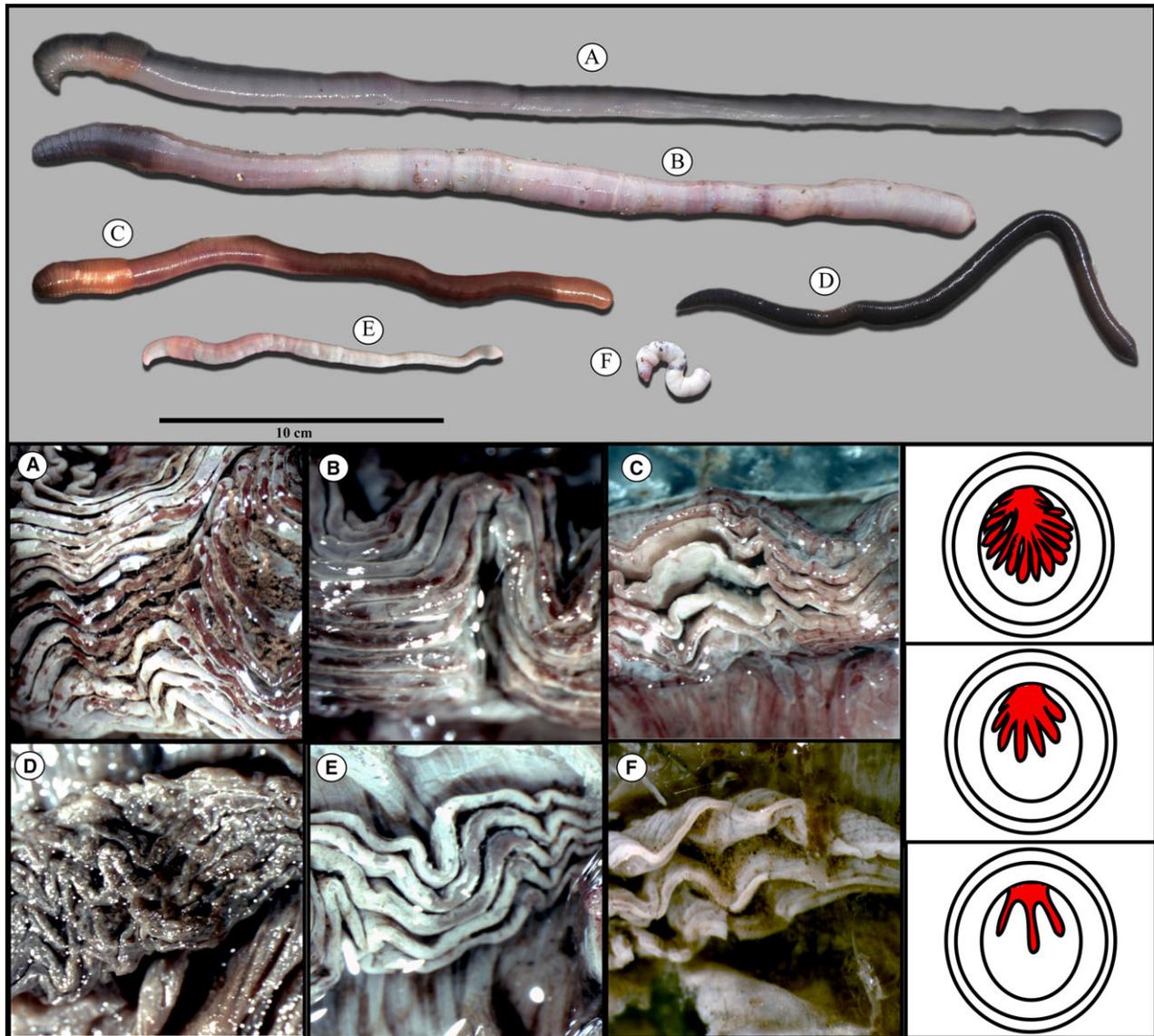
Figure 1. An example of the variability in body size and number of lamellae in Hormogastridae: (A) *Hormogaster castillana* Qiu & Bouché, 1998 (21 lamellae); (B) *Hormogaster pretiosa Michaelsen*, 1889 from Segariu (13 lamellae); (C) *Hormogaster joseantonioi* Marchán *et al*., 2014 (nine lamellae); (D) *Hormogaster redii* Rosa, 1887 (seven lamellae); (E) *Hormogaster elisae* Alvarez, 1977 (five lamellae); (F) *Ailoscolex lacteospumosus* Bouché, 1969 (three lamellae). To the right the diagrams show how the typhlosole is located in the digestive tract, with different degrees of convolution shown.

tempo and mode of evolution of this character in a phylogenetic framework.

MATERIAL AND METHODS

DATA COLLECTION

Sampling and molecular markers
Specimens were collected by hand and fixed in the field in ~96% ethanol (EtOH), with several subsequent changes of ethanol. Once in the laboratory, specimens were preserved at −20 °C. The material studied included 90 populations, 15 of which were newly sampled for this study (Appendix S1). Specimens were deposited in the Oligochaete collection of the Department of Zoology and Physical Anthropology, Universidad Complutense de Madrid (UCMLT), Spain, with vouchers UCMLT 206–230.

Total genomic DNA was extracted from ventral integument tissue samples using the DNeasy Tissue Kit (QIAGEN), with two consecutive steps of elution (70 µl of buffer each).

Seven molecular regions were amplified: mitochondrial cytochrome *c* oxidase subunit I (*COI*), *16S* rRNA, and Leu, Ala, and Ser tRNA (*16S* t-RNAs), one nuclear ribosomal gene (a fragment of *28S* rRNA), and one nuclear protein-encoding gene (histone *H3*).

Primer sequences, polymerase chain reactions (PCRs), and sequencing reactions are as described in Novo *et al.* (2011). GenBank accession numbers for the markers analysed here are shown in Appendix S1.

Morphological and ecological data

For each population, the maximum number of typhlosole lamellae was considered as the representative value (as in Qiu & Bouché, 1998b), which was constant between fully mature individuals. In addition, we tested the correlation between the number of lamellae and the surface of the typhlosole (for methodology and results, see Appendix S2) to evaluate the ability of the former to reflect the latter. Average weight and number of segments were chosen as measurements of body size, as they are less affected by the fixation process compared with other measurements, such as body length (which is usually distorted among individuals). Both measurements were only taken in adults showing no amputation (for between one and eight individuals per population). In some cases where no individual in the population fulfilled these requirements, the information was taken from Díaz Cosín, Briones & Trigo (1989), Rota (1993), and Qiu & Bouché (1998b). The weight measurements included gut content, as the degree of filling can be expected to average across individuals.

The soil factors potentially affecting earthworm distributions to the highest degree (soil texture, pH, organic carbon content, and nitrogen content; Edwards & Bohlen, 1996; Hernandez *et al.*, 2003; Hernández *et al.*, 2007) were analysed in the new localities [previously obtained values were taken from Novo *et al.* (2012) and Marchán *et al.* (2014)]. Soil texture (percentages of coarse/fine/total sand, coarse/fine/total silt, and clay) and pH were measured as described by Guitián & Carballas (1976). Anne's (1945) method, adapted for a microplate reader (590-nm Microplate, Bio-Rad) was used for organic oxidizable carbon analysis using glucose as a standard, and expressed as a percentage. The Kjeldahl method was used to measure total nitrogen content, as indicated in Page, Miller & Keeney (1982) and expressed as a percentage. Soil samples from the same locality were mixed and analysed jointly.

The values obtained are shown in Appendix S3. Only populations with both morphological and ecological data were considered for the correlation analyses.

PHYLOGENETIC RELATIONSHIPS

In order to find their phylogenetic placement inside the family, the new sequences were combined with all of the molecular information available for hormogastrids from previous studies (Novo *et al.*, 2010, 2011, 2012, 2015, 2016; Marchán *et al.*, 2014). Sequences from *Pontodrilus litoralis* Grube, 1855, *Dichogaster saliens* Beddard, 1893, *Amynthas robustus* Perrier, 1872, *Lumbricus terrestris* Linnaeus, 1758, and *Aporrectodea trapezoides* Dugès, 1828 were retrieved from GenBank to be used as outgroups, and *Prosellodrilus psammophilus* Qiu & Bouché, 1998 was newly sequenced for this work (GenBank accession numbers are listed in Appendix S1). Only one individual per population was analysed as representative for previously studied localities, as hormogastrid individuals from the same locality always clustered together. In the new populations, two individuals were included in order to ensure their genetic homogeneity.

Sequences of each individual gene were aligned in MAFFT (Katoh & Standley, 2013) with default settings and subsequently concatenated, resulting in a matrix of 2580 bp. jModelTest 2.1.3 (Darriba *et al.* 2012) was used to select the best-fitting evolutionary model using the Akaike information criterion (AIC; Akaike 1973) and Bayesian information criterion (BIC; Schwarz, 1978). GTR+I+G was selected for *COI*, *16S*, and *28S*, and GTR+G was selected for *H3*.

The phylogenetic analyses were run using the main phylogenetic hypothesis from Novo *et al.* (2016) as a constraint.

Bayesian inference of the phylogeny was estimated with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), as implemented in the CIPRES Science Gateway 3.3 (Miller, Pfeiffer & Schwartz, 2010). Unlinked nucleotide substitution models selected were specified for each gene fragment and the nucleotide substitution estimates were allowed to vary independently between each partition. Parameters were set to 40 million generations and 10 000 trees were sampled for every 4000th generation, initiating the analysis from a random tree. Two independent analyses were performed and 20% of the trees were discarded as burn-in. The remaining trees were combined to find the maximum *a posteriori* probability estimate of phylogeny.

Maximum-likelihood analyses were performed in RAxML-HPC 8.1.11, as implemented in the CIPRES Science Gateway 3.3 (http://www.phylo.org/index.php/portal), using GTR+I+G for each data partition with ten alternative runs, and estimating the support for the resulting topologies by 1000 rapid bootstrap replicates.

To generate a suitable starting tree for BEAST, the Bayesian inference tree was converted into an ultrametric tree by non-parametric rate smoothing (NPRS) using the function chronopl in the R package 'ape' (Paradis, Claude & Strimmer, 2004). We used relative timing by calibrating the tree with a value of 1 in the root for Hormogastridae. The final ultrametric tree, a requirement for the PGLS analysis, was generated with BEAST 1.7 (Drummond *et al.*, 2012) using the same calibration and the NPRS tree as the starting tree. Each partition was trimmed with GBlocks (Castresana, 2000) under the less stringent parameters, and GTR+I+G was specified as the evolutionary model for all of them. The analysis was conducted under a Yule diversification model and an uncorrelated lognormal relaxed clock. Three parallel runs were specified, each of which included 50 million generations, sampling every 5000th generation. Tree and log files were combined in LOGCOMBINER 1.7 by resampling at lower frequency every 15 000 generations, and results were visualized in TRACER 1.5 (Rambaut & Drummond, 2007). The final tree was generated by TreeAnnotator 1.7. with a burn-in of 2000.

PHYLOGENETIC COMPARATIVE ANALYSES

Before performing the analyses, all variables were normalized through a $\log_{10}$ transformation. Both the number of lamellae and the number of segments were treated as continuous characters. In order to reduce the collinearity of soil variables, a factor analysis was performed. The first three factors (comprising 74% of the total variance) were retained, and the factor scores were used as input in the analysis. This led to a final set of one dependent variable (number of typhlosole lamellae) and four predictor variables: average weight, soil texture (factor 1), soil richness (factor 2), and additional soil texture (factor 3) (for details about the definition of the factors, see Appendix S4).

As a result of the strong collinearity between the size variables (number of segments and average weight), we evaluated which one should be included in our analyses. There is a possibility of the weight of the more developed (and heavier) typhlosoles influencing the whole body weight, and thereby making both variables interdependent; however, both the average weight and number of segments showed a similar correlation with the number of lamellae, suggesting that this is not the case. In addition, the number of segments is a relatively unreliable variable because of the frequent amputation and secondary growth observed in several individuals. Therefore, we selected average weight as the best size variable and did not include the number of segments in our analyses.

A PGLS analysis was used to identify the coevolution of the number of lamellae and size, and the dependence of soil variables; the packages 'ape' and 'caper' (Paradis *et al.*, 2004; Orme, 2013) were used in the R environment.

As optimizing the three scaling parameters λ, κ, and δ simultaneously makes the biological interpretation difficult, each of them were optimized while the other two parameters were kept constant at their default values. All possible combinations of predictor variables were analysed. Akaike information criterion (AICc) values were compared, and the model with the lowest AICc was selected. Models with an increase in AICc (ΔAICc) lower than 2 were considered as having essentially equal support, according to previous studies (e.g. Symonds & Moussalli, 2011). Individual values of Pagel's λ were obtained for all of the variables analysed to study their phylogenetic signal.

RESULTS

PHYLOGENETIC RELATIONSHIPS

Both the Bayesian and maximum-likelihood inferences of the phylogenetic tree showed congruent topologies, with generally well-supported clades (Appendix S5).

The ultrametric tree obtained from BEAST is shown in Figure 2. The number of lamellae and average weights are displayed at the end of the corresponding branches to illustrate the family-wide variability.

PHYLOGENETIC COMPARATIVE ANALYSES

The best-supported PGLS models (with the lowest AICc values), plus all the models with ΔAICc < 2, are shown in Table 1. The model diagnostics can be seen in Appendix S3.

The models obtained by optimizing λ explained the least variance. Weight was positively correlated with number of typhlosole lamellae at high significance levels in all of the models, whereas soil richness showed a weak, negative significant correlation in three of the models. Lambda values were consistently high for all models.

The models in which δ and κ were optimized explained a higher percentage of variance. In both cases, weight showed positive, highly significant correlation with the dependent variable. Soil texture and soil richness had significant contributions to all models. Delta values were low, whereas kappa values were close to 1.

When considered separately, the number of typhlosole lamellae and weight showed strong phylogenetic
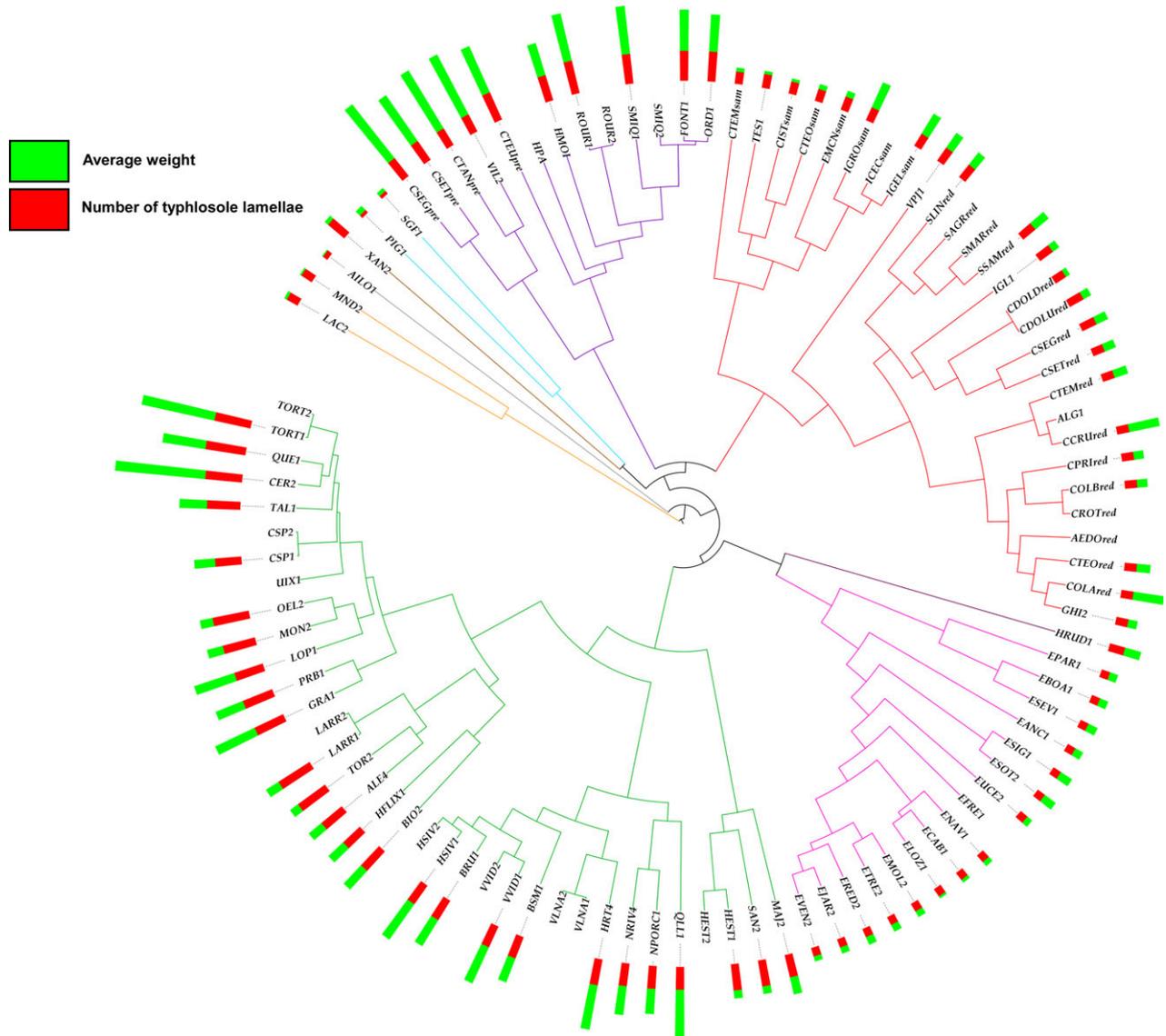
Figure 2. Ultrametric tree used as the phylogenetic input for the phylogenetic generalized least squares (PGLS) analysis. Number of typhlosole lamellae and average weight are shown as red and green bars for taxa with available information. The colour code for the branches show the main Hormogastridae clades (for details, see Appendix S4).

signal (λ = 0.971 and 0.917, respectively). Soil texture and additional texture showed moderate phylogenetic signal (λ = 0.709 and 0.573, respectively), whereas a very low phylogenetic signal was found for soil richness (λ = 0.164).

DISCUSSION

EVOLUTIONARY PRESSURES

Although the variation in number of typhlosole lamellae in Hormogastridae showed a high phylogenetic component, there was convincing evidence for weight having a strong influence in the evolution of

this character. This supports our initial hypothesis of a higher number of lamellae being advantageous as body size increases. The adaptive value of the multilamellar typhlosole could indeed be explained by its strong correlation with the increase in typhlosole surface (Appendix S2). If this increase in intestinal functional surface were an evolutionary requisite for greater body size in hormogastrid earthworms, a similar adaptation would be expected in other families. Although multiple longitudinal lamellae do not appear in other families with giant earthworms *sensu* Faria Siqueira *et al.*, (2013) (Megascolecidae, Acanthodrilidae, Glossoscolecidae *s.l.*, Kynotidae, and Microchaetidae); in the same way as in

Table 1. Phylogenetic regression models obtained by optimizing λ, δ, and κ

	λ				δ		κ	
Intercept	**0.825***	0.840***	0.825***	0.828***	0.793***	**0.793***	0.783***	**0.783***
Weight	**0.123***	0.110**	0.122***	0.123***	0.166***	**0.167***	0.185***	**0.186***
Soil texture (f1)	**−0.012*			−0.007	−0.023**	**−0.022**	−0.021*	**−0.021*
Soil richness (f2)			−0.012*	−0.012*	−0.020***	**−0.020***	−0.019***	**−0.019***
Add. soil texture (f3)			−0.001	−0.001	−0.001	−0.001		−0.001
Explained variance, r^2	**14.78%	9.66%	13.65%	15.63%	79.37%	**79.63%	80.72%	**81.18%
P value	***	**	**	**	***	***	***	***
AIC	**−180.890	−179.1283	−178.890	−179.490	−176.96506	**−178.92826	−170.9438	**−172.9386
	**λ = 0.984	λ = 0.975	λ = 0.984	λ = 0.986	δ = 0.266	**δ = 0.265	κ = 1.071	**κ = 1.073

The model with the lowest corrected Akaike information criterion (AICc) is shown in bold for each case. Coefficients for the variables in each model are shown with their statistical significance. All models with ΔAICc < 2 are also shown. Add. soil texture, additional soil texture.

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

Hormogastridae, they do show a wealth of different adaptations. For example, strongly developed and complex typhlosoles are common in the Rhinodrilidae, usually with an S-shaped section and multiple lateral foldings of a complex nature. Glossoscolecidae *s.s.* (James & Davidson, 2012) have very complex typhlosoles in the anterior third of the gut. There are exceptions like *Megascolides australis* McCoy, 1878, one of the longest known earthworms, which lives in poor soils but lacks any kind of typhlosole. It is worth noting that increased body size by means of elongation can mitigate the relative reduction of intestinal surface. One extreme example of such an adaptation is *Amynthas mekongianus* (Cognetti, 1922), with a length of around 200 cm but just 1 cm in diameter.

In Lumbricidae, in which the typhlosole shows strong development and diversity, most of the large species (*Lumbricus* sp., *Octodrilus* sp., *Scherotheca* sp.) are anecic. This lifestyle is associated with the consumption of rich food resources (mainly decaying vegetal material), which would remove the pressure for a highly convoluted intestinal surface. Interestingly, Qiu & Bouché (1998a) linked the possession of a pleated typhlosole (that folds like an accordion in the opposite axis of hormogastrid lamellae) with strong longitudinal contractility, essential for these earthworms. This longitudinal contractility is achieved to a certain degree in Hormogastridae by their ability to compress their typhlosole into tight bends.

Some aspects of typhlosole complexity have not been accounted for in this work because of the difficulty of including these aspects in the analysis. For example, it has been stated the typhlosole has a species-specific limited length (Rota, 1993). Also, the fold does not show a uniform cross section along its length: at some point the lamellae can even disappear, to then start again along some more segments. A complex relationship between body diameter and typhlosole diameter, and also typhlosole diameter and number of lamellae has also been observed (Appendix S2). We expect these features to have a minor influence in the relationship between the number of typhlosole lamellae and the variables studied, but they could merit more detailed research in the future that might provide further insight into the evolution of the organ.

It appears that soil texture and richness could have exerted a weaker but significant evolutionary pressure in the variability of the character. Models including one or both of these factors were always better supported than models including only weight as the independent variable. While the small magnitude of the correlation shown calls for careful interpretation, it can be inferred that coarse texture could

have favoured the development of more convoluted typhlosoles. From observations in *Hormogaster castillana* Qiu & Bouché, 1998, a possible sieve-like function for its extremely developed typhlosole could provide an explanation for this hypothesis. We have observed a significant difference in texture between the soil intimately associated with the lamellae and the soil in the intestinal lumen: the former contained no particles greater than 0.5 mm in diameter, whereas the latter contained a high proportion of larger particles. If the multilamellar typhlosole provides a functional separation between the coarse fraction and the fine fraction (that tends to be more nutritious), more convoluted typhlosoles could be more advantageous in coarser soils.

The same uncertainty applies to the interpretation of the influence of soil richness. Traditionally, typhlosole development has been linked with nutrient scarcity in soils, where an increased digestive/absorptive capacity would be advantageous (Perel, 1977; James & Cunningham, 1989). Our results point to the contrary, however, with the possibility of richer soils being related to an increase in typhlosole lamellae.

Unfortunately, the knowledge of the physiology and function of the multilamellar typhlosole is very limited; further studies could shed light on which of those hypotheses are correct. An unexplored possibility is that the multiple lamellae of hormogastrids could act as a highly suitable habitat for mutualistic gut wall bacteria. According to Thakuria *et al.* (2010), the morphology of the earthworm's gut should influence the establishment of bacterial communities, which are important for the digestive system of *Hormogaster elisae* Álvarez, 1977 (Trigo *et al.*, 1999).

PHYLOGENETIC SIGNAL

The strong phylogenetic signal of the morphological variables – number of typhlosole lamellae and average weight – suggests that they could be suitable as taxonomic characters for the Hormogastridae earthworms, which confirms Rota's (1993) statement on the number of lamellae. This finding could potentially help to resolve their problematic taxonomy, both in species delimitation and high-level relationships.

The phylogenetic inertia (which was lower for average weight) could explain some deviant observations across the family. For example, *Hormogaster pretiosiformis* Zicsi, 1970 and *Hormogaster pretiosiformis oroeli* Álvarez, 1971 (see MON and OEL in the tree) possess a very high number of lamellae in proportion to their small body size; this character seems to be retained from their heavier closest relatives. Their apparent miniaturization could have been decoupled from a proportional reduction in the

number of lamellae, with insufficient time elapsed to allow both characters to reach the inferred relationship. The Central Iberian and Tyrrhenian clades (comprising the cryptic lineages of *H. elisae* and of *Hormogaster redii* Rosa, 1887, *Hormogaster insularis* Bouché, 1970, and *Hormogaster samnitica* Cognetti, 1914, respectively) are extreme examples of this differential phylogenetic constraint. Although some populations showed strong variation in body size, the number of typhlosole lamellae was basically constant.

The lower phylogenetic signal of the environmental variables is consistent with the observations of Freckleton, Harvey & Pagel (2002) and Blomberg, Garland & Ives (2003). Our results would rule out phylogenetic niche conservatism for these variables, defined as the phylogenetic signal being higher than is expected to result from Brownian motion ($\lambda = 1$).

EVOLUTIONARY RATE

The values obtained for κ and δ offer insight into the evolutionary rate of change in number of lamellae across the family Hormogastridae. Kappa ≈ 1 equates character evolution being independent of the length of the branches in the phylogenetic tree: in other words, it indicates gradual evolution (Pagel, 1999). The optimal value of delta ($\delta = 0.265$) shows a different scenario, however. Delta < 1 indicates that the early evolution of a character contributed heavily to its variation, which is characteristic of an adaptive radiation (Pagel, 1999). The family Hormogastridae comprises nine deep clades, with ancient diversification dating back to the middle–late Cretaceous (Novo *et al.*, 2011) at a minimum. The early morphological radiation detected by our analyses could correspond to the divergence of these clades.

CONCLUSION

We found that increasing body weight acted as the main evolutionary pressure in the development of the multilamellar typhlosole of the Hormogastridae; however, soil texture and richness also appeared to be involved. This is an interesting example of an evolutionary novelty having a strong influence in the phylogenetic and ecological radiation of an animal family.

The analysis of phylogenetic signal allowed us to distinguish the more constrained variables (number of lamellae and weight) from the moderately constrained (soil texture) and weakly constrained (soil richness) variables. These results help to explain the present character states found in earthworm populations and their habitats.

By optimizing κ and δ parameters, we inferred a possible early adaptive radiation in the multilamellar typhlosole, which would be congruent with the evolutionary background of the family.

To our knowledge, this is the first work using phylogenetic comparative methods on earthworms (and one of the few studying invertebrates), relating morphological and environmental variability in an explicit phylogenetic context. This work highlights the appeal of implementing the phylogenetic comparative method to test evolutionary hypotheses in invertebrate taxa, which have been neglected historically by this approach. The compilation of comprehensive databases relating molecular markers, morphology, and environmental data across high-ranking clades is the first step towards such studies.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article:

Appendix S1. Locality, coordinates and GenBank accession numbers for the studied species.
Appendix S2. Correlation study between typhlosole surface and number of lamellae.
Appendix S3. Definition of the PCA factors and model diagnostics for the PGLS analysis.
Appendix S4. Morphological characters and soil variables for the different populations and localities.
Appendix S5. Bayesian and Maximum Likelihood inference of the phylogenetic tree based on the concatenated sequence.