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A strategy in searching for stress tolerance-correlated characteristics in nematodes while accounting for phylogenetic interdependence

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Summary – Biological indicators are highly relevant for assessing the condition of a soil as they are integrative; they reflect the overall impact of physical, chemical and biological changes. Indigenous soil organisms are preferable to other test organisms because the diversity and condition of indigenous soil organisms reflect both acute and chronic effects of soil disturbances. Nematodes are ubiquitous, speciose, easily extractable and present in extremely high numbers. Given the ever increasing amount of sequence data, DNA barcode-based community analysis will soon be possible and a next step would be to define objective criteria for the ecological grouping of soil nematodes. Here, we present a framework to ascertain which traits are correlated with a tolerance to stress. For this, a field study on the effects of pH and copper on nematode communities was re-analysed. Changes in abundances of individual genera were correlated with a number of potentially stress tolerance-related characteristics. The generalised least squares (GLS) method was used to account for the phylogenetic dependence of the data. Only the relationship between the ability to enter a survival stage and tolerance to copper at pH 6.1 was found to be significant, but the quantity of missing data probably had a negative impact on the analyses. This study did, however, clearly demonstrate the importance of accounting for the effects of phylogenetic dependence in the data. When the phylogeny was taken into account, we observed an average change in P value of 0.196 (and in some cases as much as 0.6) for the correlations of possible stress-related characteristics and Cu or pH tolerance. This research constitutes a proof-of-principle for a transparent method to relate stress tolerance to (ecological) characteristics. The usefulness of this powerful method should become even clearer when substantially higher numbers of individuals are analysed (as facilitated by using DNA barcodes) and when missing data are filled in.

Keywords – comparative method, interdependence, maturity index, nematodes, phylogenetic stress tolerance.

Because of their abundance and their (trophic) diversity, nematodes occupy important positions in the soil foodweb (De Ruiter *et al.*, 1998). They also display a great variability in sensitivity to environmental stress (Bongers, 1990). As such, the composition of nematode communities is considered to be an informative indicator for soil health. However, little is known about biological characteristics that underlie the very wide variation in stress (in)tolerance. Here, we propose a strategy to investigate what traits are correlated with stress tolerance in nematodes.

The monitoring of nematode communities as indicators for soil health conditions is widely applied. However, sample size and taxonomic resolution are currently dictated by practical limitations (and are respectively smaller and lower than desired). The morphology of nematodes is

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relatively conserved and, therefore, community analysis is time-consuming and requires ample expert knowledge. Recent advances in the use of molecular characteristics for the analysis of nematode communities may remove (in part) these practical obstacles (Holterman *et al.*, 2008).

Several indices can be used to describe community diversity, such as the Shannon-Wiener index (Shannon, 1948), Simpson's diversity index (Simpson, 1949) or the species richness index (*S*; the number of species present in an ecosystem). An important characteristic of the Maturity Index (MI) as proposed by Bongers (1990) is the inclusion of ecological characteristics, and, over the last decade, this MI has been widely used for nematode community analyses. The Maturity Index assigns each nematode family a so-called cp-value. This 'coloniser-persister' scale ranges from 1 to 5 and corresponds roughly to r-K strategies, with values of 1 and 2 being assigned to the most tolerant r-strategists and values of 4 and 5 to the most sensitive K-strategists. Families assigned to cp-class 1 are enrichment opportunists and, while they can outlast periods of adverse conditions, they are mainly found in food-rich conditions. For this reason they are often excluded from the MI (Bongers, 1999). The MI was demonstrated to be an effective means for assessing and quantifying the impact of soil pollution on nematode communities (Bongers & Ferris, 1999). However, families were assigned to cp-classes mainly on the basis of expert knowledge and some general observations (Bongers, 1999), and there are few clear criteria for each cp-class. In addition, it is known that genera in a single family do not always react similarly to stress (Ettema & Bongers, 1993) and, for some nematode families, a refinement of the MI down to genus, or even species, level would be appropriate. Identification of traits correlated with specific forms of stress tolerance would be helpful for the refinement of the MI or related indices.

When looking for correlations between traits using data from different species, it is important to realise that one of the basic assumptions of the comparative method, *i.e.*, the data are independent, is often violated (Felsenstein, 1985). If a strong phylogenetic correlation exists in the data, it is important to take this into account. Felsenstein designed a method, named phylogenetically independent contrasts, which takes these confounding effects into account (Felsenstein, 1985). However, this method may overestimate the effects of phylogeny (Harvey & Rambaut, 2000). Another (intermediate) approach was proposed by Pagel (1999): the generalised least squares (GLS) method.

To study traits correlated with stress tolerance in nematodes we focused on the effects of copper and pH on nematode communities. The traits involved in this study are body size, reproductive potential, (a)sexual reproduction, feeding type, the ability to form survival stages and cuticle permeability. Body size is an important life history trait which correlates with many processes (Peters, 1983). Reproduction can be expected to play a role in stress tolerance since a high reproduction rate could partially compensate for increased mortality and enhance population recovery from the stress. It is noted that stress tolerant nematodes are often small, have short generation times and produce large numbers of offspring (Bongers, 1999). Unfortunately, generation times and number of offspring are not determinable for most nematodes, since most nematodes cannot be cultured or are barely culturable. Therefore, we looked to the gonad size relative to body size as an alternative measure for fecundity. To the best of our knowledge this has never been done for nematodes, but it has been done in various other animals, such as tardigrades (Guidetti *et al.*, 2007), flat worms (Schärer *et al.*, 2005) and tree frogs (Rodrigues *et al.*, 2005, 2007). Asexual reproduction is often considered a trait for opportunists but this does not necessarily seem to be the case for nematodes (Bongers, 1999). Feeding type could also be related to stress tolerance. In the MI, most bacterial feeders belong to low cp-classes while members of cp-classes 4 and 5 are often predators or omnivores (Bongers, 1999). Entering a survival stage can help to outlast long periods of stress and, finally, changing the cuticle permeability could be an expedient to prevent pollutants from entering the body.

Until now, nematode families have been assigned to cp-classes for the Maturity Index on the basis of expert knowledge. Identifying traits which are correlated with a tolerance to stress is a first step towards defining clear objective criteria for the assigning of nematode taxa to cp-classes. Furthermore, having clear criteria will allow for the refinement of the MI from a family to a genus level index. In this study we set out a framework to identify traits important to stress tolerance. We will demonstrate the importance of taking the effects of phylogeny on the data into account. Finally, the effectiveness of our framework to identify relevant traits will be discussed.

Materials and methods

Nematode community data for copper and pH stress

To determine the tolerance of several nematode genera to copper and pH stress, data were used from a field study on the long-term effects of copper and pH on nematode communities (Korthals *et al.*, 1996). The study site, known as the Bovenbuurt pastures, is located approximately 3 km NNE of Wageningen, The Netherlands. The field soil is slightly loamy sand. After having been used as a permanent pasture for at least 30 years, in 1978 a crop rotation scheme of silage maize, starch potatoes and oats was applied. In 1982 the field was divided into 128 plots of 6 × 11 m. Four copper levels were created by applying $CuSO_4 \cdot 5H_2O$ at rates of 0, 250, 500 and 750 kg Cu ha^{-1}. Levels of pH were adjusted to 4.0, 4.7, 5.4 or 6.1. This resulted in 16 different treatments with eight replicates per treatment. In March 1992 samples were taken from each plot. A part of the sample was used to determine the pH (pH-KCl) and to determine the total quantity of copper present ($Cu\text{-}HNO_3$) and the available copper concentration ($Cu\text{-}CaCl_2$). Nematodes were extracted from another part of the soil sample. Nematodes were counted under a dissecting microscope and, after fixation in formalin, at least 150 nematodes were identified using a light microscope. For full details of field history, soil composition, sampling, pH determination and copper extraction we refer to Korthals *et al.* (1996).

Redundancy analysis

In order to investigate the change in abundance of genera related to pH or copper treatment, a detrended correspondence analysis (DCA) was performed (Ter Braak, 1995). Detrended correspondence analyses by segments revealed a gradient of 1.75 standard deviation units for pH and gradients of 1.53, 1.52, 1.33 and 1.65 for Cu at pH levels of 4.0, 4.7, 5.4 and 6.1, respectively, indicating a strong linear response of taxa. Therefore, a redundancy analysis (RDA) was performed (Ter Braak, 1995). RDA is a constrained version of a principal component analysis (PCA), meaning that it focuses on the part of the variance explained by the environmental variables only.

RDA analysis was performed on a matrix of 128 samples (eight replicates per treatment) and 71 species using CANOCO for Windows 4.5 (Ter Braak & Smilauer, 2002). Nematode abundance data were $\ln(2x + 1)$ transformed to down-weight high values and approximate a normal distribution. For the pH and Cu values the true pH (pH-KCl) and the available Cu-concentration ($Cu\text{-}CaCl_2$) were used. Five RDA were performed. One RDA was to determine the tolerance to pH; for this all the plots with a Cu-treatment were excluded from the analysis. To determine the tolerance to Cu, a separate RDA was performed for each pH-level (4.0, 4.7, 5.3, 6.1), excluding the samples for the other pH levels. The values of the first canonical axis were used as a measure for the tolerance of a genus to the stressor (pH or Cu). A strongly positive or negative value indicated that the variation in abundance was strongly explained by the pH or Cu treatment, *i.e.*, the genus under consideration was strongly affected. A value close to zero indicated that abundance was not well explained by the treatment, *i.e.*, the genus is tolerant.

The redundancy analyses (RDA) were followed by unrestricted Monte Carlo permutation (MC) tests with 499 permutations to assess the significance of the relation between each environmental variable and community composition (Verdonschot & Braak, 1994).

Nematode traits

Six nematode traits were studied for their possible correlation with tolerance to copper and pH stress. These were adult body size, relative gonad size, (a)sexual reproduction, cuticle permeability, feeding type and the ability to form a survival stage. Wherever possible, only data from species occurring in The Netherlands was used. The cp-values for the maturity index were taken from Bongers (1999).

Adult body size was determined using Andrássy's (1956) formula, using body length and width taken from Bongers (1994). A correction for tail shape was applied as described in Van der Wurff *et al.* (2007). Body size data was log 10-transformed to approximate a normal distribution of the data. Feeding types were according to Yeates *et al.* (1993). Survival stages (the ability to form dauer juveniles or resistant stages) were according to Bongers (1999). Reproduction type was based on whether a genus can reproduce asexually or not; no distinction was made between genera that can only reproduce asexually and genera that can reproduce both sexually and asexually. Most data came from Bongers (1994) and Lorenzen (1994), with additional data from the literature where required. When the mode of reproduction was not directly known the presence of males was used as a guideline. If males were unknown or rare, a species was supposed to be able to reproduce asexually. If both sexually and asexually reproducing species were present in a genus, the genus was scored as being able to reproduce asexually.

To assess the permeability of the cuticle, live nematodes were coloured with different staining agents. In a preliminary experiment (results not shown), aqueous solutions of six staining agents – Coomassie R (Merck, Whitehouse Station, NJ, USA), Coomassie G (Merck), Erytrosin B (Kodak, Hemel Hempstead, UK), Trypan blue (Merck), ponceau sodium (Merck) and acid fuchsin (Merck) – were tested with a taxonomically diverse number of species. Three staining agents were selected, one which stained many nematodes (Erytrosin B), one which only stained the nematodes with the most permeable cuticles (Coomassie R) and one which was intermediate between these two (Trypan blue). Living nematodes were transferred to a small well with one drop of staining agent diluted with demineralised water. If only natural openings were stained, such as the mouth cavity or the amphids, the nematode was scored as non-stained. Testing was done on nematodes isolated directly from environmental samples, and, whenever possible, at least five individuals were used for each nematode-stain combination. It is noted that due to the low abundance of some genera, not every combination could be (fully) tested.

To determine the relative gonad size, pictures were taken from slides in the collection at the Laboratory of Nematology (Wageningen University) using a light microscope (Zeiss Axioscope) equipped with differential interference contrast optics and a CCD camera (CoolSnap, RS Photometrics, Tucson, AZ, USA). When available, pictures from multiple species per genus were taken. For each species six individuals were used. The area of the total nematode and the gonad was measured using the program Image-Pro Express 4.0 (Media Cybernetics, Bethesda, MD, USA). Relative gonad size was expressed as the proportion of the total area of the nematode that was occupied by the gonads. Relative gonad size data were normally distributed.

Phylogenetic tree

Based on previous analyses (Holterman *et al.*, 2006, 2008) a user-tree was defined (Fig. 1). Each genus was represented by one full length small subunit ribosomal DNA (SSU rDNA) sequence (GenBank accessions in Appendix A). The Rhabditidae were represented by ten species to represent the large variation present in the family. The Diplogastridae were represented by four sequences. The alignment was created by ClustalW as applied in BioEdit v7.0.1 (Hall, 1999) and improved manually using arthropod secondary structure information (http://bioinformatics.psb.ugent.be/webtools/rRNA/secmodel/index.html in accordance with Wuyts *et al.*, 2000). MrBayes 3.1.2 was used to calculate branch lengths. The alignment was divided into a stem and loop partition and the GTR model with invariable sites and gamma correction was used. Parameter values were unlinked between partitions and the rate prior was set to variable. The topology of the user tree was fixed. The analysis was run for 3 million generations using four independent runs and four chains per run. The 'burnin' was 1 200 000 generations. For the Rhabditidae and Diplogastridae the average branch length was calculated.

Comparative analysis

For the comparative analysis the program Continuous v.1.0d13 (Pagel, 1997, 1999) was used. Dummy variables were used for the feeding types. To maximise the amount of included data (species with missing data in a trait under analysis are excluded), RDA values and traits were analysed pair-wise and a Bonferroni correction was applied to allow for the effects of multiple pair-wise testing. A standard constant variance random walk model of evolution was used. The parameter λ reveals if the data are predicted by the phylogeny and can be estimated by Continuous. When $\lambda = 0$, Continuous assumes the trait is evolving independent of phylogeny, if $\lambda = 1$, Continuous assumes there is a full correlation between the phylogeny and data (in effect it is using independent contrasts; Felsenstein, 1985). Likelihood ratio tests were performed to test if the value of λ estimated by likelihood was significantly different from 1 or 0 for each pair of RDA values and traits. Next a likelihood ratio test was performed to test if a RDA-value and a trait were significantly correlated by constraining the covariances to zero for the H_0. This was done using a λ estimated by Continuous and a λ set to zero to study the effect of using a phylogenetic correction.

Results and discussion

We have set up a framework to investigate if certain nematode traits – body size, reproductive potential, feeding type, survival stage, asexual reproduction and cuticle permeability – are correlated with tolerance for Cu or pH stress. Furthermore, the importance of allowing for the phylogenetic non-independence of the data in the analy-

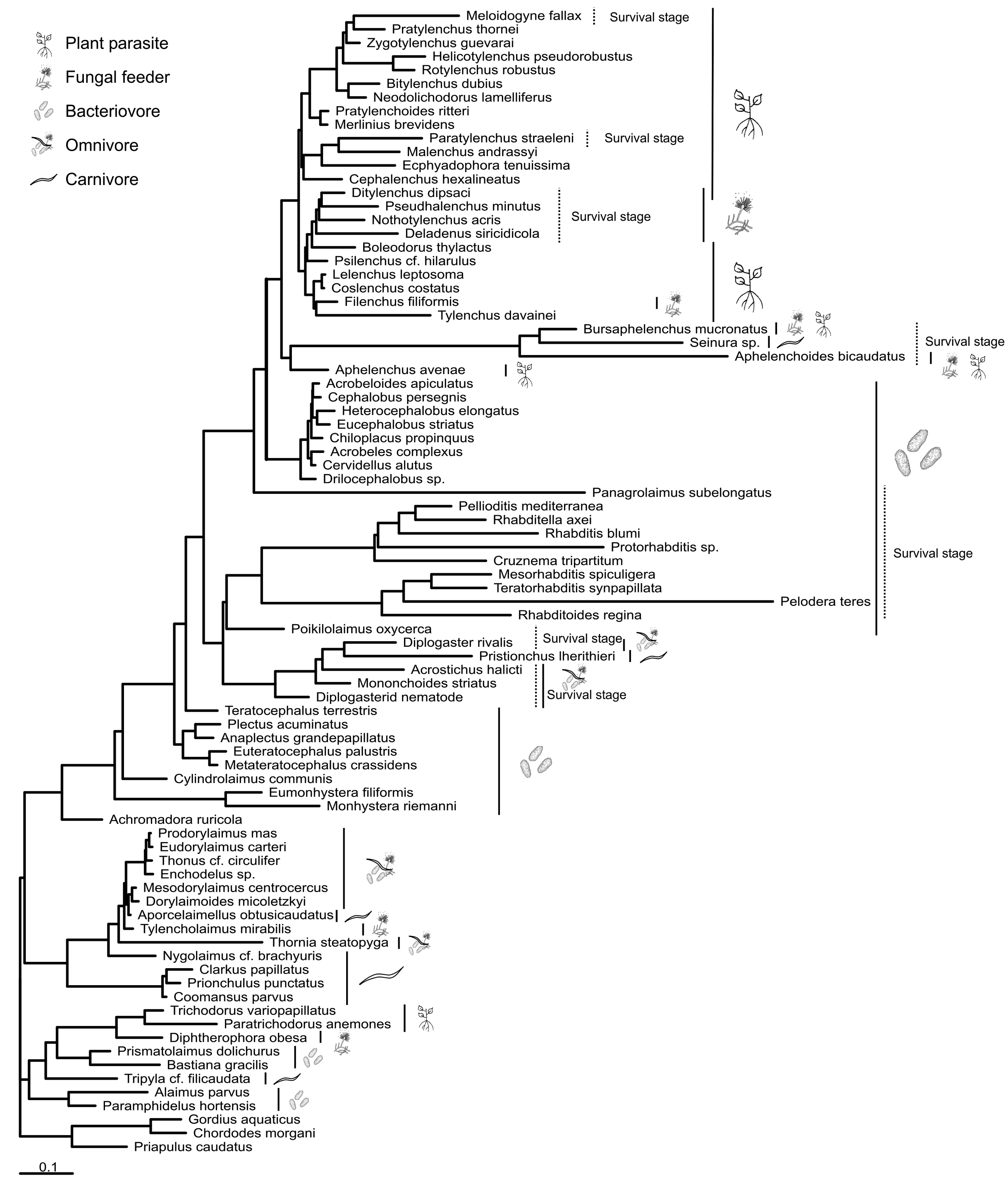

Fig. 1. *Phylogenetic tree of taxa involved in this study. Topology was based on Holterman* et al. *(2006) and Holterman* et al. *(2008); branch lengths were calculated with MrBayes 3.1.2. Feeding types and the ability to enter a survival stage are indicated on the tree.*

sis was studied. The results will be discussed in the light of improving the MI.

Nematode responses

The nematode sensitivity analyses were performed on natural communities, instead of laboratory assays, to mimic a natural response towards stressors. The field site was suitable for this, because the soil was homogenous with respect to other important soil parameters besides pH and copper, such as organic matter and percentage of small (<2 μm) soil particles (Korthals *et al.*, 1996). Although a multivariate test is not required for testing an (in principle) univariate experimental setup, we choose to use RDA since its use is extremely powerful in natural field conditions, *i.e.*, a soil dominated by multiple (co-)factors. In addition, abundances are patchy by nature and especially disturbance-sensitive groups, such as predators and omnivores typically have low abundances. Furthermore, only a relatively low number of individuals were analysed per sample.

The values of the first canonical axis of the RDA analyses are presented in Table 1 together with the data for the nematode traits. If the RDA values are positive, it means there is a positive correlation between abundance and the stress factor (pH or Cu), whereas a negative value means there is a negative correlation. RDA values close to zero (between -0.5 and 0.5) are considered not to be correlated with the treatment, *i.e.*, the species are tolerant to the stress. RDA values generally display a positive relationship with cp-values in the case of pH (a higher more neutral pH being the less stressed condition) and a negative relationship with copper (Table 1; Fig. 2). This supports the general use of cp-values at the taxonomic level of family. However, differing RDA values for species within the families Cephalobidae and Pratylenchidae show that a family-based cp-value is not always justified (Table 1). This is in accordance with earlier findings that some genera in a family may not always behave as would be expected from their cp-values (Ettema & Bongers, 1993). One of the genera Ettema and Bongers mentioned as being more abundant than expected was *Aporcelaimellus*, which in our analyses displays RDA values close to zero and therefore is more tolerant than its cp-value of 5 would suggest.

Phylogenetic dependence

As can be seen from the results (Table 2) many traits are not phylogenetically independent (λ significantly larger than zero). At the same time, most traits do not evolve entirely according to the tree topology either; λ is significantly smaller than 1 in most cases. This means that the generalised least squares (GLS) method as applied by the program Continuous is ideally suited to investigate the data for correlations between traits. A method such as independent contrasts (Felsenstein, 1985) would overestimate the effect of phylogeny (in effect it assumes that λ is 1), while the GLS method can take an intermediate approach between independent contrasts and not accounting for phylogenetic dependence at all. The importance of using a correct method for accounting for the effects of phylogeny was also demonstrated by other studies (Martins *et al.*, 2002; Stuart-Fox, 2009).

A phylogenetic dependence of the data can easily be visualised. For example, bacterial feeding has a very high λ in all cases. In the tree, almost all bacterial feeders cluster together (Fig. 1). If, by coincidence, the ancestor of these bacterial feeders also happened to be tolerant to stress, it is very possible that its descendants inherited both of these traits even if no actual physical relation between the two traits exists. An analysis of these traits without accounting for their shared ancestry would thus probably show the two traits to be correlated regardless of whether an actual relation between the two exists or not. If, on the other hand, a certain trait is spread over the tree, such as hyphal feeding (Fig. 1; λ is 0 or very close to 0 in all cases), shared ancestry is unlikely to play a role in the correlation of this trait with other traits.

Correlations between traits and stress tolerance

Correlations between stress tolerance (as represented by RDA values) and nematode traits were tested in a pairwise fashion. To demonstrate the effect of accounting for phylogeny, the same tests were performed using the maximum likelihood estimate for λ in one case and constraining λ to 0 in the other case. After applying a Bonferroni correction only a single significant correlation was found (Table 2). This was between the ability to enter a survival stage and the RDA for copper at pH 6.1 and not using a phylogenetic correction. Given that, in this case, λ was not significantly different from zero ($P = 2.44 \times 10^{-4}$; $\lambda = 0.12$), this could be considered a valid result. It certainly seems logical that the ability to enter a survival stage and survive periods of adverse conditions is correlated with a tolerance to stress. Although there are more correlations with a low P value (survival stage

Table 1. *Data for the different nematode traits and the results of the RDA analysis.*

Genus	Family	cp value	log body size (μm^3)	Relative gonad size	Feeding type	Survival stage	Reproduction	Coomassie R	Erytrosin B	Trypan blue	RDA pH 4.0-6.1	RDA Cu pH 4.0	RDA Cu pH 4.7	RDA Cu pH 5.4	RDA Cu pH 6.1
Cephalenchus	Tylodoridae	*	5.04	0.15	PP	*	Asexual	*	*	*	*	*	*	*	*
Psilenchus	Psilenchidae	*	5.4	0.22	PP	*	Sexual	–	–	–	*	*	*	*	0.091
Panagrolaimus	Panagrolaimidae	1	5.8	0.28	BF	Yes	Asexual	*	*	–	0.363	−0.12	−0.33	−0.12	0.254
Rhabditis	Rhabditidae	1	5.87	0.31	BF	Yes	Asexual	*	–	–	−0.07	−0.12	0.149	0.278	0.163
Diplogaster	Diplogastridae	1	5.74	0.36	O	Yes	Sexual	*	–	+	−0.24	−0.21	*	*	0.194
Pristionchus	Neodiplogastridae	1	6.66	0.29	P	*	Sexual	*	–	–	−0.01	−0.22	−0.16	0.348	0.407
Acrobeloides	Cephalobidae	2	5.17	0.36	BF	No	Asexual	*	–	–	0.256	−0.75	−0.6	−0.6	0.279
Cephalobus	Cephalobidae	2	5.57	0.28	BF	No	Sexual	*	*	–	0.524	−0.16	0.104	−0.13	0.345
Heterocephalobus	Cephalobidae	2	5.43	0.26	BF	No	Asexual	*	+	–	−0.2	−0.16	*	0.278	0.203
Eucephalobus	Cephalobidae	2	5.33	0.31	BF	No	Sexual	*	+	–	0.381	0.038	−0.13	0.157	0.205
Chiloplacus	Cephalobidae	2	5.86	0.26	BF	No	Asexual	*	–	–	−0.33	0.158	0.093	0.513	0.595
Acrobeles	Cephalobidae	2	5.76	0.3	BF	No	Sexual	*	+	–	0.743	−0.28	−0.45	−0.8	−0.74
Cervidellus	Cephalobidae	2	5.03	0.29	BF	No	Asexual	*	–	+	0.342	−0.61	−0.41	−0.64	−0.55
Drilocephalobus	Osstellidae	2	4.97	0.35	BF	*	Sexual	*	–	*	−0.24	−0.56	−0.34	−0.03	0.032
Plectus	Plectidae	2	5.82	0.2	BF	No	Asexual	*	–	+	−0.21	−0.64	−0.77	0.183	−0.24
Anaplectus	Plectidae	2	6.19	0.26	BF	No	Sexual	*	–	+	0.248	−0.28	−0.09	0.016	−0.34
Eumonhystera	Monhysteridae	2	5.2	0.21	BF	No	Asexual	+	+	+	*	*	*	*	−0.12
Monhystera	Monhysteridae	2	5.86	0.35	BF	No	Sexual	*	*	*	0.367	*	−0.07	−0.06	−0.31
Ditylenchus	Anguinidae	2	5.78	0.31	HF, PP	Yes	Sexual	–	–	–	0.163	−0.32	−0.2	0.228	0.313
Pseudhalenchus	Anguinidae	2	4.59	0.26	HF	Yes	Sexual	*	*	*	−0.49	−0.25	0.077	0.412	0.231
Nothotylenchus	Anguinidae	2	5.59	0.3	HF	Yes	Sexual	*	*	*	0.13	−0.02	0.04	0.391	0.319
Deladenus	Neotylenchidae	2	5.57	0.42	HF	Yes	Asexual	*	*	*	*	0.355	*	−0.09	*
Aphelenchus	Aphelenchidae	2	5.37	0.25	HF	No	Asexual	*	–	–	0.771	−0.04	−0.07	−0.24	0.117
Filenchus	Tylenchidae	2	4.54	0.17	HF, PP	No	Asexual	–	–	–	0.089	−0.5	−0.27	0.187	−0.09
Bursaphelenchus	Parasitaphelenchidae	2	5.51	*	HF, PP	Yes	Sexual	*	*	*	*	*	*	*	*
Aphelenchoides	Aphelenchoididae	2	5.2	0.21	HF, PP	Yes	Sexual	–	*	*	−0.62	−0.28	−0.03	0.557	0.645
Seinura	Seinuridae	2	5.12	0.21	P	Yes	Sexual	*	*	*	*	0.279	0.456	*	*
Paratylenchus	Paratylenchidae	2	4.71	0.23	PP	Yes	Asexual	–	–	–	−0.06	*	0.058	−0.18	0.155
Ecphyadophora	Ecphyadophoridae	2	4.15	0.14	PP	*	Sexual	*	*	*	−0.09	*	−0.14	0.235	*
Malenchus	Tylenchidae	2	4.75	0.21	PP	No	Asexual	–	+	–	*	*	*	*	*
Boleodorus	Tylenchidae	2	4.91	0.17	PP	No	Sexual	*	*	*	0.309	*	*	−0.06	−0.35
Lelenchus	Tylenchidae	2	4.45	0.18	PP	No	Sexual	*	*	*	−0.19	−0.25	0.163	−0.13	−0.05
Coslenchus	Tylenchidae	2	4.81	0.23	PP	No	Asexual	*	–	*	0.155	*	*	*	−0.24
Tylenchus	Tylenchidae	2	5.53	0.16	PP	No	Sexual	–	–	–	0.324	−0.03	−0.23	−0.38	−0.01
Achromadora	Achromadoridae	3	5.18	0.21	*	No	Asexual	–	+	+	0.008	−0.19	−0.06	*	−0.26
Teratocephalus	Teratocephalidae	3	4.89	0.09	BF	No	Asexual	*	–	–	0.077	*	*	−0.15	0.091
Euteratocephalus	Metateratocephalidae	3	5.39	0.11	BF	No	Asexual	+	*	*	*	*	*	*	*
Metateratocephalus	Metateratocephalidae	3	4.82	0.19	BF	No	Asexual	–	*	*	−0.07	*	−0.16	*	*
Cylindrolaimus	Cylindrolaimidae	3	5.52	0.24	BF	*	Asexual	–	+	*	0.27	*	−0.1	*	−0.09
Prismatolaimus	Prismatolaimidae	3	5.04	0.12	BF	No	Asexual	+	+	+	0.339	−0.12	−0.24	0.077	0.068
Bastiania	Bastianiidae	3	4.98	0.15	BF	No	Asexual	–	–	–	0.215	*	−0.16	−0.07	−0.23

Table 1. *(Continued).*

Genus	Family	cp value	log body size (μm^3)	Relative gonad size	Feeding type	Survival stage	Repro-duction	Coomassie R	Erytrosin B	Trypan blue	RDA pH 4.0-6.1	RDA Cu pH 4.0	RDA Cu pH 4.7	RDA Cu pH 5.4	RDA Cu pH 6.1
Diphtherophora	Diphtherophoridae	3	5.73	0.27	HF	No	Sexual	+	+	+	0.533	0.168	−0.52	−0.45	−0.34
Tripyla	Tripylidae	3	6.14	0.31	P	No	Sexual	*	*	*	*	*	−0.01	*	*
Meloidogyne	Meloidogynidae	3	5.56	*	PP	Yes	Asexual	–	–	–	*	*	0.152	0.24	*
Pratylenchoides	Pratylenchidae	3	5.61	*	PP	No	Sexual	*	*	*	*	0.244	*	*	*
Pratylenchus	Pratylenchidae	3	5.07	0.14	PP	No	Asexual	–	–	–	0.254	−0.81	−0.8	−0.3	−0.16
Zygotylenchus	Pratylenchidae	3	5.12	*	PP	No	Sexual	*	*	*	−0.13	*	−0.15	−0.31	*
Helicotylenchus	Hoplolaimidae	3	5.44	0.19	PP	No	Asexual	–	–	–	0.059	−0.19	−0.26	−0.11	−0.27
Rotylenchus	Hoplolaimidae	3	5.68	0.13	PP	No	Sexual	–	–	–	−0.13	−0.11	−0.14	−0.07	−0.17
Bitylenchus	Telotylenchidae	3	5.6	0.23	PP	No	Asexual	–	–	–	−0.05	−0.32	−0.53	−0.09	0.035
Neodolichorhynchus	Telotylenchidae	3	5.88	0.19	PP	No	Sexual	*	*	*	0.203	*	*	*	−0.19
Merlinius	Telotylenchidae	3	5.21	0.22	PP	No	Sexual	–	–	–	0.542	−0.42	−0.32	−0.57	−0
Alaimus	Alaimidae	4	5.56	0.06	BF	No	Asexual	+	+	+	0.389	*	−0.18	−0.29	−0.44
Paramphidelus	Alaimidae	4	5.27	*	BF	No	Asexual	*	+	*	−0.06	*	*	−0.04	−0.08
Tylencholaimus	Tylencholaimidae	4	5.51	0.12	HF	No	Asexual	–	*	+	0.017	−0.3	−0.22	−0.43	−0.16
Mesodorylaimus	Dorylaimidae	4	6.33	0.29	O	No	Sexual	*	*	+	0.39	*	*	−0.24	−0.37
Prodorylaimus	Dorylaimidae	4	6.33	0.16	O	No	Asexual	*	*	+	0.39	*	*	−0.24	−0.37
Eudorylaimus	Qudsianematidae	4	6.42	0.16	O	No	Sexual	+	+	+	0.064	−0.19	0.124	−0.19	−0.11
Thonus	Qudsianematidae	4	6.23	0.19	O	No	Asexual	+	+	–	0.155	−0.21	−0.17	−0.04	−0.22
Enchodelus	Nordiidae	4	6.63	0.16	O	No	Asexual	*	*	*	*	*	*	*	*
Dorylaimoides	Mydonomidae	4	5.83	0.2	O	No	Asexual	*	*	*	0.203	*	*	*	−0.19
Thornia	Thorniidae	4	5.92	*	O	No	Sexual	*	*	*	*	*	*	*	*
Clarkus	Mononchidae	4	6.13	0.2	P	No	Asexual	+	+	+	0.33	*	*	*	−0.36
Prionchulus	Mononchidae	4	6.81	0.23	P	No	Asexual	–	+	+	*	*	−0.08	*	*
Coomansus	Mononchidae	4	6.35	0.38	P	No	Asexual	–	+	*	−0.03	−0.2	−0.4	−0.31	−0.07
Trichodorus	Trichodoridae	4	5.74	0.16	PP	No	Sexual	*	+	–	0.355	−0.11	−0.43	−0.62	−0.35
Paratrichodorus	Trichodoridae	4	5.9	0.27	PP	No	Asexual	+	+	–	*	*	*	*	*
Aporcelaimellus	Aporcelaimidae	5	6.9	0.18	O	No	Asexual	–	+	–	0.182	−0.12	−0.37	−0.24	−0.1
Nygolaimus	Nygolaimidae	5	5.84	0.18	P	No	Asexual	+	+	+	0.155	*	−0.1	*	−0.28

BF = bacterial feeder; HF = hyphal feeder; PP = plant parasite; O = omnivore; P = predator. Asexual means asexual reproduction is possible but not necessarily the main mode of reproduction.

*Missing data.

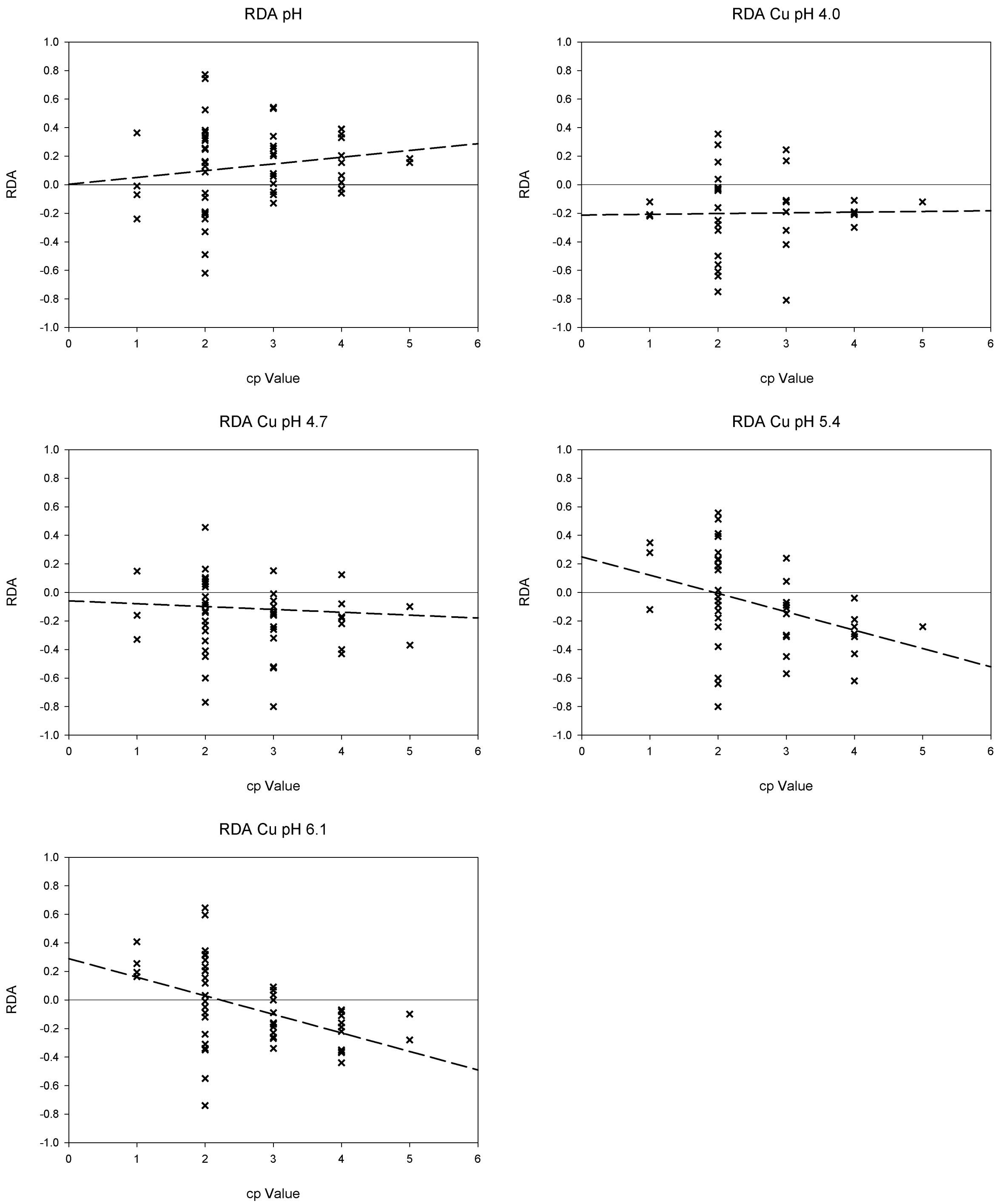

Fig. 2. *RDA values plotted against cp values with trend lines.*

with other RDAs, cuticle permeability) these are all insignificant because of the large Bonferroni correction that had to be applied, due to the pair-wise testing.

Accounting for the effects of phylogeny

Although no significant correlations were found when accounting for phylogeny, and only one significant corre-

Table 2. *Results of the comparative analysis with the program Continuous.*

RDA	Trait	λ	*P*		Correlation		Absolute difference correlation *P*-values
			λ < 1	λ > 0	λ estimated	λ = 0	
pH	log10 body size	0.38	0*	7.43e−5*	0.096	0.0996	0.004
pH	Relative gonad size	0.29	2.65e−14*	0.0482	0.817	0.7	0.117
pH	Bacterial feeder	0.97	0.0419	7.88e−15*	0.7	0.968	0.268
pH	Hyphal feeder	0	2.22e−16*	1	0.295	0.295	0.000
pH	Plant parasite	0.76	4.21e−5*	2.22e−7*	0.888	0.457	0.431
pH	Omnivore	0.56	7.33e−9*	2.85e−6*	0.271	0.175	0.096
pH	Predator	0.95	0.0173	5.06e−7*	0.886	0.731	0.155
pH	Survival stage	0.077	9.44e−7*	0.33	0.0425	0.0283	0.014
pH	Asexual reproduction	0.08	0*	0.186	0.271	0.395	0.124
pH	Coomassie R	0.27	3.88e−9*	0.00351	0.0674	0.0417	0.026
pH	Erytrosin B	0.48	3.11e−12*	8.70e−17*	0.194	0.25	0.056
pH	Trypan blue	0.16	0*	0.0462	0.699	0.65	0.049
Cu pH 4.0	log10 body size	0.38	6.40e−13*	0.0124	0.0787	0.176	0.097
Cu pH 4.0	Relative gonad size	0.46	6.08e−9*	0.0203	0.263	0.708	0.445
Cu pH 4.0	Bacterial feeder	0.93	0.00443	3.98e−6*	0.423	0.15	0.273
Cu pH 4.0	Hyphal feeder	0	1.08e−13*	1	0.982	0.982	0.000
Cu pH 4.0	Plant parasite	0.58	9.19e−7*	0.0206	0.279	0.538	0.259
Cu pH 4.0	Omnivore	0.32	4.05e−11*	0.00659	0.201	0.165	0.036
Cu pH 4.0	Predator	0.89	1.91e−3*	0.0186	0.678	0.651	0.027
Cu pH 4.0	Survival stage	0.17	6.66e−3*	0.241	0.177	0.267	0.090
Cu pH 4.0	Asexual reproduction	0.0084	0*	0.943	0.172	0.173	0.001
Cu pH 4.0	Coomassie R	0.39	1.44e−6*	0.0135	0.344	0.0412	0.303
Cu pH 4.0	Erytrosin B	0.45	4.00e−13*	1.01e−4*	0.0402	0.00324	0.037
Cu pH 4.0	Trypan blue	0.25	1.41e−14*	0.0453	0.421	0.947	0.526
Cu pH 4.7	log10 body size	0.32	2.78e−15*	0.00411	0.764	0.78	0.016
Cu pH 4.7	Relative gonad size	0.51	1.70e−9*	0.0814	0.758	0.887	0.129
Cu pH 4.7	Bacterial feeder	0.94	0.0123	1.64e−8*	0.763	0.572	0.191
Cu pH 4.7	Hyphal feeder	0	1.11e−16*	1	0.449	0.449	0.000
Cu pH 4.7	Plant parasite	0.62	7.85e−7*	2.38e−4*	0.408	0.536	0.128
Cu pH 4.7	Omnivore	0.33	3.86e−12*	0.0128	0.578	0.624	0.046
Cu pH 4.7	Predator	0.88	0.00218	6.97e−4*	0.294	0.216	0.078
Cu pH 4.7	Survival stage	0.11	1.79e−9*	0.194	0.0253	0.0272	0.002
Cu pH 4.7	Asexual reproduction	0.016	0*	0.894	0.159	0.161	0.002
Cu pH 4.7	Coomassie R	0.29	4.99e−7*	0.0167	0.572	0.566	0.006
Cu pH 4.7	Erytrosin B	0.44	3.63e−12*	4.48e−5*	0.764	0.494	0.270
Cu pH 4.7	Trypan blue	0.16	7.77e−16*	0.111	0.701	0.603	0.098
Cu pH 5.4	log10 body size	0.45	8.21e−13*	7.17e−5*	0.942	0.285	0.657
Cu pH 5.4	Relative gonad size	0.43	5.53e−9*	0.0226	0.923	0.734	0.189
Cu pH 5.4	Bacterial feeder	0.97	0.155	1.05e−12*	0.596	0.189	0.407
Cu pH 5.4	Hyphal feeder	0.041	3.53e−13*	0.621	0.299	0.247	0.052
Cu pH 5.4	Plant parasite	0.71	4.44e−5*	2.78e−6*	0.248	0.774	0.526
Cu pH 5.4	Omnivore	0.58	3.89e−5*	6.96e−7*	0.748	0.199	0.549
Cu pH 5.4	Predator	0.97	0.141	1.68e−6*	0.9	0.938	0.038
Cu pH 5.4	Survival stage	0.13	5.63e−7*	0.183	0.00942	0.00381	0.006
Cu pH 5.4	Asexual reproduction	0.069	0*	0.328	0.533	0.675	0.142
Cu pH 5.4	Coomassie R	0.38	6.72e−6*	0.00362	0.614	0.816	0.202
Cu pH 5.4	Erytrosin B	0.45	2.30e−13*	9.92e−5*	0.627	0.22	0.407
Cu pH 5.4	Trypan blue	0.14	0*	0.0796	0.678	0.462	0.216

Table 2. *(Continued).*

RDA	Trait	λ	*P*		Correlation		Absolute difference correlation *P*-values
			λ < 1	λ > 0	λ estimated	λ = 0	
Cu pH 6.1	log10 body size	0.39	1.11e−16*	5.93e−6*	0.947	0.144	0.803
Cu pH 6.1	Relative gonad size	0.34	2.11e−13*	0.00223	0.476	0.808	0.332
Cu pH 6.1	Bacterial feeder	0.99	0.316	5.55e−16*	0.994	0.428	0.566
Cu pH 6.1	Hyphal feeder	0.1	1.78e−15*	0.0867	0.0633	0.04	0.023
Cu pH 6.1	Plant parasite	0.73	1.46e−4*	1.18e−8*	0.272	0.644	0.372
Cu pH 6.1	Omnivore	0.56	7.87e−8*	3.50e−7*	0.718	0.0776	0.640
Cu pH 6.1	Predator	0.97	0.167	1.89e−8*	0.992	0.613	0.379
Cu pH 6.1	Survival stage	0.12	1.06e−7*	0.0765	0.00178	2.44e−4*	0.002
Cu pH 6.1	Asexual reproduction	0.16	0*	0.00952	0.677	0.703	0.026
Cu pH 6.1	Coomassie R	0.29	2.47e−08*	0.00182	0.429	0.0483	0.381
Cu pH 6.1	Erytrosin B	0.44	7.22e−14*	7.74e−6*	0.463	0.0325	0.431
Cu pH 6.1	Trypan blue	0.17	0*	0.0166	0.026	0.00195	0.024
Average							0.196

Significant *P* values after a Bonferroni correction (0.05/60 = 8.33e−4) are marked with an asterisk (*).

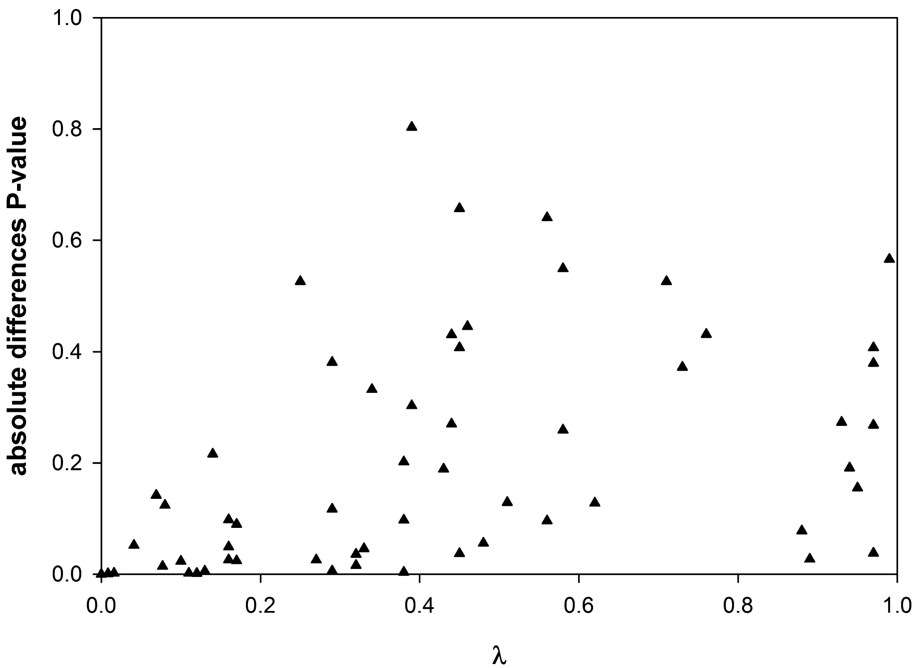

Fig. 3. *The absolute differences of the* P *values for the correlations tests with and without phylogenetic correction plotted against λ.*

lation when phylogeny was not taken into consideration, the data still demonstrate the importance of accounting for phylogeny. The average absolute difference in *P* values between the tests for correlation with and without accounting for phylogeny is 0.196 (Table 2), and the changes in the *P* values were both positive and negative. As was to be expected, this difference becomes even larger with higher λ values (Fig. 3), and testing without phylogenetic correction becomes more inappropriate. The importance of allowing for phylogenetic dependence among the data has been demonstrated by other studies (Holden & Mace, 1997; Pagel, 1999; Espinoza *et al.*, 2004; McKechnie *et al.*, 2006; Swanson & Garland, 2009); in these cases, results contradicting established theory were found because the effects of phylogeny were taken into account in the analysis. Despite its importance, only few authors have allowed for the effect of phylogeny in nematode studies (Morand, 1996; Morand & Sorci, 1998; Poulin & Morand, 2000; and, to a limited extent, Yeates & Boag, 2006). Yet the results presented here in Figure 3 clearly demonstrate the importance of accounting for the effects of phylogenetic dependence in the data when looking for correlations between traits in nematodes.

Towards experimentally derived cp-classes

What relevance do these results have when it comes to defining more objective criteria for assigning nematodes to cp-classes for the MI? First of all, they demonstrate the importance of allowing for the phylogenetic non-independence of data when looking for correlations between traits and stress tolerance. For many traits λ is significantly larger than 0 (Table 2) and, especially if λ becomes larger than 0.3, the effect on P values can be substantial (as much as 0.6; Fig. 3). Furthermore, the results also demonstrate the suitability of the GLS method for accounting for the effects of phylogeny on the data. The independent contrast method would, in most cases, overestimate the effect of phylogeny on the data.

Only one significant correlation was found between stress tolerance and nematode traits; the ability to enter a survival stage. This means that for now we cannot design clearer criteria for the cp-classes. However, it seems unlikely that none of these traits, other than the ability to enter a survival stage, is correlated with tolerance to stress. It is known that body size correlates with many other life history traits (Peters, 1983) and most of the nematodes in a low cp-class are fast reproducing bacterial feeders (Bongers & Ferris, 1999). Furthermore, the nematode stains also displayed low P values in several cases ($0.05 > P > 8.33 \times 10^{-4}$) and it stands to reason that nematodes with a permeable cuticle are more exposed to pollutants in their body than nematodes with a more or less impermeable cuticle.

Several measures can be taken to improve the chances of finding significant correlations. First of all the amount of data from field studies should be increased by analysing substantially larger numbers of individuals per sample ($\gg$150 individuals per samples) and by repeatedly sampling over a period of time. This would, at least partially, alleviate the problem of genera not being found in all treatments, resulting in RDA values being calculated for more genera. The development of molecular identification methods would be a great help in increasing the number of nematodes analysed per sample by a hundred-fold or more (Holterman *et al.*, 2008). Another possibility would be to identify the nematodes down to species level instead of genus level so that traits such as body size and relative gonad size would no longer have to be averaged over the species in a genus. However, this would require a substantial extra effort when identifying nematodes through light microscopy, and it is unlikely that molecular identification will be developed to the level of species identification of the entire nematode fauna in the near future. Furthermore, certain groups of nematodes, such as the Rhabditidae and members of the Dorylaimida, are notoriously difficult to identify, even at the genus level.

Perhaps most important is the acquisition of the missing data for the species traits, especially for the data on cuticle permeability. These experiments require living, freshly collected and identified nematodes from field samples (most nematodes are considered to be unculturable), and hence it is no surprise that the data were incomplete. Filling in the missing data and perhaps combining the results of the different stains into an index may prove to be more informative. Confirmation of some of the more speculative data – the feeding types and reproductive modes of certain genera – is also important in this regard. More importantly though, filling in the missing data means that the correlations would no longer have be tested pair-wise, but could be tested simultaneously in a single analysis. This would obviate the need for a correction for the effects of multiple pair-wise testing, such as a Bonferroni correction, which in this case study lowered the significance level from 0.05 to 0.00083.

Conclusion

As a first step towards a more objective cp-classification and to refine the Maturity Index from a family level to genus level, the traits relevant to tolerance for environmental disturbance have to be identified. We have laid out a framework to identify traits correlated with stress tolerance while taking into account the effects of phylogeny on the data. Accounting for the affects of phylogeny was demonstrated to be very important. Unfortunately, our approach is hampered by incomplete data, especially on the permeability of the cuticle and the tolerance for stress (as represented by the RDA values). Improving on the amount of available data may lead to more significant results and the identification of traits relevant to stress tolerance in the future.

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Appendix A. *GenBank accessions of the SSU rDNA sequences used in this study.*

Species	Accession No.	Species	Accession No.
Achromadora ruricola	AY593941	*Meloidogyne fallax*	AY593895
Acrobeles complexus	AY284671	*Merlinius brevidens*	AY284597
Acrobeloides apiculatus	AY284673	*Mesodorylaimus centrocercus*	AY284799
Acrostichus halicti	U61759	*Mesorhabditis spiculigera*	AF083016
Alaimus parvus	AY284738	*Metateratocephalus crassidens*	AY284686
Anaplectus grandepapillatus	AY284697	*Monhystera riemanni*	AY593938
Aphelenchoides bicaudatus	AY284643	*Mononchoides striatus*	AY593924
Aphelenchus avenae	AY284639	*Neodolichorhynchus lamelliferus*	AY284598
Aporcelaimellus obtusicaudatus	AY284811	*Nothotylenchus acris*	AY593914
Bastiania gracilis	AY284725	*Nygolaimus* cf. *brachyuris*	AY284770
Bitylenchus dubius	AY284601	*Panagrolaimus subelongatus*	AY284681
Boleodorus thylactus	AY593915	*Paramphidelus hortensis*	AY284739
Bursaphelenchus mucronatus	AY284648	*Paratrichodorus anemones*	AF036600
Cephalenchus hexalineatus	AY284594	*Paratylenchus straeleni*	AY284630
Cephalobus persegnis	AY284662	*Pellioditis mediterranea*	AF083020
Cervidellus alutus	AF202152	*Pelodera teres*	AF083002
Chiloplacus propinquus	AY284677	*Plectus acuminatus*	AF037628
Chordodes morgani	AF036639	*Poikilolaimus oxycerca*	AF083023
Clarkus papillatus	AY284748	*Pratylenchoides ritteri*	AJ966497
Coomansus parvus	AY284766	*Pratylenchus thornei*	AY284612
Coslenchus costatus	AY284581	*Priapulus caudatus*	Z38009
Cruznema tripartita	U73449	*Prionchulus punctatus*	AY284746
Cylindrolaimus communis	AY593939	*Prismatolaimus dolichurus*	AY593957
Deladenus siricidicola	AY633447	*Pristionchus lheritieri*	AF036640
Diphtherophora obesa	AY284838	*Prodorylaimus mas*	AY593946
Diplogaster rivalis		*Protorhabditis* sp.	AF083001
Diplogastrid nematode	AY284689	*Pseudhalenchus minutus*	AY284638
Ditylenchus dipsaci	AY593911	*Psilenchus* cf. *hilarulus*	AY284593
Dorylaimoides micoletzkyi	AY284830	*Rhabditella axei*	AY284654
Drilocephalobus sp.	AY284680	*Rhabditis blumi*	U13935
Ecphyadophora tenuissima		*Rhabditoides regina*	AF082997
Enchodelus sp.	AY284792	*Rotylenchus robustus*	AJ966503
Eucephalobus striatus	AY284666	*Seinura* sp.	AY284651
Eudorylaimus carteri	AJ966484	*Teratocephalus terrestris*	AY284683
Eumonhystera filiformis	AY593937	*Teratorhabditis synpapillata*	AF083015
Euteratocephalus palustris	AY284684	*Thonus* cf. *circulifer*	AY284795
Filenchus filiformis	AY284592	*Thornia steatopyga*	AY284787
Gordius aquaticus	X80233	*Trichodorus variopapillatus*	AY284841
Helicotylenchus pseudorobustus	AY284606	*Tripyla* cf. *filicaudata*	AY284730
Heterocephalobus elongatus	AY284668	*Tylencholaimus mirabilis*	EF207253
Lelenchus leptosoma	AY284584	*Tylenchus davainei*	AY284588
Malenchus andrassyi	AY284587	*Zygotylenchus guevarai*	AF442189