Earthworm species of the genus *Eisenia* can be phenotypically differentiated by metabolic profiling

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Abstract The universality of low molecular weight metabolites allows rapid and straightforward investigation of the biochemistry of genetically uncharacterised species. Thus ex vivo metabolic profiling in combination with multivariate data analysis (metabonomics) offers great potential in comparative biology. Here we present the first use of high resolution nuclear magnetic resonance (NMR) spectroscopy to distinguish closely related animal species via their metabolic phenotype (metabotype). We have profiled the three *Eisenia* (Oligochaeta, Lumbricidae) species *Eisenia fetida*, *Eisenia andrei* and *Eisenia veneta* using tissue extracts and coelomic fluid analysis. The low molecular weight biochemical profiles of tissue extracts were highly conserved for all three species, with *E. fetida* and *E. andrei* being more similar to each other than to *E. veneta*. However the metabolic profiles of the coelomic fluid of the different species were highly distinctive – the NMR spectra allowed unequivocal identification of species. Multivariate statistics were also used to quantify these spectral differences and to enable simplified graphical visualisation of species similarity. These results show that two morphologically undistinguishable species (*E. fetida* and *E. andrei*) differ markedly in their biochemical profiles despite apparently occupying the same ecological niche, and indicate that metabolic phenotype profiling can be used as a powerful functional genomics tool. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Nuclear magnetic resonance spectroscopy; Metabolic profiling; Functional genomics; Chemotaxonomy; Phenotype; *Eisenia*

1. Introduction

Soil invertebrates such as earthworms are appropriate models in which to study evolutionary problems. Their limited speed of dispersion (approximately 10 m per year unaided [1]) means that locally isolated populations may develop, and, if there is sufficient selection pressure and local niche variability, such isolation is likely to result in speciation [2]. Understanding of the nature and extent of earthworm speciation is currently hindered by taxonomic issues. Earthworm taxonomy currently relies primarily on examination of external characteristics, such as position of the sexual organs, form of the prostomium, and pattern of the setae [3]. However, because there are few external visible morphological characteristics, it is likely that the true number of species is underestimated based on these methods alone [4]. Hence, examination of phenotypic differences at the biochemical level has been used to confirm the existence of speciation in morphologically 'challenging' taxa. '*Eisenia fetida*' (Savigny) possesses two colour variants which are otherwise morphologically indistinguishable. These were recognised as potential species by André [5], but gel electrophoresis of proteins was required in order to demonstrate that these were indeed two different species, as shown by the distribution of esterases [6–8]. These are now formally classified as *E. fetida* and *Eisenia andrei* (Bouché) [3]. There is an ongoing interest in the extent of interrelatedness and phenotypic differences between two such similar species, and the consequent genetic and ecological significance [9–11].

Chemotaxonomic analysis is an alternative approach for the identification of phenotypic differences between species that is commonly applied for plants and for microbes, yet is practically unused for animals. Biochemical profiles, or 'fingerprints', of low molecular weight metabolites such as polar lipids, fatty acids, quinones, sugars, polar acids, and polyamines can be employed for species differentiation [12]. Chemotaxonomy in plants and microbes has most often used chromatographic and mass spectrometric techniques for analysis of particular metabolite groups. An alternative to these methods is to use high resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy, which can give a non-selective but specific analysis of the low molecular weight metabolites of a biological sample that requires neither physical separation nor derivatisation of the analytes [13,14]. Combination of metabolite profiling with multivariate data interpretation and pattern recognition, or metabonomics [15], can be used to detect and quantify very subtle shifts between profiles. For example, a recent study has shown that two phenotypically normal strains of laboratory mouse can be distinguished on the basis of the ^{1}H NMR spectroscopic profiles of their excreted urinary metabolites [16]. An earthworm biofluid of interest is the coelomic fluid, which supports many important biological and biochemical processes [17]. The coelomic fluid of *Eisenia veneta* (Rosa) has recently been characterised at the metabolite level, and shown to contain a range of metabolites that would be of potential use in biochemical studies [18].

In this paper, we demonstrate the utility of NMR-based

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metabonomics as a chemotaxonomic tool to confirm the taxonomic separation of two earthworm species. In this study the closely related and morphologically non-differentiated species *E. fetida* and *E. andrei* are compared to the less closely related *E. veneta* [8] using whole-worm tissue extract and coelomic fluid metabolite profiles.

2. Materials and methods

2.1. Earthworms

E. veneta were taken from a culture maintained at CEH Monks Wood, UK. Three separate sub-populations of each of *E. fetida* and *E. andrei* were obtained; the source of each sub-population and number of individual worms taken from each is given in Table 1. Fully clitellate adults (*E. veneta*) and a mixture of clitellate adults and large juveniles (*E. andrei* and *E. veneta*) were allowed to void their gut contents on moist filter paper for 48 h in the dark at 15°C, followed by sample preparation, described below.

2.2. Preparation of samples for NMR spectroscopy: coelomic fluid

Coelomic fluid was extracted by electric stimulation. A worm was placed in 0.5 ml of 0.1% NaCl solution in a Petri dish, and a potential difference of 9 V applied across the worm for 20–30 s, causing it to extrude coelomic fluid and coelomocytes. The fluid was then quick-frozen in liquid nitrogen and stored at −40°C until use. After thawing and centrifugation (10 min, 17 000×*g*) to remove coelomocytes and suspended solids, the supernatant (440 μl) was added to 60 μl of ^2H_2O which contained NaN_3 (2000 mg l^{-1}), to suppress microbial contamination, and sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-propionate (TSP; 500 mg l^{-1}), and to 100 μl of 400 mM sodium phosphate buffer, pH 7.4. The ^2H_2O provided a field-frequency lock for the spectrometer and the TSP was used as a chemical shift reference (δ 0.0).

2.3. Preparation of samples for NMR spectroscopy: tissue extracts

Tissue extracts were made only for *E. veneta* and for *E. fetida* and *E. andrei* from sub-population 1, i.e. tissue extracts were not made for *E. fetida* and *E. andrei* from sub-populations 2 and 3. After extraction of coelomic fluid, worms were quick-frozen in liquid nitrogen. They were then homogenised from frozen into 50% v/v acetonitrile/water solution (*E. veneta*: 3 ml; *E. andrei* and *E. fetida*: 1 ml) using a mortar and pestle. The homogenate was then centrifuged (10 min, 17 000×*g*) and the supernatant lyophilised and stored until use. The lyophilised residue was then reconstituted in a 50 mM pH 7.4 sodium phosphate buffer solution made up in 95% v/v ^2H_2O that also contained TSP (5 mg l^{-1}) and NaN_3 (275 mg l^{-1}). Two millilitres of solution was used to reconstitute the residue for *E. veneta* and 1 ml for *E. andrei* and *E. fetida*.

2.4. ^1H NMR spectroscopy

All samples were measured at 300 K and 600 MHz using a Bruker Avance DRX600 spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm broad-band inverse or triple axis inverse probe (coelomic fluid samples) or with a flow-injection probe with a 120 μl flow-cell volume (tissue extracts). Spectra were acquired over a spectral width of 8.4 kHz into 32 K data points, resulting in an acquisition time of 1.95 s with an additional relaxation delay of 1.5 s allowed between free induction decays (FIDs). A standard NOESY-PRESAT pulse sequence (Bruker BioSpin, Rheinstetten, Germany) was used to achieve suppression of the water resonance. 256 FIDs were summed for the coelomic fluid samples and 128 FIDs were summed for the tissue extracts. The summed data were then zero-filled to 64 K data points and an exponential apodisation function equivalent to 0.3 Hz line broadening applied, followed by Fourier transformation. Spectra were referenced to TSP at δ 0.0 and manually corrected for phase and baseline prior to data analysis.

2.5. Data analysis

Spectra were converted to numerical data suitable for multivariate analysis using the software package AMIX 2.1 (Bruker Analytik, Rheinstetten, Germany), which divides the spectrum into a number of chemical shift regions of equal width, or segments, and integrates the total signal within each segment. For the tissue extracts spectra were quantified between δ 9.0–5.0 and δ 4.5–0.5, i.e. excluding the area around the suppressed water resonance centred at δ 4.70, and for the coelomic fluid samples (set 1 only, for *E. veneta* and sub-population 1 for *E. andrei* and *E. fetida*) between δ 9.5–5.5 and δ 4.5–0.5. A segment width of 0.04 ppm was used, resulting in 200 variables per spectrum for both coelomic fluid and tissue extract samples. For the second set of samples (i.e. including all sub-populations of worms) the aromatic region only of the coelomic fluid spectra was quantified. The spectra were divided into 0.01 ppm regions between δ 9.5 and 6.5, giving 300 variables per spectrum.

Principal components analysis (PCA) was carried out using Simca-P 8.0 (Umetrics, Umeå, Sweden) on data normalised to total signal intensity by expressing each datum as a percentage of the average value for a row (individual spectrum). This is analogous to normalising concentrations by bodyweight, and the purpose is to prevent separation of samples in multivariate space on the basis of total signal intensity, which will be related to the size of the worm. The effect of different data scaling was investigated using both data that were mean-centred but unscaled and that were mean-centred and scaled to unit variance. Hierarchical cluster analysis was carried out in S-PLUS 2000 (Insightful, Bagshot, UK) and used the principal component (PC) scores for the first 30 PCs (data scaled to unit variance) as input, which accounted for 85% of the variance in the data (adjusted R^2). The standardised input variables were analysed using a Euclidean metric and average linkages.

3. Results

We carried out initial experiments on one set (sub-population) of each worm species, to determine if there were any observable differences. Representative sets of six spectra, chosen at random, of the three different species are shown in Fig. 1 (for the tissue extracts) and in Fig. 2 (for the coelomic fluid samples). While there were clear inter-individual variations between spectra for the tissue extracts (Fig. 1), particularly in the maltose resonance visible at δ 5.40, there were no apparent consistent species differences observed from direct spectral inspection – the same major metabolites are present in the tissue extracts for all of the different species. In contrast, the coelomic fluid spectra show unequivocal and consistent species differences (Fig. 2). This is particularly marked in the high frequency region (δ 9.5–6.5) of the spec-

Table 1
Source of different sub-populations of earthworms used

Sub-population	Source	Numbers
E. veneta	culture maintained at CEH Monks Wood	10
E. fetida 1	Blades Biological[a]	24
E. andrei 1	Blades Biological[a]	18
E. fetida 2	hand-collected from wild[b]	10
E. andrei 2	culture maintained at CEH Monks Wood	12
E. fetida 3	bait shop[c]	13
E. andrei 3	bait shop[c]	10

[a]Blades Biological Supplies, Edenbridge, UK.
[b]Site near Avonmouth, UK. Ordnance Survey grid reference ST568826.
[c]Stan Jay, Huntingdon, UK. Mixed culture of *E. fetida* and *E. andrei* supplied, and individual colour morphs sorted by hand.

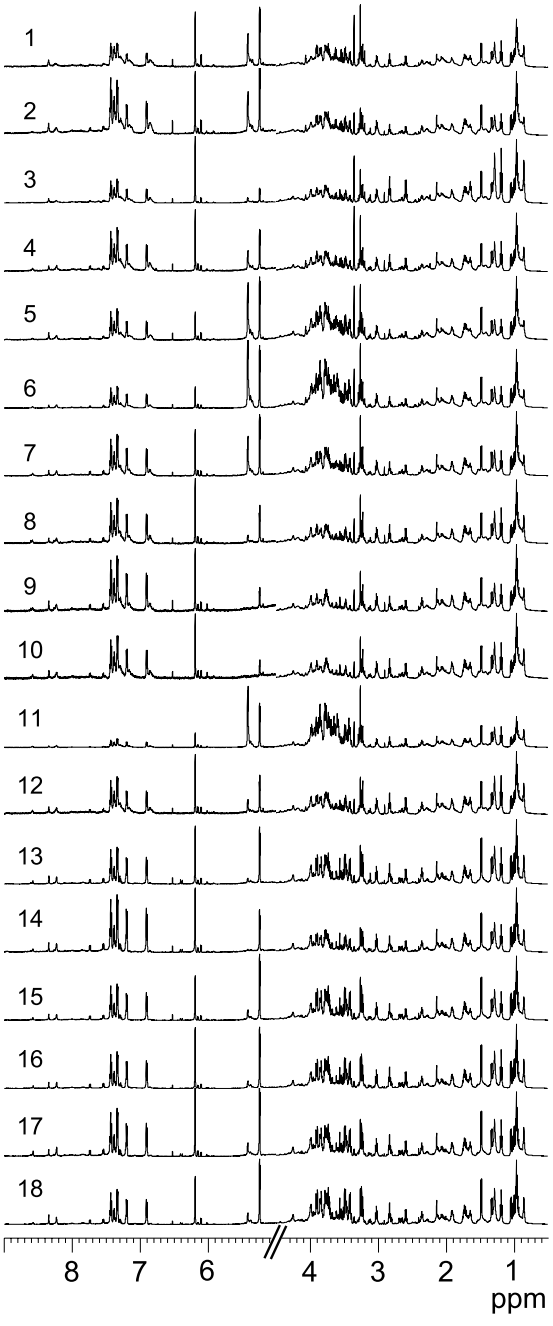

Fig. 1. 600 MHz ^{1}H NMR spectra of tissue extracts of three earthworm species, genus *Eisenia*. Left hand side: aromatic region. Right hand side: aliphatic region. The vertical scale for the aromatic region has been expanded relative to the aliphatic region to permit clearer viewing of the resonances. Spectra 1–6: *E. fetida*. Spectra 7–12: *E. andrei*. Spectra 13–18: *E. veneta*.

trum. For instance, one of these assigned metabolites is nicotinamide mononucleotide [18], which is present only in *E. veneta* coelomic fluid, and not found in the coelomic fluid of the other two species.

In order to further assess the level of metabolic differences between species, PCA was undertaken on the two types of sample. PCA of the tissue extract spectra of the three sets of species together shows that *E. veneta* is completely distinguished from the other two species based on spectral profiles in PC 2. There is some degree of difference between *E. fetida* and *E. andrei* within PCs 1 and 2, but the two species clusters overlap considerably within PC space (Fig. 3A). It was found that animal body weight (and by implication development stage, as the smallest worms were the juveniles) did not affect the spectral profiles: there was no correlation between body weight and PC scores (PCs 1 and 2, PCA of individual species data analysed separately) for any species in either matrix – P was >0.2 in all cases. Furthermore there were no apparent differences between the spectral profiles of adult and juvenile worms.

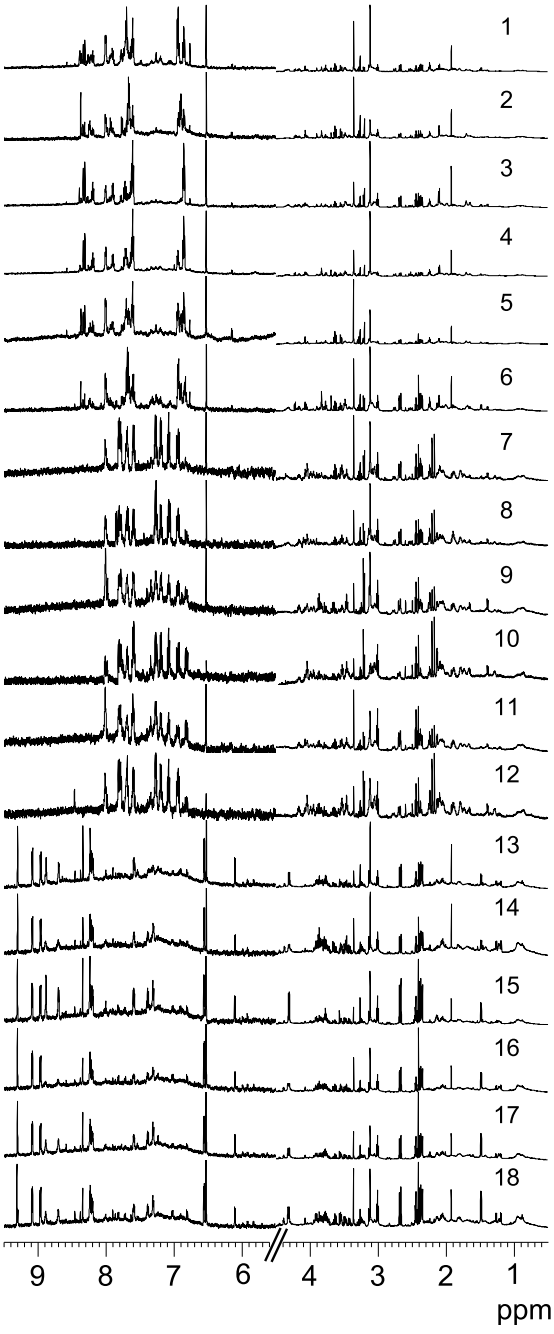

Fig. 2. 600 MHz ^{1}H NMR spectra of coelomic fluid of three earthworm species, genus *Eisenia*. Left hand side: aromatic region. Right hand side: aliphatic region. The vertical scale for the aromatic region has been expanded relative to the aliphatic region to permit clearer viewing of the resonances. Spectra 1–6: *E. fetida*. Spectra 7–12: *E. andrei*. Spectra 13–18: *E. veneta*.

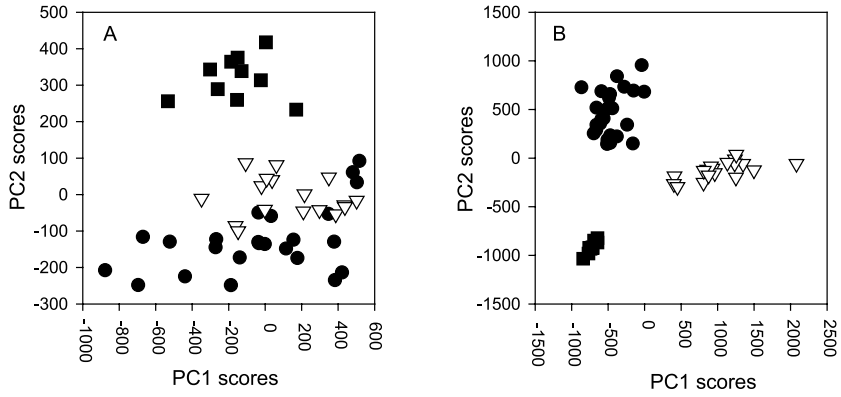

Fig. 3. PCA score plots of data from ^{1}H NMR spectra (data mean-centred but not scaled). A: Tissue extract spectral data, 74% of variance in dataset explained by PCs 1 and 2. B: Coelomic fluid spectral data, 67% of variance in dataset explained by PCs 1 and 2. ● *E. fetida*, ▽ *E. andrei*, ■ *E. veneta*.

For the coelomic fluid samples, species differences between the biochemical profiles species were more pronounced. All three species form separate clusters with no overlap within PCs 1 and 2 (Fig. 3B). When the *E. fetida*/*E. andrei* data were analysed separately they formed two distinct clusters separated along PC 1, i.e. the largest proportion of the variance within these data is explained by species difference (data not shown). Visual inspection of the spectra confirms that the profile for each species is readily distinguishable from the other two (Fig. 2) based on the coelomic fluid results.

These species differences are based upon individuals taken from a single sub-population. This means that it is not possible to differentiate true species difference from the contribution of possible confounding environmental factors (for instance food supply, overall water status). To confirm the species difference initially observed, we profiled further two sub-populations of *E. fetida* and *E. andrei* with different environmental histories (Table 1). This gave a total of three different sub-populations tested for these two species. Only *E. fetida* and *E. andrei* were used in this second set of tests as this gave a more stringent test of metabolite-level species difference than would inclusion of the more distantly related and morphologically less similar species *E. veneta*. Spectra were acquired for the coelomic fluid only, as this biofluid showed the most marked species differences in the initial study. Detailed data analysis was then based on the high frequency spectral region (δ 9.5–6.5). The use of this restricted spectral region was because the greatest species differences for the initial set of sub-populations were observed in aromatic metabolites (Fig. 2), and we were testing the specific hypothesis that species differences would be conserved between sub-populations.

3.1. All sub-populations

Analysis of coelomic fluid profiles for the two additional strains indicated that the characteristic species differences were maintained for all sub-populations. For all worms, it was possible to assign each spectrum a priori to the correct species without knowledge of the sample class. For purposes of visualisation, multivariate methods are here used to give a graphical representation. PCA, an unsupervised and hence unbiased technique, shows that the spectra fall into two natural clusters with no overlap. These separate individuals exactly according to species (Fig. 4A). A spectral width of 6 Hz resolution rather than 24 Hz resolution was used for data reduction in order to improve distinction between aromatic metabolites; however PCA using 24 Hz resolution also separated the species along PC 1 (not shown). Furthermore, using

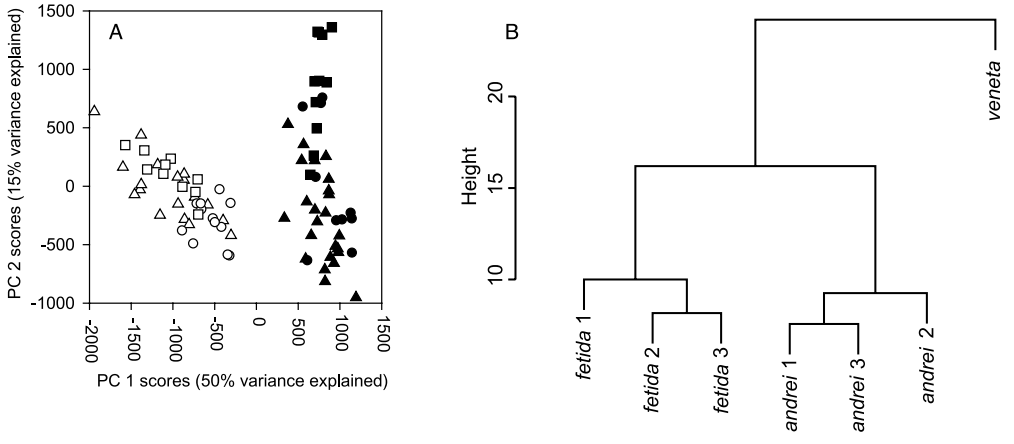

Fig. 4. A: PCA score plot for PC 1 against PC 2 of aromatic region of ^{1}H NMR spectral data (mean-centred but not otherwise scaled) of *E. fetida* and *E. andrei* taken from three different sub-populations. Filled symbols: *E. fetida*. Open symbols: *E. andrei*. Sub-population 1: triangles. Sub-population 2: circles. Sub-population 3: squares. B: Hierarchical cluster analysis showing relative similarity of sub-populations. The data for *E. veneta* have been included in the analysis.

hierarchical cluster analysis, the relative similarity of the sub-populations as a whole can be presented as a dendrogram (Fig. 4B). From this it is clear that the differences between sub-populations are much smaller than the differences between species.

4. Discussion

The traditional taxonomic classification of earthworms, based on morphological characteristics, is known to underestimate the true number of earthworm species [4]. Earthworms possess relatively few distinct morphological characters useful for taxonomy, and consequently earthworm taxa are divided into multispecies 'complexes', such as the *Aporrectodea caliginosa* complex.

Bøgh [19] reported that gel electrophoresis of three to four different enzymes was potentially a suitable technique for routine taxonomic classification of earthworms. Some species, such as the *A. caliginosa* complex, showed high variability, as would be expected if these are in fact a composite of several species. Species identification based on only a small number of proteins clearly requires a high degree of care and cross-comparison between different populations, and in addition may be subject to technical limitations when the differences in migration are slight or the gels are not technically perfect. Modern developments in proteomics may circumvent many of these problems, and are likely to lead to enormous advances in comparative biology [20]. However, proteomic analysis is not a routine tool for taxonomic studies and is time-consuming and labour-intensive. In addition, it may be difficult to distinguish proteome-level changes caused by habitation of different ecotypes or by unrelated environmental impacts from inherent taxonomic differences. Metabolic regulation of enzymatic pathways means that enzyme activity is not closely related to protein concentration per se, and there are thus likely to be problems with inferring differences in biochemical function from proteomic or genomic data alone [21,22].

High resolution ^{1}H NMR spectroscopy provides information that may be complementary to the data gained from genomic and/or proteomic studies. We have shown in the current study that this can be used to give valuable information about species differences, even when the test case is two closely related species. Because the analysis is non-selective, there are no 'target' groups of analytes and hence no potential discrimination against individual compounds or compound classes. This means that even previously uncharacterised analytes can be detected. The differences between species showed up most clearly in the high frequency region of the NMR spectra of coelomic fluid samples (between δ 6.5 and δ 9.5). Some of the compounds characteristic of a particular species with resonances in that region have been assigned, e.g. nicotinamide mononucleotide in *E. veneta* [18]. Although several aromatic metabolites have not yet been assigned, particularly for *E. fetida* and *E. andrei*, valid information on biochemical differences between species is still obtained [23], as in this case.

The species differences were much clearer in the coelomic fluid biochemical profiles than for the whole-worm tissue extracts, even though the coelom receives excretory products of metabolism prior to their excretion through nephridiopores [1], and thus might be expected to contain a generally similar pattern of metabolites across different species. The results obtained in this study imply that there is a considerable degree of homeostatic control over the major low molecular weight metabolites in coelomic fluid. The metabolite species differences that were detected may well have implications for ecotoxicity tests involving *E. fetida* or *E. andrei*. The OECD earthworm acute toxicity test, required for all high production volume chemicals, allows either of the two species to be used [24]. These data show not only that these are two distinct species, as was known from previous studies, but that there are clear differences between them at the metabolic control level, which might affect their toxicologic response to xenobiotics. However, the information obtained from the tissue extract spectra might well also prove to be useful for delineating evolutionary affinities. To test this, a wider number of species would need to be studied in order to compare metabolic profile data with accepted taxonomies.

4.1. Implications of Eisenia species differences

E. fetida and *E. andrei* are not only closely related but are very similar ecotypes (epigeic) which fill almost the same ecological niche, although *E. andrei* may have slightly higher rates of growth and cocoon production [9,10]. Consequently they have been used as a model species pair to test evolutionary and ecological hypotheses at a molecular level [11]. We have shown for the first time that not only are there clear biochemical species differences, but these are so extensive that the metabolites found in coelomic fluid are remarkably dissimilar. The metabolite differences are not just a matter of degree (i.e. consistently varying concentrations of the same metabolites), but of kind: different metabolites, some of which are unknown, are restricted to the different species. Identification of these metabolites will contribute to our understanding of soil invertebrate biology and comparative biochemistry by delineating functional differences at the biochemical level. ^{1}H NMR spectroscopy could be used to help unravel species complexes in earthworms and other soil invertebrates. The data produced give complementary information on a different level of biological organisation to other post-genomic (transcriptomic or proteomic) methods – some differences may be more obvious at the metabolomic level. Another potential application would be the investigation of the biochemical profiles of sexually immature earthworms, which cannot currently be assigned to species level using standard keys [3].

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