European Journal of Soil Biology 74 (2016) 76–80

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Isolation and characterization of eight polymorphic microsatellites markers for the earthworm *Lumbricus terrestris*

Dima Souleman [a, b, c], Fabien Grumiaux [a, c, d], Hélène Frérot [a, b], Franck Vandenbulcke [a, c, *], Maxime Pauwels [a, b]

[a] Université de Lille, F-59000 Lille, France
[b] Laboratoire Evolution, Ecologie et Paléontologie, UMR CNRS 8198, Bâtiment SN2, F-59655 Villeneuve d'Ascq cedex France
[c] LGCgE, EA4515, Univ Lille1, Fonctionnement des Ecosystèmes Terrestres Anthropisés, SN3, Cité Scientifique, F-59655 Villeneuve d'Ascq, France
[d] Université Lille Nord de France, Ecole Supérieure du Professorat et de l'Education (ESPE), Site d'Arras, 7 bis rue Raoul François, BP 30927, F-62022 Arras Cedex, France

ARTICLE INFO

Article history:
Received 8 September 2015
Received in revised form
17 March 2016
Accepted 22 March 2016

Handling Editor: C.C. Tebbe

Keywords:
Lumbricus terrestris
Microsatellites
Genetic diversity
Heterozygosity

ABSTRACT

The earthworm *Lumbricus terrestris*, which is an ecologically important sentinel species for soils widely distributed throughout Northern Europe, has never been involved in deep population genetic studies. In order to promote future studies, we report here the isolation and characterization of 8 new polymorphic microsatellite loci isolated from sequencing of a microsatellite-enriched genomic library. Microsatellite markers were tested on 192 field collected individuals. Allelic richness per locus varied from 4.921 to 24.876, with a mean value of 6.125 alleles per locus. The observed heterozygosity (Ho) ranged from 0.132 to 0.839, whereas the expected heterozygosity (HE) ranged from 0.407 to 0.926. Developed markers should be helpful for accurate estimation of population structure, dispersal and gene flow among populations.

1. Introduction

In terrestrial ecology, one of the most studied zoological groups is the Annelida, Oligochaeta group. These organisms play key roles in most continental ecosystems, represent an important part of the soil macrofauna, and are integral to the maintenance of the structure and the fertility of soils. By drilling, earthworm can create both horizontal and vertical burrows, which can be very deep in the soil. These burrows are pores through oxygen and water can enter and carbon dioxide can leave the soil. They increase soil fertility by mixing the soil layers and are considered as ecosystem engineers by agronomists [1,2]. Earthworms from the Lumbricidae family are also good candidates for biomonitoring [3,4] because they are largely distributed, easy to sample and may be used to assess the effect of soil contamination through the measure of biomarkers [5,6]. Usually, earthworms are categorized into three ecological groups, i.e. epigeic, endogeic and anecic species. This categorization is based on the locality inside the soil and on the burrowing attitude [7]. Among Lumbricidae, the species *Lumbricus terrestris* is typically an anecic that lives in permanent burrows and come to the surface to feed. It is one of the largest species among Northern Europe earthworms. It is also one of the easiest to identify based on external characteristics when exhibiting a clitellum (breeding form). As a result, *L. terrestris* is an attractive sentinel species which is often used (possibly in combination with other earthworms species) to assess environmental impact of soil contamination, land-use change and fragmentation of habitats [7,8].

In comparison with ecological properties, the population genetic of the species has been poorly investigated and we know almost nothing about the distribution of genetic diversity within the species. This situation is noticeably illustrated by the recent discovery, through sequencing of the Cytochrome Oxidase I gene (COI) that, in Europe, *L. terrestris* likely includes a cryptic species named *L. herculeus* [9]. This situation may result from a lack of appropriate molecular tools. In this context, the development of molecular genotyping markers, such as microsatellites, would be

* Corresponding author. Université de Lille, Sciences et Technologies, Laboratoire de Génie Civil et géo-Environnement, LGCgE EA4515, Bât. SN3 – Bureau 03 59655 Villeneuve d'Ascq, France.
E-mail address: franck.vandenbulcke@univ-lille1.fr (F. Vandenbulcke).
URL: http://www.lgcge.fr

http://dx.doi.org/10.1016/j.ejsobi.2016.03.009
1164-5563/

very useful for accurate estimation of population genetic structure, levels of within-population genetic diversity or among-population gene flow. Microsatellites are neutral, codominant, highly polymorphic markers. They have been shown as highly suitable markers for population genetics [10]. Some microsatellite markers have been previously developed for other earthworm species: eight markers in *Lumbricus rubellus* [11], sixteen markers in *Eisenia fetida* [12], eight markers in *Allolobophora chlorotica* [13] and eleven markers in *Aporrectodea longa* [14]. Ten markers have also been proposed for *L. terrestris* [15]. Surprisingly, however, developers used only three of them in a subsequent study [16]. We ourselves failed in obtaining satisfying results testing these markers on individuals we investigated (data not shown).

In this context, the aim of our study was thus to use a recently developed high-throughput method for isolating microsatellite markers [17] to increase the number of microsatellite markers available for population genetic studies of *L. terrestris*. We also performed multiplexing of selected markers to make high-throughput genotyping easier. We tested the efficiency and level of polymorphism of proposed markers in a high number of individuals collected from the Nord-Pas de Calais Region in France.

2. Materials and methods

2.1. Sampling

Twelve European individuals of *L. terrestris* have been used for the development of a microsatellite-enriched genomic library. They were collected in northern Nord-Pas de Calais Region (50°52′5.92″N/1°51′21.18″E and 50°51′40.92″/1°51′5.55″), southern France (Valence 44°58′44.4″N/4°55′37.199″E) and Avignon 43°54′57.6″N/4°52′40.8″E) and in southern Poland (50°01′38.0″N/20°15′02.9″E). Allyl isothiocyanate (AITC) was used to extract earthworms from their subterranean burrows [18]. Collecting individuals in different parts of the species distribution in Europe was supposed to favor the selection of microsatellite loci whose mutational dynamics does not depend on any reference genotype. This may help to develop markers whose levels of polymorphisms are sufficient at various geographic scales.

Selection of microsatellites and validation of the selected microsatellites were performed on 192 individuals collected from 44 sampling sites Nord-Pas de Calais Region, France (maximum distance among sites: 3 km, see Table S1). Each sample site was made of 1 m^2 quadrat. AITC was used to extract earthworms. Worms have been identified with the taxonomic key of Bouché (1972) [19] using characteristics such as the prostomium shape, the shape and the location of the clitellum (saddle-shaped on segments 32–37) and the location of the tuberculata pubertatis (segments 33–36).

2.2. DNA extraction

Earthworms were cleaned and stored in ethanol 96% until use. Total genomic DNA was isolated using NucleoSpin® 96 Tissue (Macherey Nagel) kits that allows for the purification of multiples of 96 samples. Small part of the end of each earthworm tail was used to extract genomic DNA. Then, DNA extracts were 1:20 diluted before use.

2.3. COI barcoding and analysis

Given that two cryptic species were identified in the morpho-species *L. terrestris* [9], the taxonomical identity of our samples was checked using COI sequencing. COI was amplified using universal DNA primers LCO1490 and HCO2198 [20]. However, in order to ensure that amplification failure was not due to primer-template mismatches, an additional amplification was performed using redesigned amplification primers (LCO1490_Lt_F: ACTCAACTAATCACAAAGATATTGG; HCO2198_Lt_R: TATACTTCTGGGTGACCAAAGAATCA) from the complete *L. terrestris* mitochondrial genome sequence (GenBank NC001673.1). Standard protocols were used for amplification and sequencing reactions [9]. The Millipore-Multiscreen purification kit was used for amplicon purification and PCR fragments were sequenced using the BigDye TerminatorKit 3.1 (Applied Biosystems) and run on an ABI-3130 capillary sequencer (Applied Biosystems). All sequences were checked manually with CodonCode Aligner Version 5.1.4. Sequence analysis was performed as suggested in Ref. James et al. [9].

2.4. Microsatellite library

A stoichiometric mixture of the 12 European DNAs was performed to produce a microsatellite enriched genomic library. Library was constructed by the Genoscreen Company (Lille) by coupling multiplex microsatellite enrichment isolation techniques with the 454 GS-FLX Titanium pyrosequencing [17]. Enrichment was performed using probes containing the following microsatellite motifs: TG, TC, AAC, AAG, AGG, ACG, ACAT, ACTC. Overall, 5931 sequences containing a single microsatellite motif were found. This allows the identification of 244 independent microsatellite loci candidates. Amplification primer pairs were designed for each in silico.

2.5. Microsatellite amplification

For 20 microsatellite loci, one primer pair was chosen for *in vitro* Polymerase chain reaction (PCR) amplification trials. Microsatellite loci and primer pairs were chosen depending on the repeated motif (only di-nucleotides and tri-nucleotides motifs were selected to avoid very large allele size range), maximizing the number of repeats of the motif observed in the library (considering that loci with higher number may also show higher allelic richness in natural populations) and selecting for expected amplicon size so as to allow multiplexing. Amplification trials were first performed on a subset of individuals sampled in the Nord − Pas de Calais region (n = 16) so as to ensure that selected microsatellite markers show the highest rate of positive results (to limit the possibility of null alleles), high PCR amplification yields (estimated through peak levels), low allele drop-out, and no multiple peak profiles (stutter artifacts). We used a four-step procedure for validation. Firstly, PCR amplifications were conducted in 15 μl reaction including 0.3 μl of both forward and reverse primers (10 mM), 1.5 μl of 10X DreamTaq Buffer (containing 20 mM Tris-HCl (pH 8.0),1 mM DTT, 0.1 mM EDTA, 100 mM KCl, stabilizing agent and 50% (v/v) glycerol), 1.2 μl of dNTPs (2.5 mM), 0.3 μl of BSA, 0.075 μl DreamTaq polymerase and 3 μl of diluted DNA. PCR were designed with initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C denaturation, 1 min at 60 °C annealing, 1 min at 72 °C extension, followed by a final extension of 5 min at 72 °C. PCR products (yield, amplicon size) were checked using gel electrophoresis in 1% agarose gels and ethidium bromide staining. GeneRulerTM 100 bp DNA ladder was used to determine fragment size. Secondly, the 20 markers were tested separately using labeled forward primers. Then, 1.5 μl of each PCR product was mixed with 9.75 μl of formamide (Applied Biosystems) and 0.25 μl of Genescan-500 LIZ size standard, and analyzed through electrophoresis on an ABI3130 Genetic Analyzer (Applied Biosystem). Only eight microsatellite markers show both consistent amplification results and genetic variation among individuals. During this step, five microsatellite markers available in the literature for *L. terrestris* [15,16] and that could be multiplexed

were also tested. Only one of them (LT163) gave satisfying results. Thirdly, the eight microsatellite markers were multiplexed and co-amplified in two subsets of loci called Mplx1, Mplx2. Each multiplex contains four markers which are directly labeled on the forward primer with different fluorescent loading dye (6-FAM, NED, PET, or VIC, Applied Biosystems (Table 1). PCRs were performed for multiplex adjust under the following conditions: denaturation step at 95 °C, for 15 min, 5 cycles including (45 s at 95 °C, 5 min at 68 °C, and 60 s at 72 °C), 5 cycles including (45 s at 95 °C, 1 min at 58 °C, and 60 s at 72 °C), 20 cycles including (45 s at 95 °C, 30 s at 47 °C, and 60 s at 72 °C), and extra extension step of 30 min at 60 °C, using QIAGEN Multiplex PCR Kit. After, 1.5 μl of PCR product was mixed with 9.75 μl of formamide (Applied Biosystems) and 0.25 μl of Genescan-500 LIZ size standard, and analyzed by DNA sequencer (Applied Biosystem). Fourthly, the two developed multiplexes were tested using the same PCR conditions on a set of 192 samples coming from 44 locations in northern France (Nord-Pas de Calais).

In order to discuss the possibility of cross-species transfer of developed microsatellite markers, the latter were also tested on a set of 16 genotypes from each of the *Lumbricus castaneus* and *Lumbricus rubellus* species. DNA extraction and amplification procedures were the same as above described.

2.6. Microsatellite data analysis

Fragment analysis and allele size determination were carried out using GENEMAPPER 3.7 software (Applied biosystem). Depending on marker peaks' position and allowing an offset of up to 0.8 bases among peaks at a same position, a set of detected alleles was determined for each locus. The association between genotypes at pairs of loci was tested using the log-likelihood ratio G-statistic in FSTAT version 2.9.3. FSTAT version 2.9.3 was also used to determine genetic diversity parameters such as, the number of alleles and the FIS coefficients per locus. Then null allele frequency for each locus was estimated using the EM algorithm implemented in the FreeNA software [21]. The observed heterozygosity (Ho) and expected heterozygosity (HE) were established for each locus using GeneClasse2 [22] [21].

3. Results and discussion

Over the 192 tested individuals, 145 gave positive COI amplification results (Accession numbers GenBank KU888473–KU888617) and 177 gave positive amplification results for at least one microsatellite marker (Table S1). Individuals that gave negative results for 8 (n = 15), 7 (n = 5) or 6 (n = 7) microsatellite markers were generally not amplified by COI primers (n = 13, n = 5 and n = 3, respectively, see Table S1). However, all COI sequences we obtained, including sequences of individuals with not optimal genotyping results, felt in the *L. terrestris* cluster identified in Ref. James et al. [9] (Fig. S1). This suggests that the cryptic species *L. herculeus* does not exist in the geographic area we sampled and that, whereas developed microsatellite markers appeared to be highly specific (see below), amplification failing is not due to incorrect species identification. As a result, we believe that the absence of amplification for resulted from insufficient quality of DNA after an extended storage period in ethanol 96%.

Statistical analyses using Fstat version 2.9.3 indicate that the eight validated loci are highly polymorphic, especially loci Lt17, Lt9, Lt12, Lt163, Lt3 which exhibited a high level of allelic diversity (Table 1). By means of 192 individuals, a total of 116 alleles were identified overall. The number of alleles ranged from 5 at the locus Lt8 to 25 at the locus Lt9. The tests for genotypic disequilibrium were non-significant for all pairs of loci.

Among the eight loci, two (locus Lt17 and locus Lt163) showed relatively low estimated null allele frequencies. On the other hand, independently of allelic richness, the estimated null allele frequencies were higher at the other six loci. Estimated null allele frequencies may be higher than true frequencies for several reasons related to the population genetic model used for the estimation. Indeed, null allele frequencies are estimated assuming that individuals belongs to a panmictic population and that heterozygote deficiencies from expected proportions are only due to the presence of null alleles. However, given the current absence of knowledge about *L. terrestris* populations genetics, we cannot ensure that sampled individuals belonged to a unique population. Moreover, it is very likely that mating systems of earthworms showing hermaphrodism and assortative mating over short geographic distances differ from panmixia [23]. In general, earthworms are considered as a cross-fertilization hermaphrodite and they are rarely self-fertilizing. However, some species make an exception of this reproduction strategy. For example, spermatozoids of *E. fetida* were able to fertilize ova from the same individual [24]. Besides, genetic diversity may be affected by the presence of parthenogenesis in earthworms. Accordingly, in our study, levels of overall observed heterozygosity were always lower than levels of overall expected heterozygosity (Table 1) and FIT values varied among

Table 1
Identification of 8 polymorphic microsatellite loci for *L. terrestris*. A: alleles number; NA: estimated null allele frequency; H_O: observed heterozygosity; H_T: expected heterozygosity; F_{IT}: fixing index. ***: Significant value.

Locus	PCR product size	Primers	Repeat motif	Loading dye	Percentage of positive results	Allele size range	Most frequent allele size	A	NA	H_O	H_T	F_{IT}
Lt3	127	F: CAAAGATGCGAACAAACGTC R: CGCTTGATACATAATAGCACGG	(ca)8	PET	66%	115–175	129	11	0.25	0.305	0.753	0.596***
Lt8	134	F: TCATCGTCATCGTCCTCATC R: CATCATTATTATCGTGAAAGCGA	(tca)8	VIC	73%	125–189	143	5	0.25	0.187	0.575	0.675***
Lt16	187	F: ACTTGTGCGACACGATTGAA R: TTTCCTCGAGTCTTCTTCGC	(gag)10	6-FAM	86%	169–226	171–177	6	0.28	0.256	0.742	0.655***
Lt19	129	F: CTAAGCAGAACACCTTGGGC R: ACGTTGTCTGGCGTTTTAGC	(gt)7	NED	79%	170–243	204	6	0.22	0.132	0.407	0.674***
Lt17	210	F: TCAAGCGCTCAAAGATGTCA R: TGGAAACTGGTATCTCGGATG	(ca)7	VIC	77%	197–264	211	21	0.04	0.810	0.906	0.107
Lt9	141	F: ACTTGACACAGCGCCAAAG R: CGCTTGATACATAATAGCACGG	(ca)8	NED	68%	110–193	141	25	0.24	0.385	0.846	0.546***
Lt12	212	F: TGCATTGTGAGAGCAATTGTG R: TGAGATATGATAGCGGAGTGAA	(aca)11	6-FAM	79%	190–282	215	19	0.33	0.205	0.803	0.744***
Lt 163	171	F:GCCGGAGCGTTAGGAGCGATAG R:GGATACGCCCGACTCACCACTAA	(tgc)12	PET	81%	138–216	150	23	0.05	0.839	0.926	0.094

markers from 0.094 (Lt163) to 0.744 (Lt12) and were significantly positive for 6 of 8 loci, suggesting a non-panmictic mating system. However, such result may also result from population genetic structure in our sampling area, such as a Wahlund effect [25].

Tests for cross-species amplification of microsatellite markers revealed that they were highly specific to *L. terrestris*. Indeed, except for Lt163, that successfully amplified from 15 *L. castaneus* individuals and Lt3, that successfully amplified from 9 *L. rubellus* individuals, amplification success for other markers concerned less than half of the 16 tested individuals from each species. This specificity interrogates the capacity of developed markers to amplify other *Lumbricus* species, including *L. herculeus*. Indeed, COI barcodes revealed that *L. herculeus* was not included in our study.

The high variability of the microsatellites loci suggests that the developed markers will have a sufficient degree of polymorphism for population genetic studies. The study of population genetic structure can be useful to investigate effective population sizes as well as the respective effects of genetic drift, migration and selection in the evolution of populations. For example, population genetic data allow discussing how genotypes from distinct populations with contrasted ecology are genetically connected. High levels of neutral genetic differentiation would suggest genetic divergence among populations, whereas low levels could reveal a favourable background for the detection of outlier loci involved in local adaptation [26]. Population genetic surveys should be particularly important for species that, like *L. terrestris*, are used as sentinel species in ecotoxicological studies. Indeed, *L. terrestris* naturally occurs in anthropized habitats, such as metal-polluted sites. The evolution of adaptive divergence among metallicolous and non-metallicolous populations cannot be excluded. Such divergence may affect biological parameters that are used as biomarkers to assess the impact of a contaminant on physiology, since divergent genotypes may behave differentially in front of a specific contaminant [5]. This possibility is hardly taken into account in ecotoxicological studies. Currently, in most biomarker trials, various *L. terrestris* genotypes are generally used without any knowledge about their respective evolutionary history, and, *a fortiori*, the possibility of genetic differences in their phenotypic response. Such knowledge should be made available from population genetic data. In addition, from a more fundamental point of view, the availability of microsatellite markers may also be appropriate to discuss the demographic relationships among the two cryptic species that have been recently detected in *L. terrestris* [9].

In the present study, we report the isolation and characterization of eight polymorphic microsatellites markers for the earthworm *Lumbricus terrestris* which can be used to investigate the population sizes as well as the respective effects of genetic drift, migration and selection in the evolution of populations.

Acknowledgments

Dima Souleman was supported by a fellowship offered by Syrian Ministry of Higher Education.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejsobi.2016.03.009.

References

[1] C.A. Edwards, J. Dominguez, E.F. Neuhauser, Growth and reproduction of *Perionyx excavatus* (Perr.) (Megascolecidae) as factors in organic waste management, Biol. Fertil. Soils 27 (1998) 155–161, http://dx.doi.org/10.1007/s003740050414.

[2] R. Kautenburger, Genetic structure among earthworms (*Lumbricus terrestris L.*) from different sampling sites in western Germany based on random amplified polymorphic DNA, Pedobiologia 50 (2006) 257–266, http://dx.doi.org/10.1016/j.pedobi.2006.02.005.

[3] J. Römbke, S. Jänsch, W. Didden, The use of earthworms in ecological soil classification and assessment concepts, Ecotoxicol. Environ. Saf. 62 (2005) 249–265, http://dx.doi.org/10.1016/j.ecoenv.2005.03.027.

[4] J.C. Sanchez-Hernandez, Earthworm biomarkers in ecological risk assessment, in: D.G.W. Ware, D.M. Whitacre, L.A. Albert, P. de Voogt, C.P. Gerba, O. Hutzinger, et al. (Eds.), Rev. Environ. Contam. Toxicol., Springer, New York, 2006, pp. 85–126. http://link.springer.com/chapter/10.1007/978-0-387-32964-2_3 (accessed 05.06.15).

[5] M. Pauwels, H. Frérot, D. Souleman, F. Vandenbulcke, Using biomarkers in an evolutionary context: lessons from the analysis of biological responses of oligochaete annelids to metal exposure, Environ. Pollut. 179 (2013) 343–350, http://dx.doi.org/10.1016/j.envpol.2013.05.005.

[6] C. Pelosi, S. Joimel, D. Makowski, Searching for a more sensitive earthworm species to be used in pesticide homologation tests – a meta-analysis, Chemosphere 90 (2013) 895–900, http://dx.doi.org/10.1016/j.chemosphere.2012.09.034.

[7] Y. Capowiez, N. Dittbrenner, M. Rault, R. Triebskorn, M. Hedde, C. Mazzia, Earthworm cast production as a new behavioural biomarker for toxicity testing, Environ. Pollut. 158 (2010) 388–393, http://dx.doi.org/10.1016/j.envpol.2009.09.003.

[8] E. Lapied, E. Moudilou, J.-M. Exbrayat, D.H. Oughton, E.J. Joner, Silver nanoparticle exposure causes apoptotic response in the earthworm *Lumbricus terrestris* (Oligochaeta), Nanomed 5 (2010) 975–984, http://dx.doi.org/10.2217/nnm.10.58.

[9] S.W. James, D. Porco, T. Decaëns, B. Richard, R. Rougerie, C. Erséus, DNA barcoding reveals cryptic diversity in *Lumbricus terrestris L.*, 1758 (Clitellata): resurrection of *L. herculeus* (Savigny, 1826), PLoS One 5 (2010), http://dx.doi.org/10.1371/journal.pone.0015629.

[10] E. Guichoux, L. Lagache, S. Wagner, P. Chaumeil, P. Léger, O. Lepais, et al., Current trends in microsatellite genotyping, Mol. Ecol. Resour. 11 (2011) 591–611, http://dx.doi.org/10.1111/j.1755-0998.2011.03014.x.

[11] G.L. Harper, S. Cesarini, S.P. Casey, A.J. Morgan, P. Kille, M.W. Bruford, Microsatellite markers for the earthworm *Lumbricus rubellus*, Mol. Ecol. Notes 6 (2006) 325–327, http://dx.doi.org/10.1111/j.1471-8286.2005.01219.x.

[12] C.M. Somers, K. Neudorf, K.L. Jones, S.L. Lance, Novel microsatellite loci for the compost earthworm *Eisenia fetida*: a genetic comparison of three North American vermiculture stocks, Pedobiologia 54 (2011) 111–117, http://dx.doi.org/10.1016/j.pedobi.2010.11.002.

[13] L. Dupont, F. Lazrek, D. Porco, R.A. King, R. Rougerie, W.O.C. Symondson, et al., New insight into the genetic structure of the *Allolobophora chlorotica* aggregate in Europe using microsatellite and mitochondrial data, Pedobiologia 54 (2011) 217–224, http://dx.doi.org/10.1016/j.pedobi.2011.03.004.

[14] H. Strunk, A. Hochkirch, M. Veith, T. Hankeln, C. Emmerling, Isolation and characterization of eleven polymorphic microsatellite markers for the earthworm *Aporrectodea longa* (Ude), Eur. J. Soil Biol. 48 (2012) 56–58, http://dx.doi.org/10.1016/j.ejsobi.2011.11.004.

[15] T.P. Velavan, H. Schulenburg, N.K. Michiels, Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris L.*), Mol. Ecol. Notes 7 (2007) 1060–1062, http://dx.doi.org/10.1111/j.1471-8286.2007.01776.x.

[16] T.P. Velavan, S. Weller, H. Schulenburg, N.K. Michiels, High genetic diversity and heterogeneous parasite load in the earthworm *Lumbricus terrestris* on a German meadow, Soil Biol. Biochem. 41 (2009) 1591–1595, http://dx.doi.org/10.1016/j.soilbio.2009.03.026.

[17] T. Malausa, A. Gilles, E. Meglécz, H. Blanquart, S. Duthoy, C. Costedoat, et al., High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries, Mol. Ecol. Resour. 11 (2011) 638–644, http://dx.doi.org/10.1111/j.1755-0998.2011.02992.x.

[18] E.R. Zaborski, Allyl isothiocyanate: an alternative chemical expellant for sampling earthworms, Appl. Soil Ecol. 22 (2003) 87–95, http://dx.doi.org/10.1016/S0929-1393(02)00106-3.

[19] Phylogenetic species delimitation of the earthworms Eisenia fetida (Savigny, 1826) and Eisenia andrei Bouché, 1972 (Oligochaeta, Lumbricidae) based on mitochondrial and nuclear DNA sequences, (n.d.). http://www.sciencedirect.com/science/article/pii/S003140560500020X (accessed 02.03.16).

[20] O. Folmer, M. Black, W. Hoeh, R. Lutz, R. Vrijenhoek, DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates, Mol. Mar. Biol. Biotechnol. 3 (1994) 294–299.

[21] M.-P. Chapuis, A. Estoup, Microsatellite null alleles and estimation of population differentiation, Mol. Biol. Evol. 24 (2007) 621–631, http://dx.doi.org/10.1093/molbev/msl191.

[22] S. Piry, A. Alapetite, J.-M. Cornuet, D. Paetkau, L. Baudouin, A. Estoup, GENECLASS2: a software for genetic assignment and first-generation migrant detection, J. Hered. 95 (2004) 536–539, http://dx.doi.org/10.1093/jhered/esh074.

[23] M.N. Darío, J. Díaz Cosín, Reproduction of earthworms: sexual selection and parthenogenesis. Biology of Earthworms, 2010, pp. 69–86, http://dx.doi.org/10.1007/978-3-642-14636-7_5.

[24] J. Domínguez, A. Velando, M. Aira, F. Monroy, Uniparental reproduction of *Eisenia fetida* and *E. andrei* (Oligochaeta: Lumbricidae): evidence of self-insemination, Pedobiologia 47 (2003) 530–534.

[25] P. Garnier-Géré, L. Chikhi, Population subdivision, Hardy–Weinberg

Equilibrium and the Wahlund Effect, in: eLS, John Wiley & Sons, Ltd, 2001. http://onlinelibrary.wiley.com/doi/10.1002/9780470015902.a0005446.pub3/abstract (accessed 2.03.16).
[26] A.P. Hendry, M.T. Kinnison, M. Heino, T. Day, T.B. Smith, G. Fitt, C.T. Bergstrom, J. Oakeshott, P.S. Jørgensen, M.P. Zalucki, G. Gilchrist, S. Southerton, A. Sih, S. Strauss, R.F. Denison, S.P. Carroll, Evolutionary principles and their practical application, Evol. Appl. 4 (2011) 159–183, http://dx.doi.org/10.1111/j.1752-4571.2010.00165.x.