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APPLICATION OF DNA BARCODING IN TAXONOMY AND PHYLOGENY: AN INDIVIDUAL CASE OF COI PARTIAL GENE SEQUENCING FROM SEVEN ANIMAL SPECIES

M. Drohvalenko[1], A. Mykhailenko[1], M. Rekrotchuk[1], L. Shpak[1], V. Shuba[1], R. Trokhymchuk[1], S. Utevsky[1], O. Zinenko[1*]

[1]*V. N. Karazin Kharkiv National University,*
Svobody sq., 4., Kharkiv, 61022 Ukraine
E-mail: oleksandrzinenko@gmail.com
[*]*Corresponding author*

Application of DNA Barcoding in Taxonomy and Phylogeny: an Individual Case of COI Partial Gene Sequencing from Seven Animal Species. Drohvalenko, M., Mykhailenko, A., Rekrotchuk, M., Shpak, L., Shuba, V., Trokhymchuk, R., Utevsky, S., Zinenko, O. — A part of the COI mitochondrial barcoding gene was sequenced from seven species of different taxonomical groups: *Ambystoma mexicanum* (Amphibia, Ambystomatidae), *Darevskia lindholmi, Lacerta agilis exigua* (Reptilia, Lacertidae), *Erinaceus roumanicus (*Mammalia, Erinaceidae*), Macrobiotus* sp. *1* and *2* (Eutardigrada, Macrobiotidae) and *Cameraria ohridella* (Insecta, Gracillariidae*)*. The sequences were compared with available sequences from databases and positioned on phylogenetic trees when the taxa had not yet been sequenced. The presence of Mexican axolotls in herpetoculture in Ukraine was confirmed. The partial COI genes of the Crimean rock lizard and an eastern sub-species of the sand lizard were sequenced. We demonstrated the presence of two tardigrade mitochondrial lineages of the *Macrobiotus hufelandi* group in the same sample from the Zeya Natural Reserve in the Far East: one was nearly identical to the Italian *M. macrocalix*, and the other one is similar to *M. persimilis* and *M. vladimiri*. We also confirmed the presence of the invasive haplotype "A" of the horse chestnut leaf miner in Ukraine, in line with the hypothesized route of invasion from Central Europe.
Key words: cytochrome oxidase subunit one, barcoding, animals, Ukraine, *Ambystoma mexicanum, Darevskia lindholmi, Lacerta agilis exigua*, *Erinaceus roumanicus*, *Macrobiotus* sp.*, Cameraria ohridella.*

Introduction

The idea of using certain variable parts of a genome as a unique identifier of species has been widely recognized since the first publication in 2003 (Hebert et al., 2003). Currently, global initiatives on the barcoding of life contain about 6 million records, including 188 000 barcodes obtained from animals (Ratnasingham, Hebert, 2007, 2013). With advanced technologies and a drastic drop in the price of sequencing, the wide and practical application of DNA barcoding promises to be a simple, fast and precise method to identify species from poorly known groups or in case of difficulty with morphological identification. Furthermore, it bridges the gap between morphological and molecular identification of taxa, especially in taxonomically diverse groups where shortage of qualified experts, known as the taxonomic impediment exists (de Carvalho et al., 2007). In spite of great advances of the method, the barcoding of animal biodiversity on Earth is very hard to achieve. Areas outside high-income first world countries, remote regions and biodiversity hotspots are poorly covered. Hence, many species are still lacking molecular barcodes. We argue that there is a need for more local efforts in filling the global list of barcoded species.

Ambystoma mexicanum (Shaw, 1789) (Amphibia, Ambystomatidae) also known as the axolotl or the Mexican salamander is a neotenic salamander species and is a part of the *Ambystoma tigrinum* complex. As of 2010, wild axolotls in natural habitats in Mexico City were nearly extinct due to urbanization, water pollution and introduction of invasive fish species, such as the carp and the tilapia (Zambrano et al., 2010). The species is currently listed in CITES ("Appendices I, II and III". CITES Secretariat. CITES. Geneva, Switzerland: Convention on International Trade in Endangered Species of Wild Fauna and Flora; Checklist of CITES Species checklist.cites.org, *Ambystoma mexicanum*) and is critically endangered in the wild according to the IUCN assessment (Zambrano et al., 2010). Since 1998 its density in Lake Xochimilco has decreased drastically from thousands per square kilometer to almost zero in 2013 (Voss et al., 2015). At the same time, the axolotl is present in large numbers in captivity, as a popular pet and a model organism. Given its endangered status in the wild, the captive population may be critical for preserving the species' genetic diversity. Reliable identification to avoid confusion of axolotls with tiger salamanders, is also needed. We have sequenced the COI gene from a captive individual from Ukrainian herpetoculture in order to confirm its identity.

Darevskia lindholmi (Szczerbak, 1962) (Squamata, Lacertidae) is a rock lizard species with distribution mostly in the Caucasus. *Darevskia* spp. are famous because of the presence of parthenogenetic forms in the genus. *D. lindholmi* was found in Crimea and is the only genus representative in Ukraine. Cytochrome b sequencing and protein allozyme analysis (MacCuloch et al., 2000; Tarkhnishvili et al., 2016) show that *D. lindholmi* is the sister species of *D. brauneri* (Méhely, 1909). The Crimean *D. lindholmi* has a unique type of Caucasian lizard microsatellite allele (CLat IV; along with other types, Ciobanu et al., 2003; Grechko et al., 2006). The source and the pattern of colonization of the Crimean Peninsula are not completely clear. Hybridization and multiple colonization events, which had led to the origin of this species, were discussed by Kukushkin et al. (2017). Obtaining the barcode sequence and clarification of the phylogenetic position of *D. lindholmi* may help to reconstruct this in the future.

Lacerta agilis exigua Eichwald, 1831 (Squamata, Lacertidae) is one of the 9 subspecies of the sand lizard *Lacerta agilis* Linnaeus, 1758 (Kalyabina et al., 2001; Kalyabina-Hauf and Ananjeva, 2004). *L. a. exigua* with four other subspecies forms *Lacerta agilis* eastern subspecies group (Kalyabina et al., 2001; Kalyabina-Hauf and Ananjeva, 2004; Andres et al., 2014). In eastern and central Ukraine, this subspecies forms a narrow contact zone with *L. a. chersonensis* Andrzejowski, 1832. The contact zone was traced using morphological characters (Zinenko et al., 2005). However, no COI gene sequences have been published yet. Mitochondrial DNA sequences may be used as additional molecular marker in the hybrid-zone studies.

The phylogeny of European hedgehog species, relying on the evolution of 12S mitochondrial gene (He et al., 2012), depicts *Erinaceus europaeus* Linnaeus, 1758, *Erinaceus concolor* Martin, 1837 and *Erinaceus roumanicus* Barrett-Hamilton, 1900 (Mammalia, Erinaceidae) as a monophyletic clade. Despite the fact that hedgehogs are common in Europe, the number of barcoding gene sequences available in open databases is relatively small. Mitochondrial COI sequences of *E. roumanicus* and *E. concolor* have never been published. Considering that Ukraine could be a possible contact zone between Eastern European taxa (Bogdanov et al., 2009) and their distribution is uncertain, rapid and reliable methods of identification could be very useful.

Water bears or tardigrades (Parachaela, Macrobiotidae) are small, ubiquitous (less than 1 mm) organisms, which can tolerate a wide range of abiotic parameters in an inactive stage. The systematics of the group is under development and a significant number of new species are described every year (Lee et al., 2017; Perez-Pech et al., 2017; Kaczmarek & Michalczyk, 2017; Kaczmarek et al., 2017). However, tardigrades are a challenge to taxonomists because of their small size, unknown magnitude of intraspecific variation and often a lack of experts who are able to identify species. Tardigrades, therefore, have a strong need for a unified and reliable method of identification and the creation of a good barcode reference library, in which molecular data must be integrated with reliable morphological taxonomic knowledge (Cesari et al., 2013). Being small and light in weight, they can potentially travel long distances, resulting in their very diffuse, potentially even global distribution (Pilato, 2001). Recent studies argue that this is not the case, at least at all times (Guil et al., 2009), and that the issue could be partly elucidated using information on barcoding genes sequences.

The horse-chestnut leaf miner *Cameraria ohridella* Deschka & Dimić, 1986 (Lepidoptera, Gracillariidae) is a dangerous pest of horse-chestnut (*Aesculus hippocastanum* L., Sapindaceae) in Europe. It has been expanding the native range in the Balkans since the end of 20th century (Lees et al., 2011) and had colonized Ukraine by 2007 (Zerova et al., 2007; Guglya, Zinenko, 2008). Among the 25 different mitochondrial haplotypes, only three haplotypes took place in the invasion (Valade et al., 2009). Currently, no material from Ukraine has been studied, the origin and composition of local lineages of *C. ohridella* in Ukraine are not known.

The study was undertaken as an educational project, where students, under faculty guidance, performed the necessary manipulations: DNA sampling, depositing or morphological identification of organisms followed by the obtaining and submitting DNA barcoding sequences. Thus, we were promoting the concept of barcoding life-forms and at the same time facilitating individual research. During the study, we have tested the universal primers, optimized reaction conditions and sequenced the barcoding gene COI from various (pet, invasive, native) species, which were previously out of the focus of barcoding. Based on the obtained sequences, we were able to identify the species or the interspecific lineages using bioinformatics or phylogenetic methods.

Material and methods

The samples were conserved in ethanol or taken fresh (vertebrates). Entire larva specimen (e. g. *C. ohridella*) or several specimens (e. g. water bears) were taken. A list of accession numbers of the sequences, taxa and sample origin is given in table 1.

DNA from the tardigrades was extracted in January 2017, one year after sampling. We utilized standard methods of tardigrade extraction: a piece of the moss was soaked in water for two hours and the tardigrades were picked up from the sample using a stereomicroscope. We also obtained some tardigrade eggs from the same sample as they were necessary for species identification. Some of the extracted tardigrades were used for DNA analysis. The others from the same sample were used for making permanent slides for subsequent microscopy and were mounted in Faure's medium.

The preliminary examination of the permanent slides under the microscope had shown the presence of a single morphotype of *Macrobiotus* aff. *hufelandi* in the text and table (*Macrobiotus* species belonging to the so called '*hufelandi* group') tardigrades. We had made several attempts to isolate DNA from individual specimens of tardigrades as recommended by Cesari at al., 2011, but none of them was successful. Therefore, we extracted DNA from 3 pools of 5 tardigrade specimens torn using a needle.

Since species of the *M. hufelandi* group are practically indistinguishable under the magnification of a stereomicroscope, our material for the single DNA analysis contained individuals of different species. Later, a more thorough examination of the eggs revealed two different species of *M. hufelandi* group in one sample. One of them was later identified as *Macrobiotus* cf. *macrocalix* Bertolani & Rebecchi, 1993. The second one was *Macrobiotus* sp. not identifiable to the species level.

We used high salt DNA extraction method (Aljanabi and Martinez, 1997). Extracted DNA was dissolved in water and stored at –18 ° C. Amplification was performed in 25 μL volume, using master mix which contained DNA, Taq polymerase, dNTPs, primers and 10x buffer. Primers for the COI region were

LCO1490: GGTCAACAAATCATAAAGATATTG and

HCO2198: TAAACTTCAGGGTGACCAAAAAAT — shorter by one and two base pairs from 3' end than primers which were originally proposed by Folmer et al. (1994). PCR products were obtained using 'Tercik' and 'Biometra' thermocyclers. PCR conditions were optimized by changing annealing temperature from 55 to 47 °C, until clear single band was visible on 1 % agarose gel electrophoregram. Thermocycling conditions for the reaction were as follows: denaturation for 5 min, 36 cycles of denaturation for 60 sec at 94 °C, annealing for 90 sec at 47 °C and elongation for 60 sec at 72 °C, terminal elongation was set for 5 min at 72 °C. PCR products were purified using spin column and then sequenced by commercial sequencing service.

Chromatograms were manually edited and trimmed. In case of water bears, when several specimens were pooled in one sample, the chromatogram contained multiple double peaks, which were first manually coded using IUPAC ambiguity codes and then phased using DNASP 5.0 with COI sequences of other *Macrobiotus* spp. retrieved from GenBank. All other samples did not contain any ambiguities and when aligned in a reading frame against the COI reference sequences, did not contain any stop codons or indels.

Table 1. Material and accession numbers of samples sequences

Sample ID	Species	Locality	Date of collection	Collector	Accession number
OZ17	*Darevskia lindholmi*	Ukraine, Crimea, Chufut Kale	2012	Oleg Kukushkin	MG458880
OZ27	*Lacerta agilis exigua*	Ukraine, Kharkiv Region, Zmiiv District, Gaidary village, Biological station of Kharkiv University	1.07.2016	Oleksandr Zinenko	MG815780
OZ31	*Erinaceus roumanicus*	Ukraine, Kharkiv Region, Zmiiv District, Gaidary village, Biological station of Kharkiv University	1.07.2016	Tatiana Atemasova	MH329869
OZ39	*Cameraria ohridella*	Ukraine, Khmelnitskyi Region, Kamiyanets-Podilskyi	1.08.2016	Oleksandr Zinenko	MH059571
RF 1.2	*Macrobiotus* gr. *hufelandi (?)*	Russia, Amur Oblast, Zeya Nature Reserve	2016	Anna Suvorova	MH042875
AR1	*Ambystoma mexicanum*	Ukraine, Kharkiv, captive bred	2017	Roman Trokhymchuk	MH791446

Table 2. Parameters of phylogenetic tree reconstruction

Taxa names	Best substitution model under BIC	LnL	MrBayes settings
Lacerta agilis exigua and *Darevskia lindholmi*	HKY	–2392.312	lset applyto = (all) nst = 6 rates = invgamma; mcmc ngen = 1000000
Macrobiotus hufelandi group	GTR + G + I	–3870.424	lset applyto = (all) nst = 6 rates = invgamma; mcmc ngen = 2000000 samplefreq = 100 printfreq = 100 diagnfreq = 1000
Erinaceus roumanicus	Pos 1 and 2 — K80+I; pos 3 — HKY+G	–741.046	charset Subset1 = 1-286\3 2-286\3; charset Subset2 = 3-286\3; partition PartitionFinder = 2:Subset1, Subset2; set partition = PartitionFinder; lset applyto = (1) nst = 2 rates = propinv; prset applyto = (1) statefreqpr = fixed(equal); lset applyto = (2) nst = 2 rates = gamma; prset applyto = (all) ratepr = variable; unlink statefreq = (all) revmat = (all) shape = (all) pinvar = (all) tratio = (all); unlink brlens = (all); mcmc ngen = 1000000;

Sequences were exported in the FASTA format and similar sequences were retrieved from GenBank and BOLD databases (Ratnasingham and Hebert, 2007). To resolve phylogenetic relationships within studied genera, COI sequences from every species within the group were taken, with the addition of one species outside the genus as an outgroup.

The sequences were initially identified using BLAST search. If BLAST search was able to find identical or closely identical sequences (in the case of *Ambystoma, Cameraria* samples), further analysis was not performed. In the case of *Erinaceus* sp., *Macrobiotus* sp., *Lacerta* sp. and *Darevskia* sp., samples were identified at the genus level using BLAST and reference sequences from each genus were included into further analyses. The reference and query sequences of the genus were aligned using ClustalW in BioEdit 7.1.9 (Hall, 1999) and Mega 7.0.26 (Kumar et al., 2016). Optimal substitution model was calculated in Mega 7.0.26 and Partition Finder 2.0. Topology of phylogenetic trees was constructed in MrBayes 3.2.6 (Bayesian inference – BI) or Mega 7.0.26 (Maximum Likelihood, ML). Trees topology was shown in FigTree 1.4.2. Parameters of tree construction are given in table 2.

Results

The sequence (337 bp) of the captive bred axolotl did not show any differences from the published GenBank sequences AY65999 1and AJ584639 of the axolotl. It had two mutations, which differentiate it from *Ambystoma andersoni* Krebs and Brandon, 1984.

According to our 539 base pairs sequence of COI gene, *Darevskia lindholmi* is in a monophyletic clade with other available sequences of lizards from the *Darevskia* genus (*D. unisexualis* and *D. valentini*), *Eremias* and *Ophisops* (fig. 1). *Podarcis*, *Lacerta* and *Apathya* genera are shown to be sister clades. The partial COI (630 bp) sequence from a morphologically identified *L. a. exigua* was published for the first time. Although at least two large scale phylogeographic studies were published (Kalyabina-Hauf and Ananjeva, 2004; Andres et al., 2014), the authors used different mitochondrial loci. Ukrainian COI sequence was clustered with other representative of the species *L. agilis* (fig. 2), but not identical to them, as it would be expected since GenBank sequences belong to the Central European subspecies group L. *a. agilis*, in contrast to *L. a. exigua* from the Eastern subspecies group (fig. 1).

The short *E. roumanicus* COI fragment of 288 bp from Kharkiv Region is clustered first with additional sequences of this species and then with sister species *E. europeus*. *E. roumanicus* and *E. europeus* are nested within a larger cluster which includes *E. amurensis* sequences (posterior probability 1) (fig. 2).

After phasing the Tardigrada sequences we have obtained two 480 base pairs sequences of *Macrobiotus* sp. There were 87 ambiguities in the complete sequence submitted to the GenBank, and 80 ambiguities in the final sequence used for further analyses. After phasing,

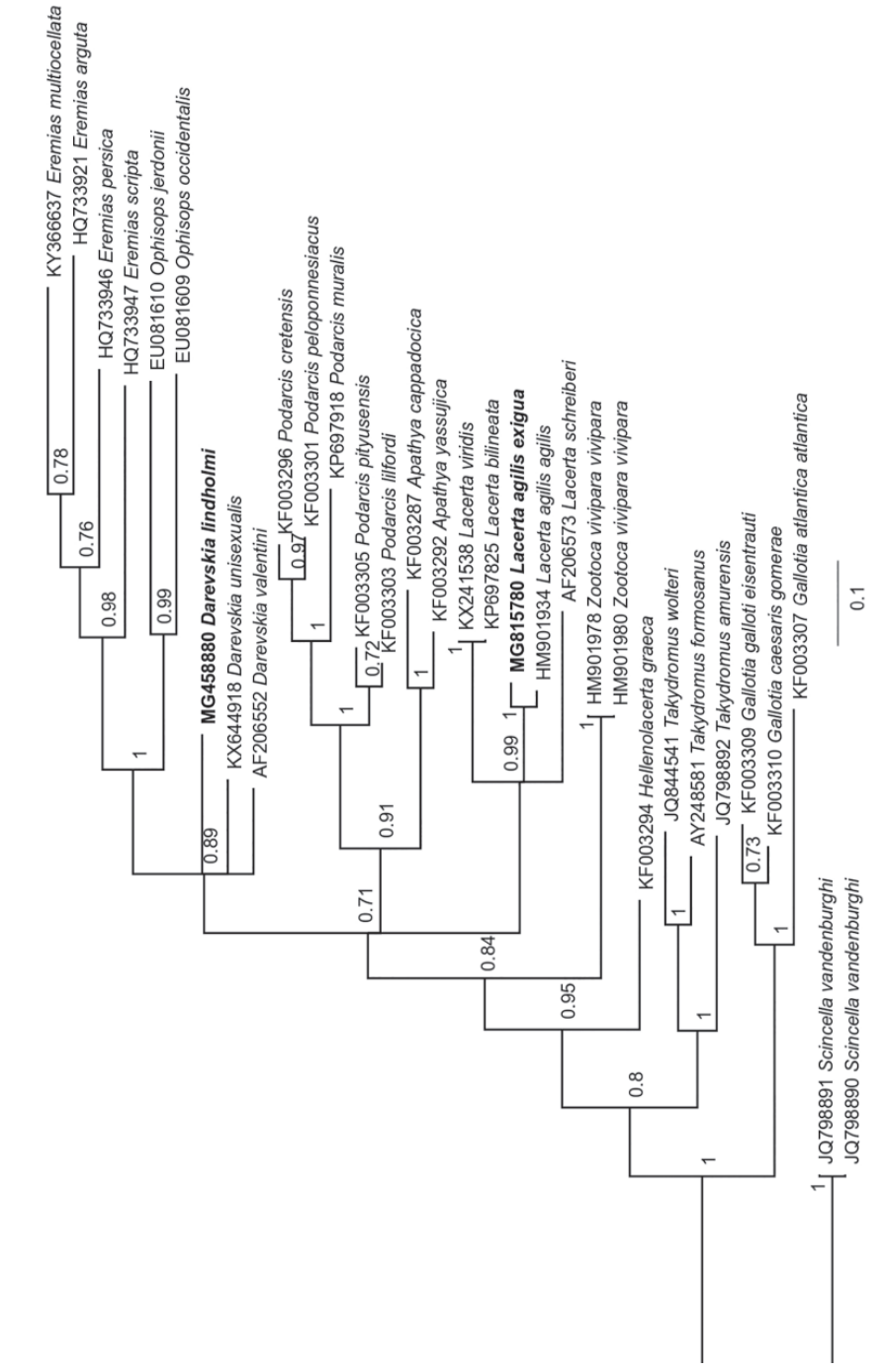

Fig. 1. Phylogenetic position of *D. lindholmi* and *L. a. exigua*, Bayesian inference phylogenetic tree. Sequences obtained by us are written in bold.

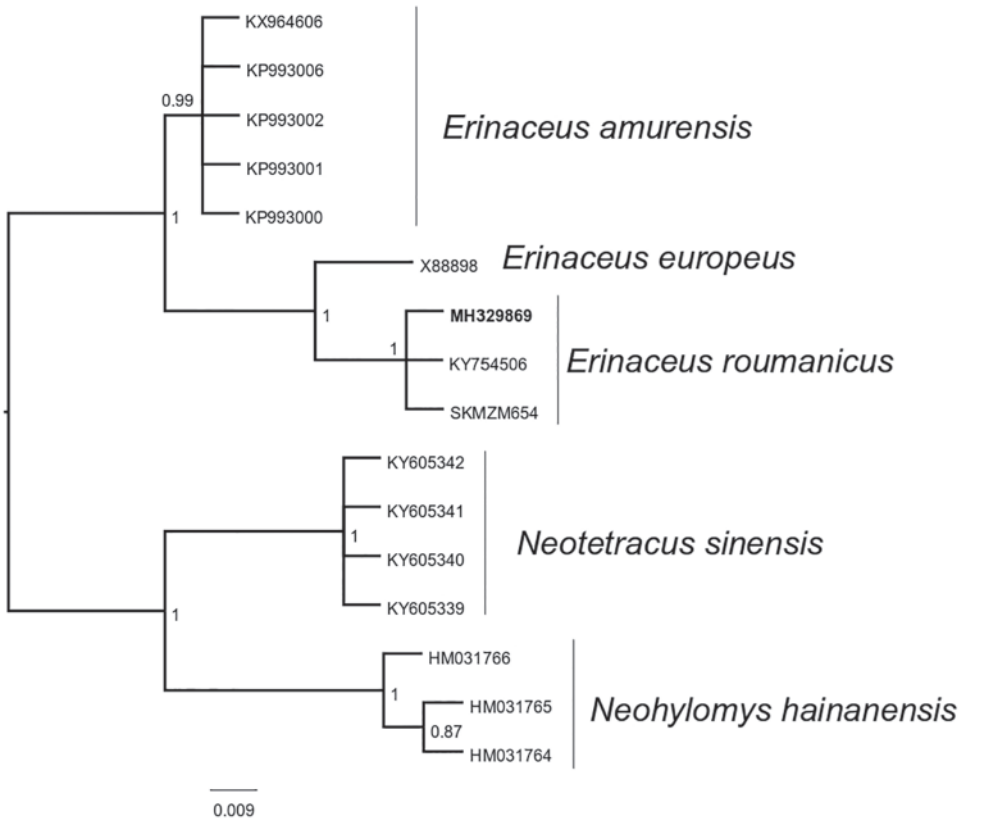

Fig. 2. Phylogenetic position of *E. roumanicus*, Bayesian inference phylogenetic tree. Sequences obtained by us are written in bold.

all ambiguities were resolved with 1.0 probability assignment. The two resulting sequences cluster either with *M. macrocalix* FJ176217 from Italy (p-distance to the reference sequence = 0.009) or with *M. vladimiri* JX 683811 from Portugal and *M. persimilis* EU244608 from Germany (p-distance = 0.020).

The sequence of *C. ohridella* from Kamyanets-Podilsky contained 505 base pairs and had one substitution comparing to invasive haplotype A, found in the Balkans, Central and Western Europe (Valade et al., 2009; Lees et al., 2011).

Discussion

Short fragment of the COI gene has confirmed the identity of the captive bred of *A. mexicanum* from Ukraine. Recent radiation of the *Ambystoma* genus, small length of the sequence, which contains only a few substitutions and is not species-specific, lead to the conclusion that one mitochondrial gene is not sufficient to trace the origin or define any intraspecific relations, but can help differentiate between the most closely related sister species *A. mexicanum* and *A. andersoni.*

We also have shown that the universal primers and COI sequences from *E. roumanicus* and *L. a. exigua* could be used for their rapid identification. Furthermore, it can be used to identify the maternal lineage in the individuals from the hybrid zones between *L. a. exigua* and *L. a. chersonensis* (Zinenko et al., 2005) or between *Erinaceus* species in Eastern Europe and the Caucasus. In both cases, sampling localities are within the distribution ranges of particular taxa and could be unambiguously associated with *L. a. exigua* (see Kalyabina-Hauf and Ananjeva, 2004; Zinenko et al., 2005) or *E. roumanicus* (He et al., 2012), therefore, they can be used as a reference.

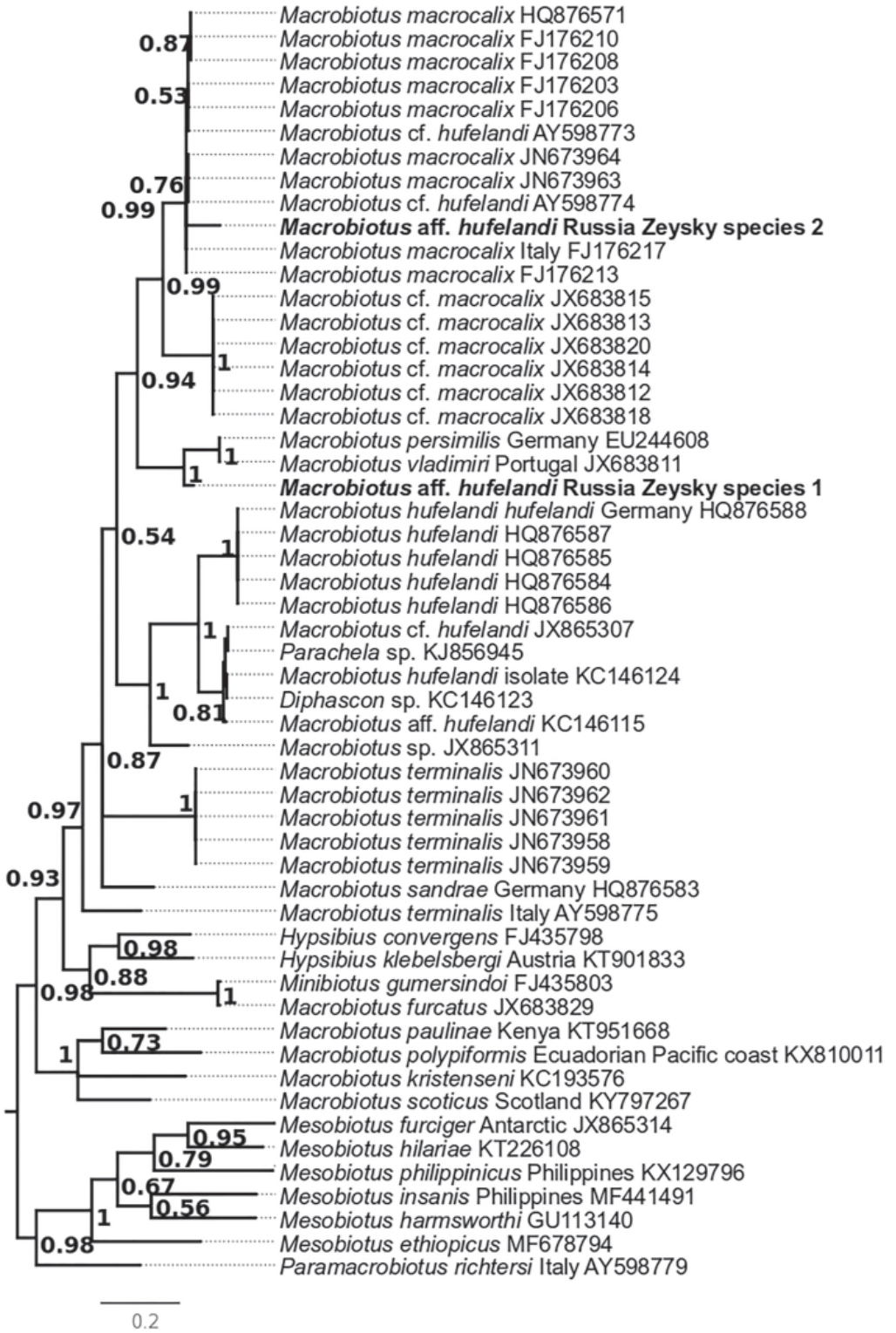

Fig. 3. Phylogenetic position of *Macrobiotus* sp., Bayesian inference phylogenetic tree. Sequences obtained by us are written in bold.

The Lacertidae tree with the COI sequence of *D. lindholmi* was compared to a previously described Lacertidae phylogeny (Kapli et al., 2011). The latter included the representatives of 40 genera and the sequences of 6 genes (including COI). Our tree differs from it by topology on the level of genera. Statistical support of nodes in the tree provided by this study was lower due to too short sequence of the COI fragment, which lacked phylogenetically informative substitutions. Our tree reconstruction had high support at the level of genera, but higher systematics levels above the genus level appeared to be more tangled compared to other authors. Hence, we should use more loci and longer sequences to investigate relations among *Darevskia* as well as other genera of lacertid lizards. Intraspecific relations could be revealed using denser sampling of closely related species from the Caucasus, which were not available at the time of the study.

Our chosen sampling strategy and sample processing of Tardigrada was successful, however we would recommend this approach only for those localities with well-known fauna of Tardigrada or in metabarcoding studies. Our sampling area was probably too remote to host the exact lineages of European *Macrobiotus* species, whose sequences from GenBank were taken for comparison. Nevertheless, the genetic distance between the obtained phased sequences and the nearest sequences on the tree were rather small, confirming the high dispersal abilities of water bears. Finally, the presence of two mitochondrial lineages from different species of the same genus in one environmental sample is rather interesting. On this stage we are too far away from understanding the ecology of these species. Mitochondrial markers alone are not enough to understand if they truly coexist as separate taxa or whether gene flow is possible between them (fig. 3).

The finding of a widespread invasive haplotype of *C. ohridella* in Ukraine confirms the high invasive potential of this particular lineage, which has been shown to substitute even other non-invasive haplotypes in the natural range of the species in the Balkans (Lees et al., 2011). Invasion of this haplotype to Ukraine is consistent with the vehicle-mediated dispersal of *C. ohridella* and the Central European origin of the species in Ukraine (Zerova et al., 2007).

Standard methods of DNA barcoding could be used to answer various research questions. We have successfully utilized them to elucidate taxonomic or intraspecific position of *A. mexicanum*, *Macrobiotus* sp. and *C. ohridella*. Newly obtained partial gene COI sequences from *L. a. exigua*, *D. lindholmi* and *E. roumanicus* could be used to study their distribution patterns, hybridization and phylogeography in Eastern Europe.

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