New records of leeches of the genus *Limnatis* (Hirudinea, Praobdellidae) from the South Caucasus and Central Asia: phylogenetic relationships of Eurasian and African populations

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Abstract

New records of leeches of the genus Limnatis *(Hirudinea, Praobdellidae) from the South Caucasus and Central Asia: phylogenetic relationships of Eurasian and African populations.* Leeches of the genus *Limnatis* Moquin–Tandon, 1827 infest mucous membranes of various mammals, including humans and domestic ungulates. The type species of the genus *L. nilotica* (Savigny, 1822) was initially thought to occur throughout the Western Palaearctic, from North Africa to the Middle East and Central Asia. It was later found that *L. paluda* (Tennent, 1859) is a widespread Western Asian species. However, the South Caucasus and vast areas of Central Asia have not been explored sufficiently in terms of leeches of the genus *Limnatis*. We recorded *L. paluda* from Azerbaijan and Uzbekistan for the first time. We also carried out the first molecular characterisation of *L. nilotica* herein. We found a deep genetic differentiation (8 %) between the Western Asian *L. paluda* and North African (Moroccan) *L. nilotica* based on their *COI* sequences. This finding corroborates a previous morphology–based hypothesis on their separate species assignments. The low genetic diversity of *L. paluda* is explained by the recent colonisation of arid landscapes of Western Asia.

Key words: Annelida, *Limnatis paluda*, *Limnatis nilotica, COI*, Genetic diversity

Resumen

Nuevos registros de sanguijuelas del género Limnatis *(Hirudinea, Praobdellidae) en el Cáucaso meridional y Asia central: relaciones filogenéticas de las poblaciones eurasiáticas y africanas*. Las sanguijuelas del género *Limnatis* Moquin–Tandon, 1827 infestan las mucosas de varios mamíferos, incluidos los seres humanos y los ungulados domésticos. Se creía que la especie tipo del género, *L. nilotica* (Savigny, 1822), estaba presente en todo el paleártico occidental, desde África del norte hasta Oriente Medio y Asia central. Posteriormente, se observó que *L. paluda* (Tennent, 1859) es un especie ampliamente distribuida en Asia occidental. Sin embargo, no se han hecho estudios suficientes sobre el género *Limnatis* en la zona del Cáucaso meridional ni en buena parte de Asia central. Registramos *L. paluda* por primera vez en Azerbaiyán y Uzbekistán. La primera caracterización molecular de *L. nilotica* tuvo lugar allí. Las secuencias del gen citocromo oxidasa (COI) permitieron constatar que existe una profunda diferenciación genética (del 8 %) entre *L. paluda*, de Asia occidental, y *L. nilotica*, de África del norte (Marruecos). Ello corrobora la hipótesis basada en la morfología que se asignaba especies diferentes. La escasa diversidad genética de *L. paluda* se explica por la reciente colonización de territorios áridos de Asia occidental.

Palabras clave: Anélidos, *Limnatis paluda*, *Limnatis nilotica*, COI, Diversidad genética

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Introduction

The genus *Limnatis* Moquin–Tandon, 1827 comprises bloodsucking leeches that occur in the south–western Palaearctic. They infest mucous membranes of various organs such as the pharynx, nasopharynx, oesophagus, larynx, trachea, bronchial tubes and female genital organs in humans and domestic mammals, including horses, cattle, camels, deer, and dogs (Kaburaki, 1921; Moore, 1927; Almallah, 1968; Arenas et al., 1993; Boye and Joshi, 1994; Al–Ani and Al–Shareefi, 1995; Ağin et al., 2008; Bahmani et al., 2012, 2014; Negm–Eldin et al., 2013; Rajaei et al., 2014; Raele et al., 2015). Moreover, leeches of the genus *Limnatis* can also be parasitic on amphibians (Lukin, 1976). The members of this genus are therefore important in terms of medicine, veterinary science, and parasitology. Taking global climate change into account, it is clear that they can pose a potential invasive threat by shifting their ranges, as has already happened with many species (Parmesan and Yohe, 2003).

According to the current view on the classification, the genus contains three species, *Limnatis bacescui* Manoleli, 1972, *Limnatis nilotica* (Savigny, 1822) and *Limnatis paluda* (Tennent, 1859) (Nakano et al., 2015). The type species of the genus, *L. nilotica*, was first described by the French zoologist Jules Cesar Savigny from Egypt under the name *Bdella nilotica* (Savigny, 1822). Later, in 1827, Moquin–Tandon renamed Savigny's *Bdella nilotica* to *Limnatis nilotica* in his seminal monograph (Moquin–Tandon, 1827). The second species is *Limnatis bacescui* Manoleli, 1972 from Romania (South–Eastern Europe), where it was described and is currently known only from its type locality (Manoleli, 1972). The third member of the genus, *L. paluda*, was described by Tennent (1859) as *Haemopis paludum* from Sri Lanka (= Ceylon). Its taxonomic status was revised by Moore (1927) so that the species was transferred to the genus *Limnatis* and its name was changed to *L. paluda* (Tennent, 1859).

Traditionally, praobdellid leeches, found both in North Africa and Western Asia, were identified as *L. nilotica*. Representatives of this species had been recorded for Kazakhstan and Central Asia (Lukin, 1976), and southern Iran (Grosser and Pešić, 2006). Subsequently, Phillips and Siddall (2009) and Nakano et al. (2015) found that the leeches of the genus *Limnatis* of Israel, Afghanistan and Kazakhstan should be assigned to *L. paluda*.

Despite the long history of previous studies, vast areas of Central Asia and the Caucasus have not been explored sufficiently in terms of their leeches of the genus *Limnatis*. There are no records based on molecular data concerning the species identity of those leeches in the South Caucasus and Central Asian countries except in Kazakhstan (Nakano et al., 2015), long known as regions of the *Limnatis* range (Lukin, 1976). Furthermore, North African leeches of the genus *Limnatis* have never been characterised based on their DNA sequences. The differentiation between the Western Asian *L. paluda* and the North African *L. nilotica* has relied on morphological and geographical considerations (Moore, 1938). For this reason we aimed to identify leeches of the genus *Limnatis* collected in Uzbekistan (Central Asia) and Azerbaijan (the South Caucasus) and to clarify taxonomic and phylogenetic relationships between North African and Western Asian leech populations of the genus *Limnatis* using both morphological and molecular characters.

Material and methods

Sample collection

Leeches were collected during field trips in Uzbekistan, Azerbaijan, and Morocco (table 1). These samples were anesthetized in 10 % ethanol, fixed, and preserved in 96 % ethanol for further examination using both morphological and molecular methods. The specimens are stored in the collection of invertebrate animals at the Department of Zoology and Animal Ecology, V. N. Karazin Kharkiv National University.

Morphological examination

Identification was carried out using a stereomicroscope Konus Crystal–45. Photo documentation was done using a USB HDCE–50B camera. We relied on Moore (1938) to find morphological features distinguishing *L. nilotica* and *L. paluda.*

DNA extraction, amplification and sequencing

Using molecular methods we analysed four specimens of *L. paluda* collected in Uzbekistan, one specimen from Azerbaijan and one from Morocco assigned to *L. nilotica*. A small piece of tissue from the posterior part of the body was taken for DNA extraction. Genomic DNA was isolated using a GeneElute Mammalian Genomic DNA Minprep Kits.

The mitochondrial cytochrome *c* oxidase subunit I (*CO1*) fragment was chosen as a standard animal DNA barcode gene region (Hebert et al., 2003) and amplified using following primers (Folmer et al., 1994): LCO1490, 5′–GGTCAACAAATCATAAAGATATTGG–3′ (forward) and HCO2198, 5′–TAAACTTCAGGGTGACCAAAAAATCA–3′ (reverse) by applying 5 cycles of 30 s at 94 °C, 1 min 30 s at 45 °C and 1 min at 72 °C, 35 cycles of 30 s at 94 °C, 45 s at 51 °C and 1 min at 72 °C, and 1 cycle of 5 min at 72 °C after an initial 3 min denaturation step at 94 °C. Alternatively, another PCR protocol (Utevsky et al., 2021) was implemented.

PCR products were cleaned using two enzymes, Exonuclease I and Shrimp alkaline phosphatase (SAP) (Fermentas, Thermo Fisher Scientific, USA). Exonuclease I (0.2 µl) and SAP (1 µl) were added to 10 µl of the PCR product. The mixture was then incubated for 45 min at 37 °C followed by 15 min incubation at 80 °C. The cleaned PCR product was then sequenced in both directions by Macrogen

Table 1. Information on specimens examined: COI, *COI* sequence code; N, number of specimens.

Tabla 1. Información sobre los ejemplares examinados: COI, código secuencias COI; *N, número de especímenes.*

Species	Date	COI	N	Locality	Collector
L. paluda	15 VI 2005	LN	19	A mountain stream, Urgut District, Samarqand Region, Uzbekistan	A. Abdullaev
L. paluda	7 VI 2007	m20, m21	4	Biological Station (University of Samarqand), Samarqand Region, Uzbekistan	S. Utevsky
L. paluda	9 VIII 2016	T10	1	41°01'19.48" N, 48°41'45.50"E, a warm spring, Hashi, Quba District, Azerbaijan	A. Manafov
L. nilotica	22 IX 2019	U59	4	33°58'01.4"N, 3°02'19.0"W, Ain Tafrent spring, Debdou, Morocco	Y. Mabrouki

Inc. (the Netherlands) using the same primers as at the amplification stage. The chromatograms of sequences were processed in ChromasPro 1.32 (Technelysium Pty., Queensland, Australia). The length of the newly generated *COI* sequences was 650–661 bp.

Phylogenetic analysis

To reveal phylogenetic relationships of the Caucasian, Central Asian and North African leeches of the genus *Limnatis*, all available *COI* sequences of leeches assigned to that genus, sequences of Nearctic and Neotropic praobdellid leeches plus members of other families of Hirudiniformes and the erpobdellid leech *Trocheta danastrica* Stschegolew, 1938 were chosen for analysis and downloaded from GenBank (table 2).

The *COI* sequences were unambiguously aligned using MUSCLE algorithm in MEGA X. The final dataset contained a total of 1,302 positions. The alignment was checked for stop codons by translating it to amino acids using MEGA X (Kumar et al., 2018). Best–fit models of molecular evolution were determined for each codon position under the Bayesian information criterion using KAKUSAN4 (Tanabe, 2011): HKY85 with gamma distribution (+G) for the first codon position and GTR+G for the second and third positions. Phylogenetic relationships were assessed by Bayesian inference using MrBayes v3.2.7a (Ronquist and Huelsenbeck, 2003) as implemented in the CIPRES Science Gateway (Miller et al., 2010, accessible at www.phylo.org). Searches were performed in two parallel runs with eight chains each for ten million generations, sampled every 100th generation. After the first 25% of the sampled trees were discarded, the final topologies were consented following the 50% majority rule.

In addition, using MEGA X, we calculated the number of uncorrected base differences per site (based on p–distances) between species–level clades of the genus *Limnatis*.

Results

Morphology

The specimens collected in Azerbaijan and Uzbekistan were identified as *L. paluda* based on their morphological characters. The leeches range from 12.6 to 95.4 mm in length. The specimen from Azerbaijan is 48.9 mm in length. The body is flattened, indistinctly separated into the trachelosome and the urosome. Some of the specimens have a well–defined clitellum. The oral sucker is confluent with the trachelosome. The caudal sucker is wide and constitutes 0.86 of the maximum width of the urosome. The oral sucker has a sulcus on its inner surface. The anus is inconspicuous. Mid–body segments are five–annulated. The gonopores are separated by five annuli. The leeches have five pairs of eyes that line up, creating a parabolic arc pattern. Papillae are weakly developed. Live leeches are greenish dorsally with no dark pattern. Lateral margins of the body are orange. The venter is bluish black (fig. 1A, 1B).

The Moroccan leeches, which were assigned to *L. nilotica*, are 7.7–13.0 mm in length. The posterior sucker constitutes 0.76 of the maximum body width. The dorsal coloration pattern includes six longitudinal rows of black dots and short lines. Pigmentation was largely bleached due to preservation in ethanol. The specimens do not have a well discernible sulcus. The gonopores are separated by five annuli (fig. 1C).

Table 2. Collection sites and sequence accession data for COI sequences analysed: GenBank, GenBank accession number and specimen code. (Species names are presented according to this study).

Tabla 2. Lugares de recolección y datos sobre las accesiones de las secuencias COI analizadas: GenBank, número de acceso y código de la muestra. (Los nombres de las especies se indican de acuerdo con este estudio).

Taxon	GenBank	Country	Reference
Limnatis nilotica (Savigny, 1822)	MZ318072, U59	Morocco	This study
Limnatis sp.	GQ368754	Namibia	Phillips and Siddall (2009)
Limnatis sp.	AY763152	Croatia	Trontelj and Utevsky (2005)
Limnatis paluda (Tennent, 1859)	KY989474	Iran	Darabi–Darestani et al. (2021)
L. paluda	KY989473	Iran	Darabi–Darestani et al. (2021)
L. paluda	KY989472	Iran	Darabi–Darestani et al. (2021)
L. paluda	KY989471	Iran	Darabi–Darestani et al. (2021)
L. paluda	GQ368755	Afghanistan	Phillips and Siddall (2009)
L. paluda	AB981656	Kazakhstan	Nakano et al. (2015)
L. paluda	AB981654	Kazakhstan	Nakano et al. (2015)
L. paluda	AY425452	Israel	Borda and Siddal (2004)
L. paluda	MZ318071, T9	Uzbekistan	This study
L. paluda	MZ318070, T10	Azerbaijan	This study
L. paluda	MZ318067, LN	Uzbekistan	This study
L. paluda	MZ318068, m20	Uzbekistan	This study
L. paluda	MZ318069, m21	Uzbekistan	This study
Limnobdella mexicana Blanchard, 1893	GQ368758	Mexico	Phillips and Siddall (2009)
L. mexicana	GQ368756	Mexico	Phillips and Siddall (2009)
L. mexicana	GQ368757	Mexico	Phillips and Siddall (2009)
L. mexicana	GQ368759	Mexico	Phillips and Siddall (2009)
Myxobdella sinanensis Oka, 1925	LC192132	Japan	Nakano et al. (2017)
M. annandalei Oka, 1917	GU394014	India	Phillips et al. (2010)
Pintobdella chiapasensis (Caballero, 1957)	GU394015	Mexico	Phillips et al. (2010)
Tyrannobdella rex Phillips et al., 2010	GU394016	Peru	Phillips et al. (2010)
Hirudo orientalis Utevsky and Trontelj, 2005	EF405599	Uzbekistan	Utevsky et al. (2007)
Hirudo nipponia Whitman, 1886	AY763153	Korea	Trontelj and Utevsky (2005)
Whitmania laevis (Baird, 1869)	KT693113	India	Chatterjee et al. (2017)
Aliolimnatis michaelseni (Augener, 1936)	GQ368738	Guinea–Bissau	Phillips and Siddall (2009)
Hirudinaria manillensis (Lesson, 1842)	AY425449	Puerto Rico	Borda and Siddall (2004)
Haemopis sanguisuga (Linnaeus, 1758)	AF462021	Sweden	Siddall (2002)
Semiscolex similis Oceguera–Figueroa, 2005	AY425457	Bolivia	Borda and Siddall (2004)
Patagoniobdella fraternal Ringuelet, 1976	AY425459	Chile	Borda and Siddall (2004)
Oxyptychus braziliensis (Pinto, 1920)	AY425455	Brazil	Borda and Siddall (2004)
Macrobdella decora (Say, 1824)	MH672573	North America	Müller et al (2019)
M. decora	EU100095	USA	Borda et al. (2008)
Philobdella gracilis Moore, 1901	DQ097218	USA	Phillips and Siddall (2005)
Philobdella floridana (Verrill, 1874)	DQ097219	USA	Phillips and Siddall (2005)
Haemadipsa sylvestris Blanchard, 1894	AF003266	Vietnam	Siddall and Burreson (1998)
Trocheta danastrica Stschegolew, 1938	MT013043	Ukraine	Khomenko et al. (2020)

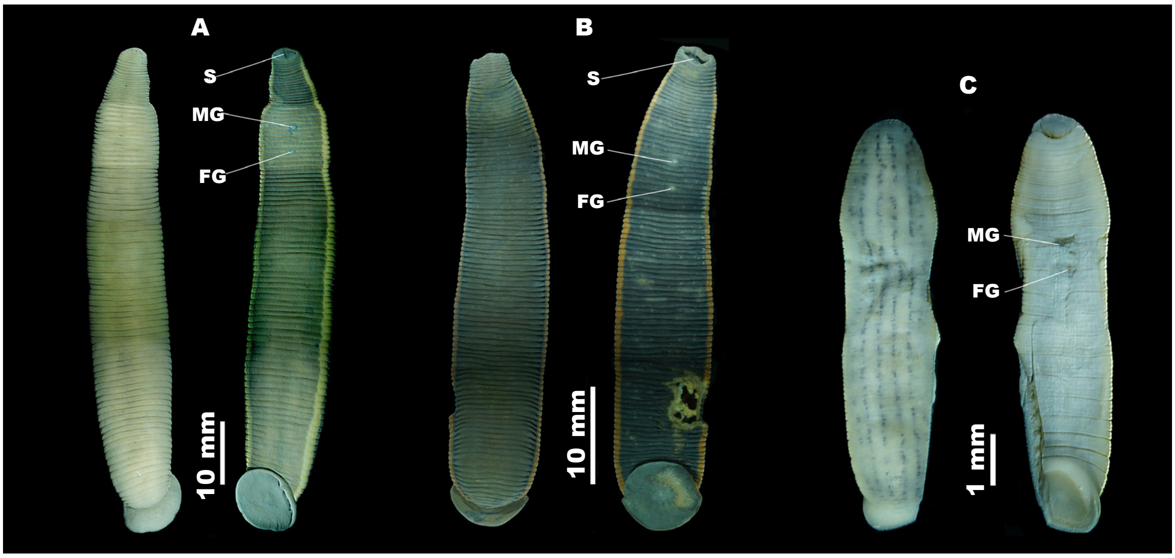

Fig. 1. External characters of leeches of the genus *Limnatis*: A, *Limnatis paluda* from Uzbekistan (Samarqand Region); B, *Limnatis paluda* from Azerbaijan; C, *Limnatis nilotica* from Morocco: FG, female gonopore; MG, male gonopore; S, sulcus. (Dorsal sides are depicted on the left and ventral sides, on the right for each specimen).

Fig. 1. Caracteres externos de las sanguijuelas del género Limnatis*: A,* Limnatis paluda *procedente de Uzbekistán (región de Samarcanda), barra de escala de 10 mm; B,* Limnatis paluda *procedente de Azerbayán; C,* Limnatis nilotica *procedente de Marruecos: FG, gonoporo femenino; MG, gonoporo masculino; S, surco. (La imagen izquierda de cada ejemplar muestra la vista dorsal y la imagen derecha, la vista ventral).*

Phylogeny and genetic differentiation

Phylogenetic analyses show the family Praobdellidae is a well–supported monophyletic group with a posterior probability of 1.00. The genus *Limnatis* is also a well–supported clade. All Middle Eastern, Caucasian and Central Asian samples joined a clade with a posterior probability of 0.78. This monophyletic group matches *L. paluda.* The phylogenetic structure of the clade is simple and shallow. The Namibian *Limnatis* sp. is sister to the clade of *L. paluda* with a posterior probability of 0.98. This group of Namibian and Western Asian leeches is sister to the clade consisting of the Moroccan *L. nilotica* and Croatian *Limnatis* sp. The latter clade is supported by a posterior probability of 0.62 (fig. 2).

The number of base differences per site from averaging sequence pairs within the clade of *L. paluda* is as low as 0.0030 ± 0.0012. Uncorrected distances between the Western Asian *L. paluda*, Balkan *Limnatis* sp., North African *Limnatis* sp. and South African *Limnatis* sp. exceed 0.06 (table 3), suggesting species–level differences between those populations.

Discussion

Morphological examination of the leeches of the genus *Limnatis* suggests the specimens collected in Azerbaijan and Uzbekistan should be assigned to the Middle Eastern and Central Asian *L. paluda*. This identification is based on the morphological features as follows: in contrast to the North African *L. nilotica*, Central Asian and South Caucasian leeches of the genus *Limnatis* are characterised by the monotonous green coloration with no dark dots or lines on the dorsum. The dorsal coloration pattern consisting of black stripes, lines and dots is characteristic of the North African *L. nilotica* (Moore, 1938). The Moroccan specimens have the typical coloration of *L. nilotica.*

The phylogenetic analysis corroborated the morphological identification and revealed a deep differentiation between North African and Western Asian leeches of the genus *Limnatis.* Molecular characterisation of the North African *Limnatis*, which is currently assigned to *L. nilotica* in the strict sense, was performed herein for the first time. All Middle Eastern, Caucasian, Central Asian and Afghan leeches joined the clade of *L. paluda* (fig. 2). This corroborates independent species statuses of the North African and Western Asian populations, both of which had been assigned by some classical authors (Lukin, 1976) to *L. nilotica*. The Namibian *Limnatis* is sister to the *L. paluda* clade and belongs to an unidentified species, which may represent the little–known taxonomical diversity of the genus *Limnatis* in Africa (Moore, 1938). The Moroccan and Croatian leeches are in sister relationships. While the Moroc-

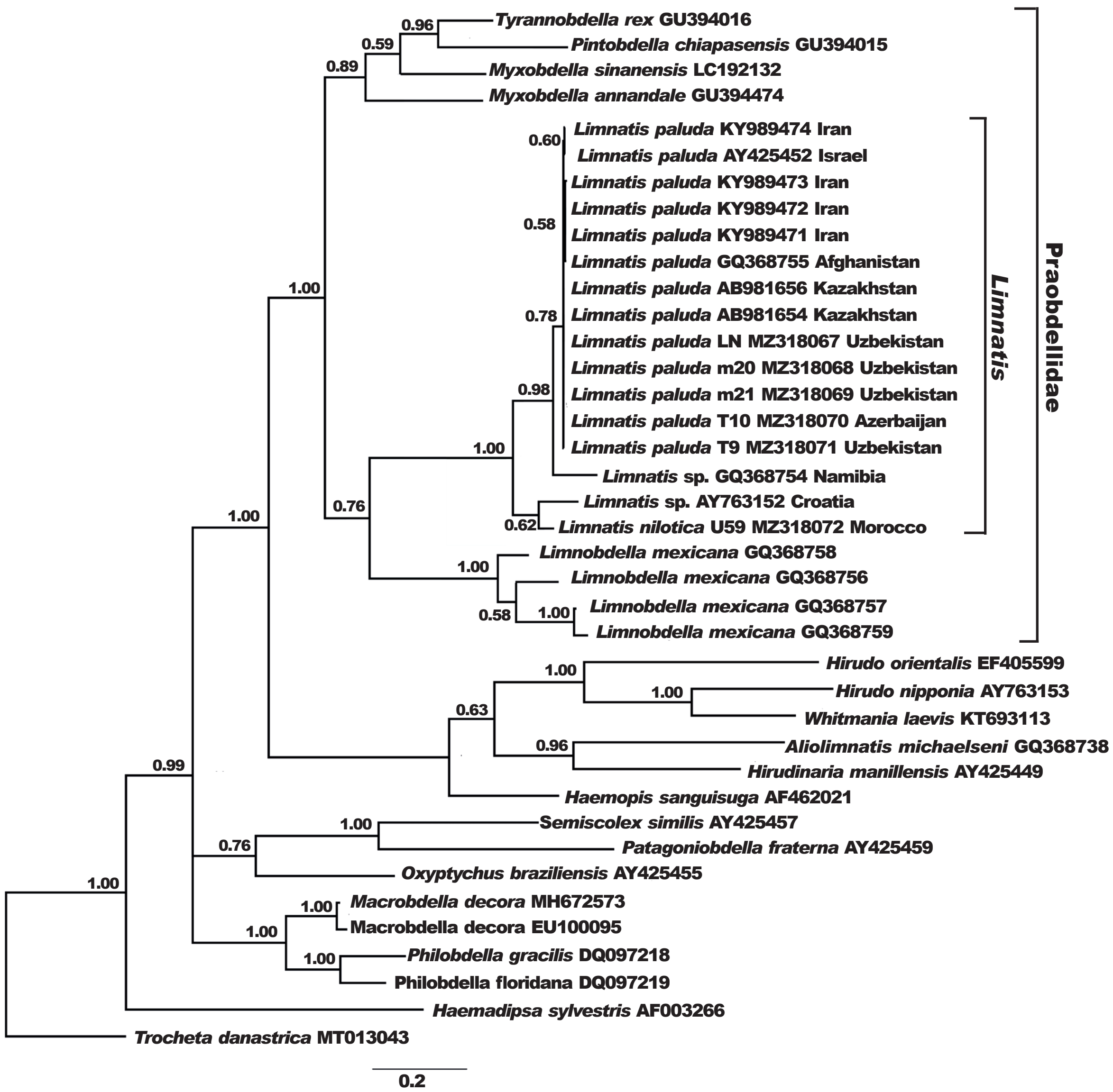

Fig. 2. Bayesian phylogenetic tree of arhynchobdellid taxa, including the genus *Limnatis* and other members of the family Praobdellidae, based on *COI* sequences. Posterior probabilities are shown for clades. The tree is rooted at *Trocheta danastrica.*

Fig. 2. Árbol filogenético bayesiano de los taxones de arrincobdélidos, incluidos el género Limnatis y otros miembros de la familia de los praobdélidos, basado en las secuencias de COI*. Se muestra la probabilidad posterior de los clados. El árbol está enraizado en Trocheta danastrica.*

can specimen should be assigned to *L. nilotica*, the Croatian leech is another unidentified species. The Moroccan–Croatian clade is sister to the clade of *L. paluda* plus the Namibian leech. The phylogenetic relationships imply that the taxonomy of the genus *Limnatis* is far from complete. Further studies of Southern European and South African populations of the genus *Limnatis* (fig. 3) are needed for proper identification. Moreover, the conspecific relationships of Sri Lankan and Western Asian leeches, which are currently assigned to *L. paluda* (Moore, 1927; Phillips and Siddall, 2009; Nakano et al., 2015), should be substantiated using fresh specimens and molecular data. Biogeographically, the occurrence of the same leech species in Sri Lanka and in Western Asia appears questionable.

Despite the vast range of *L. paluda,* the species was found to have low genetic diversity and a shallow phylogenetic structure. This may be explained by the recent colonisation of arid landscapes in Western

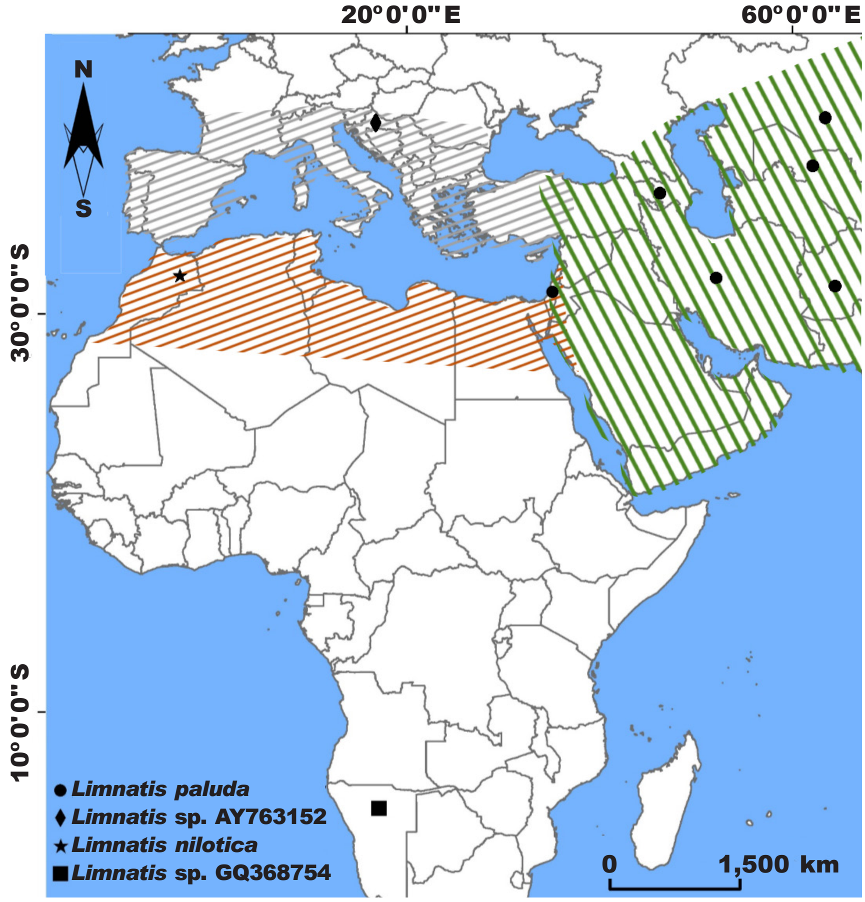

Fig. 3. Geographical distribution of leeches of the genus *Limnatis*: red, *L. nilotica*; green, *L. paluda*; grey, South European *Limnatis* sp.

Fig. 3. Distribución geográfica de las sanguijuelas del género Limnatis*: en rojo,* L. nilotica*; en verde,* L. paluda*; en gris,* Limnatis *sp. del sur de Europa.*

Asia. The range expansion could be attributed to the parasitism of these leeches on their ungulate hosts that appear to be able to transmit their parasites over long distances (Nakano et al., 2015). The eastern medicinal leech *Hirudo orientalis* Utevsky and Trontelj, 2005 is another instance of the rapid colonization of that area, which caused comparable genetic consequences (Trontelj and Utevsky, 2012). Migrations of nomads and their livestock throughout vast territories of the Middle East, Caucasus and Central Asia or

Table 3. Estimates of evolutionary divergence based on p–distances between *COI* sequences of leeches of the genus Limnatis and their standard errors.

Tabla 3. Estimación de la divergencia evolutiva a partir de la distancia entre las secuencias (p–distance) de COI *de las sanguijuelas del género Limnatis y su error estándar.*

	Limnatis paluda	*Limnatis* sp. Namibia	*Limnatis* sp. Croatia
Limnatis sp. Namibia	0.0723 ± 0.0107		
Limnatis sp. Croatia	0.0947 ± 0.0115	0.1192 ± 0.0137	
Limnatis nilotica Morocco	0.0795 ± 0.0103	0.1028 ± 0.0126	0.0628 ± 0.0093

other human activities in that area (as was discussed in Nakano et al., 2015) could contribute to shaping the genetic structure of *L. paluda.* Obviously, more studies are needed to clarify the evolutionary history and to elaborate a robust classification of the leeches of the genus *Limnatis*.

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