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Phylogeny and revision of Erpobdelliformes (Annelida, Arhynchobdellida) from Mexico based on nuclear and mithochondrial gene sequences.

Filogenia y revisión de los Erpobdelliformes (Annelida, Arhynchobdellida) de México, con base en secuencias de ADN nuclear y mitocondrial.

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Abstract. The phylogenetic relationships of the suborder Erpobdelliformes, a group of non-sanguivorous leeches, were investigated with the use of mitochondrial cytochrome *c* oxidase subunit I, mitochondrial 12S rDNA and nuclear 18S rDNA. The resulting hypothesis indicates that Erpobdellidae and Salifidae are monophyletic and each other closest relatives. We detect, for first time in leeches, intra-specific variation of similar amount than inter-specific variation. We formally resurrect the name *Erpobdella mexicana*, proposed by Dugès for Mexican specimens, and recommend the use of the name *Erpobdella ochoterenai* rather than *Erpobdella microstoma* for Mexican specimens. We record an invasive species of the family Salifidae: *Barbronia arcana* in Mexico, representing the first record of the species outside Australia, first record of the family in Mexico and third in the New World.

Key words: Hirudinea, leeches, Erpobdellidae, Salifidae, *Erpobdella*, *Barbronia* , COI, 12S, 18S, México, Barcoding of life.

Resumen. Se estudian las relaciones filogenéticas del suborden Erpobdelliformes, un grupo de sanguijuelas no hematófagas de vertebrados, con base en secuencias de la subunidad I del citocromo c oxidasa del ADN mitocondrial, 12S ADNr del ADN mitocondrial y 18S ADNr del ADN nuclear. La hipótesis resultante señala que las familias Salifidae y Erpobdellidae son monofiléticas y hermanas entre sí. Se detecta por primera vez en sanguijuelas variación interespecífica de magnitud similar a la variación interespecífica. Formalmente se restablece el nombre empleado por Dugès: *Erpobdella mexicana* para las formas mexicanas, así como se argumenta sobre el uso del nombre *Erpobdella ochoterenai* en lugar de *Erpobdella microstoma* para las formas mexicanas. Se registra a una especie invasora de la familia Salifidae en México: *Barbronia arcana*, el cual constituye el primer registro de la especie fuera de Australia, primer registro de la familia en México y tercero en el continente americano.

Palabras clave: Hirudinea, sanguijuelas, Erpobdellidae, Salifidae, *Erpobdella*, *Barbronia*, COI, 12S, 18S, México, Código de Barras genético.

Introduction

Erpobdelliform leeches are macrophagous predators of aquatic invertebrates including arthropods, mollusks and annelids, having abandoned the blood feeding habits of their ancestors (Siddall and Burreson, 1998; Apakupakul et al., 1999; Trontelj et al., 1999; Borda and Siddall, 2003). Members of the Erpobdellidae Blanchard, 1894, common in North America and Europe, are characterized by their possession of multiple testisacs per segment. The other family of erpobdelliforms, Salifidae Johansson, 1910, are common in Asia, Africa and Australia, and typically are characterized by their possession of pharyngeal stylets, few testisacs per somite, gastropore and/or post-cephalic eyespots (Sawyer, 1986). Genera in Erpobdellidae (*Erpobdella, Dina, Mooreobdella, Trocheta, Nephelopsis, Motobdella* and *Croatobranchus*) were established principally on annulation pattern, presence or absence of preatrial loops in the male reproductive system, presence or absence of gastric caeca and of body appendages. Recent phylogenetic studies based on morphology and DNA sequence data showed that a radical revision of the family was required because the morphological characters used to distinguish groups are not informative with respect to phylogenetic affinities (Trontelj and Sket, 2000; Siddall, 2002). For this reason, Siddall (2002) formally synonymized all the genera of Erpobdellidae with *Erpobdella*. Molecular and morphological phylogenetic

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analysis of Arhynchobdellidae (Borda and Siddall, 2003) confirmed the sister relationship between Erpobdellidae and Salifidae, although a single 18S rDNA sequence of *Barbronia weberi* was available to represent the family Salifidae.

In Mexico, three erpobdellid species have been found: *Erpobdella triannulata* Moore, 1908; *Erpobdella ochoterenai* (Caballero, 1932), described as *Herpobdella* (sic) *ochoterenai*, transferred to the genus *Mooreobdella* by Sawyer and Shelley (1976) due the absence of preatrial loops in the male reproductive system, and later transferred to *Erpobdella* by Siddall (2002). López-Jiménez (1985) considered that *Erpobdella ochoterenai* should be considered as a junior synonym of *Mooreobdella microstoma*, criteria followed by Badillo-Solís et al. (1998); and finally, *Erpobdella punctata* (Leidy, 1870). Mexican specimens of *E. punctata* were described as *Nephelis mexicana* Dugès 1876. Specimens of *Nephelis mexicana* were deposited in the United States National Museum and in the Musée d´Histoire Naturelle of Paris. Moore (1898) studied specimens of the first collection and considered that *Nephelis mexicana* is synonym of *Dina quadristriata*. Soos (1966) considered both: *Nephelis mexicana* and *Dina quadristriata* as synonyms of *Dina lineata*. Ringuelet (1976) revised the material from both collections and concluded that Mexican specimens correspond to *Erpobdella punctata* and latter named them as *Erpobdella punctata mexicana* (Ringuelet, 1981). The subspecific status of Mexican specimens is based on the presence of a curve of each ejaculatory duct previous to the respective cornua (horn, seminal vesicle). Oka (1932) recorded *Herpobdella lineata* and *Herpobdella octoculata* from Mexico but Caballero (1937) considered both records as *Herpobdella punctata*.

No native salifid species is known to occur in Mexico, but recently, *Barbronia weberi* (Blanchard, 1897), an invasive leech from Asia, was recorded in Brazil and USA (Pamplin and Rocha, 2000; Rutter and Klemm, 2001). The aim of this study is to investigate the taxonomic validity and phylogenetic affinities of Mexican Erpobdelliformes using molecular data.

Material and methods

We collected specimens from ten localities from 2003 to 2005, belonging to four species of Erpobdelliformes (Scientific Collecting License FAUT0056 to VLR). All specimens were found attached to submerged rocks and plants, collected by hand and fixed in 4% formalin or 96% ethanol, stored in 70% ethanol. Voucher specimens are deposited in the "Colección Nacional de Helmintos" (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México. We re-analysed the phylogenetic relationships of Erpobdelliformes with the newly collected material, using sequences of two mitochondrial and one nuclear gene. Sequences of mitochondrial cytochrome *c* oxidase subunit I, mitochondrial 12S and nuclear 18S rDNA of ten specimens from Mexico were generated in the present study. Sequences from 13 species of Erpobdelliformes from previous phylogenetic analyses were included in the present analyses. Outgroup taxa were selected based on previous phylogenetic hypotheses (Siddall, 2002; Borda and Siddall, 2003); they comprise species of the Hirudiniformes (*Cylicobdella coccinea, Haemopis sanguisuga, Macrobdella decora*) and *Americobdella valdiviana* (Table 1).

DNA extraction and purification. Specimens were stored in 100% ethanol until used for DNA extraction. Tissue from the caudal sucker was used in order to minimize the possibility of contamination from prey DNA found in the gastric and intestinal region. Standard phenol-chloroform extraction methods were used to recover DNA from specimens. Laboratory protocols followed Hillis et al. (1996) and Palumbi (1996).

Nuclear and mithochondrial DNA sequence amplification. PCR amplifications of nuclear 18S rDNA, mitochondrial 12S rDNA and partial cytochrome *c* oxidase subunit I (COI) were used for the molecular phylogenetic study. To obtain 18S rDNA fragments, the primers pairs "AL","CY" and "BO" were used yielding three overlapping double stranded DNA fragments of approximately 600 base pairs (bp) each. (Apakupakul et al., 1999). Primers used to amplify 18S rDNA, 12S and COI are shown in Table 2. Amplification reactions contained 0.625 units of Amplificasa (Biogenica), 2.5 µl of 10X buffer, 1.5 mM of magnesium chloride 20X, 2 mM of each dNTP (8 mM total), 1 µm of each primer, 1 µm of template and distilled, sterilized water to 25 µl. Reactions were accomplished with thermocycler Mastercycler® gradient 5331 (Eppendorf Scientific).

The following amplification protocols were used: 18S-heated to 94 °C for 5 min, followed by 35 cycles of 94 °C (15 s), 44 °C (20 s), and 70 °C (90 s) and a final extension at 72 °C for 7 min; 12S-heated to 94 °C for 5 min, followed by 30 cycles of 95 °C (1 min), 52 °C (1 min), and 70 °C(1 min) and final extension at 72 °C for 7 min; and COI heated to 94 °C for 5 min, followed by 15 cycles of 94 °C (45 s), 47 °C (45 s), and 72 °C (45 s), then 25 cycles of 94 °C (20s), 45 °C (20 s), and 72 °C (30 s) and final extension at 72 °C for 6 min. The QIAquick PCR Purification Kit protocol (Qiagen) was used to purify amplification products.

DNA sequencing. Amplification products were sequenced in both directions. Each 10µl sequencing reaction mixture included 2µl BigDye (Applied Biosystems), 2µl of Dye 'extender' buffer (1 M Tris, pH 9; 25 mM $MgCl_2$), 0.25 µl of 10 µM primer and 3 µl of gene amplification product. Samples were sequenced in a thermocycler Mastercycler® gradient 5331 (Eppendorf Scientific). Samples were purified in Centrisep Spin Columns (Princeton separations) and electrophoresed in an ABI Prism 310 sequencer.

DNA sequence alignment. Sequence of complementary strands were edited and reconciled with Sequence Navigator

Table 1. Sequences included in this study, sampling sites and Genbank accesion numbers; + specimens collected in this study.

Taxon	Locality	12S	18S	COI
Barbronia arcana +	Amacuzac river, Morelos, Mexico	DQ235588	DQ235608	DQ235598
Barbronia weberi	Lake Milstatt, Austria	-	AF099951	-
Erpobdella bucera	Michigan, USA	AF462026	AF115998	AF116024
Erpobdella costata	Georgia, USA	AY425442	AY425478	AY425460
Erpobdella dubia	Michigan, USA	AF462022	AF115997	AF116023
Erpobdella japonica	Korea	AF462023	AF116000	AF116026
Erpobdella lineata	Denmark Fakse/Falster	AF099952	AF099950	-
Erpobdella melanostoma	Michigan, USA	AF462027	AF115999	AF116025
Erpobdella mestrovi	Croatia	-	AF272842	-
Erpobdella obscura	Ontario, Canada	AF462028	AF116004	AF003276
Erpobdella ochoterenai+	Xochimilco, México City	DQ235586	DQ235606	DQ235593
Erpobdella ochoterenai+	Totolcingo, Tlaxcala, Mexico	DQ235593	DQ235613	DQ235603
Erpobdella ochoterenai+	Ameca river, Jalisco, Mexico	DQ235589	DQ235609	DQ235599
Erpobdella ochoterenai+	La Vega, Jalisco, Mexico	DQ235590	DQ235610	DQ235600
Erpobdella octoculata	France	AF099954	AF116001	AF003274
Erpobdella punctata	Ontario, Canada	AF462024	AF116002	AF003275
Erpobdella punctata mexicana+	Atlangatepec, Tlaxcala, Mexico	DQ235591	DQ235611	DQ235601
Erpobdella punctata mexicana+	La Olla dam, Guanajuato, Mexico	DQ235587	DQ235607	DQ235597
Erpobdella punctata mexicana+	Fuentes Brotantes, México City	DQ235585	DQ235605	DQ235595
Erpobdella testacea	France	AF462025	AF116003	AF116027
Erpobdella triannulata+	Catemaco lake, Veracruz, Mexico	DQ235592	DQ235612	DQ235602
Erpobdella triannulata+	El Espino, Tabasco, Mexico	DQ235594	DQ235614	DQ235604
Americobdella valdiviana	Chile	AY425407	AY425461	AY425443
Cylicobdella coccinea	Bolivia	AY425462	AY425362	AY425444
Haemopis sanguisuga	Sweden	AF099960	AF09941	AF462021
Macrobdella decora	Michigan, USA	AY425431	AF116007	AF003271

Table 2. Primers used for PCR amplification and sequencing.

Gene	Primer name	Primer sequence
18s rDNA	A	5´-AACCTGGTTGATCCTGCCAGT-3´
	L	5´-CCAACTACGAGCTTTT-3´
	C	5´-CGGTAATTCCAGCTC-3´
	Y	5´-CAGACAAATCGCTCC-3´
	B	5´-TGATCCTTCCGCAGGTTCACCT-3´
	O	5´-AAGGGCACCACCAG-3´
COI	LCO1490	5´-GGTCAACAAATCATAAAGATATTGG-3´
	HCO2198	5´-TAAACTTCAGGGTGACCAAAAAATCA-3´
12S rDNA	12S-AI	5´-AAACTAGGATTAGATACCCTATTAT-3´
	12-BI	5´-AAGAGCGACGGGCGATGTGT-3´

(Bioedit). Alignments of the 18S rDNA and 12S rDNA gene sequences were acomplished using Clustal W. 18S sequences vary from 1804 to1859 bp, the resulting alignment was 1888 positions. 12S rDNA sequences vary from 334 to 367 bp, the resulting alignment was 381 positions. Alignment of 649 bp of COI was done by eye across all taxa because there were no insertions or deletions.

Phylogenetic analyses. Parsimony analyses were performed using PAUP* 4.0b10 (Swofford, 2000). Heuristic search used 1000 replicates of random taxon addition and tree-bisection-reconnection branch swapping. All charachters were unweighted and non-additive. Bootstrap values were obtained with PAUP* 4.0b10 (Swofford, 2000). AutoDecay ver. 4.0 (Eriksson, 1998) was used to calculate Bremer support values (Bremer, 1988). Consistency and retention indices were calculated with PAUP* (Swofford, 2000).

Results

Material examined. Four leeches from Laguna Texhuil, Xochimilco, Mexico City (CNHE 5328), 28 July 2004; 5 specimens from Totolcingo lake, Tlaxcala (CNHE 5326), 22 August 2002; 13 specimens from Ameca River, Jalisco (CNHE 5327), 19 September 2002; 79 specimens from Ameca River, near La Vega dam, Jalisco (CNHE 5325), 9 November 2003. Each specimen with one pair of labial eyespots and two pairs of bucal eyespots. A mid-dorsal black line along the body occurs in almost all cases; in some specimens, an additional pair of marginal lines along the body are visible. Male gonopore on XII b_2/a_2, but in some specimens are displaced to b_2. Female gonopore between somites XII and XIII. Three or three and a half annuli between gonopores. Male reproductive system without preatrial loops. This suite of morphological features is consistent with Caballero´s (1932) description of *Herpobdella ochoterenai.*

Nine leeches from Atlangatepec lake, Tlaxcala (CNHE 5323), 21 August 2002. Six specimens from Parque Nacional Fuentes Brotantes, Tlalpan, Mexico, D. F. (CNHE 5324), 8 April 2004. Four leeches from La Olla dam, Guanajuato (CNHE 4702, 5354), 6 February 2003. Each specimen with one pair of labial eyespots and two pairs of bucal eyespots. One pair of paramedian dorsal black lines. Male gonopore on somite XII b_2/a_2, Female gonopore on XII b_5/b_6. Two annuli between gonopores. Male reproductive system provided with a preatrial loop to ganglion XI and a curve of each ejaculatory duct previous to the respective cornua. This suite of morphological features is consistent with the description of Dugès of *Nephelis mexicana.*

Four leeches from Catemaco Lake, Veracruz (CNHE 5330), 9 August 2002. Five leeches from El Espino, Tabasco (CNHE 4701, 5355). Each specimen with one pair of labial eyespots and two pairs of bucal eyespots. A wide mid-dorsal black line along the body occurs and an additional pair of marginal lines along the body are visible in specimens from Veracruz. Specimens from Tabasco, with one pair of paramedial dorsal black lines. Male gonopore on somite XII b_1/b_2. Female gonopore on XII b_5/b_6. Three annuli between gonopores. Male reproductive system provided with a preatrial loop to ganglion XI. This suite of morphological features is consistent with Moore´s (1908) description of *Erpobdella triannulata* and redescription by López-Jiménez (1985).

Three leeches form Amacuzac River, Morelos (CNHE 5342), 14 September 2003. Each specimen with one pair of labial eyespots and two pairs of bucal eyespots. One pair of diffused marginal dark lines. Two copulatory pores on X/XI and XIII/XIV respectively. Male gonopore on somite XII b_1. Female gonopore on XII/XIII. Without pharyngeal stylets. A pair of lateral postcaeca in the posterior part of the caeca. This suite of morphological features is consistent with the redescription of *Barbronia arcana* of Govedich et al. (2002)

Molecular data. Genetic divergence among distant populations (> 500 km) of *E. ochoterenai* ranges between 8.6-11.6 % in COI, 0.2-0.3% in 18S and 4.7-9.4% in 12s, while divergence among sequences of sister species, easily distinguishable based on morphological characters, like *Erpobdella punctata* and *E. melanostoma,* is 14.2% in COI, 0.8% in 18s and 5.5% in 12s. The same phenomenon is observed among populations (separated >150 km from each other) of *E. mexicana* (divergence ranging from 4.2-11.9% in COI, 0-0.2% in 18s and 4.1-10.5% in 12s).

Parsimony analysis of 649 nucleotides of COI resulted on a single tree of 1236 steps, CI= 0.38 and RI=0.34. This unique tree failed to recognize the monophyly of Erpobdelliformes. Parsimony analysis of 18S rDNA alone (1888 characters) resulted in 97 equally most-parsimonious trees, each of which had 720 steps; CI=0.74; RI=0.76. The strict consensus of the 97 trees recognized the major groups of Erpobdelliformes, but showed no resolution in terminal taxa. Parsimony analysis of 12S rDNA alone resulted in 3 trees, of 699 steps, CI=0.57 and RI=0.34. The strict consensus of those trees recognized the major groups of Erpobdelliformes. Parsimony analysis of all available data, 2918 characters from the three molecular data sets yielded one most-parsimonious tree (Figure 1); 2720 steps long, CI=0.52 and RI=0.52.

Discussion

The result of the phylogenetic analysis of Erpobdelliformes from Mexico based on two mitochondrial and one nuclear gene sequences, using *Americobdella valdiviana* and three Hirudiniformes as outgroups, support the phylogenetic results of Siddall (2002) and Borda and Siddall (2003). These authors found two clades of North American erpobdellids, one of them bearing two pairs of labial eyespots, while species included in the other present only one pair, a plesiomorphic condition for Erpobdelliformes. Mexican Erpobdellidae,

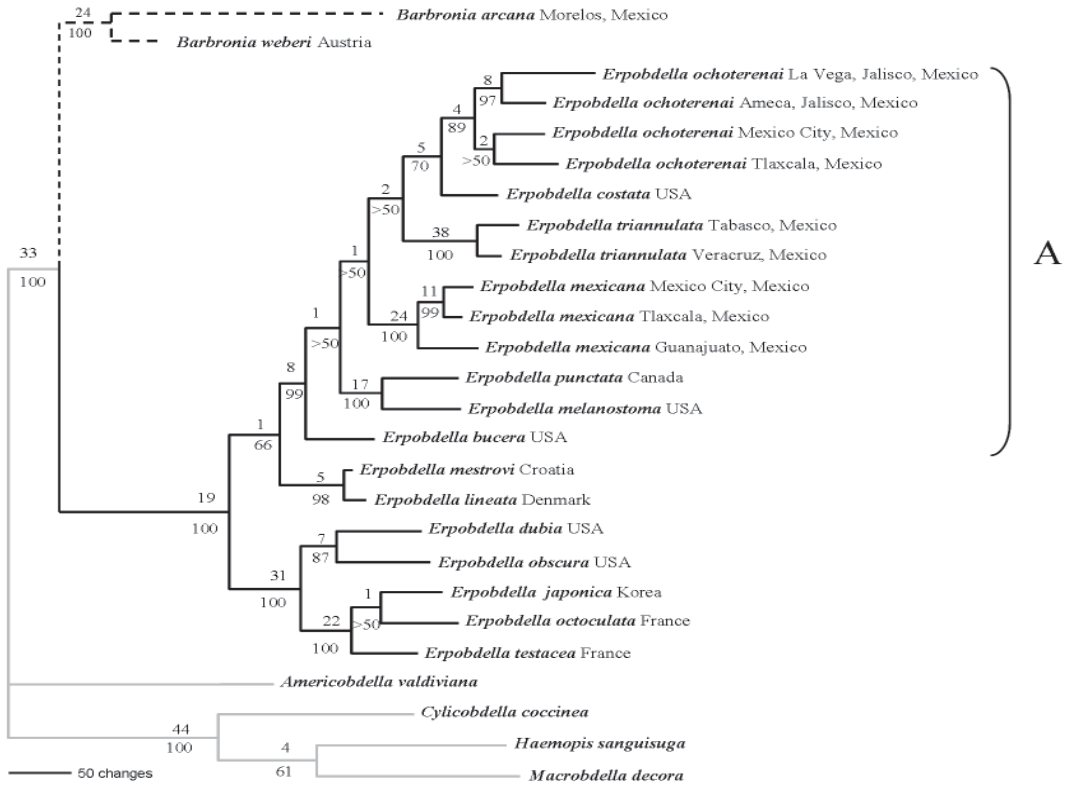

Figure 1. Optimal tree resulting from parsimony analysis of the combined 18S, 12S and COI sequences. Numbers above and below nodes indicate Bremer support and Bootstrap values respectively. Branch length is proportional to amount of change. Outgroup taxa in grey. Dotted lines indicate the family Salifidae and family Erpobdellidae on solid dark lines. (A) Members of the family Erpobdellidae from North America, bearing one pair of labial eyespots.

which also exhibit one pair of labial eyespots, group within the later (Figure 1), previously the genus *Mooreobdella*, but for which there is no obvious morphological synapomorphy (Siddall, 2002).

Three samples of *Erpobdella punctata mexicana* from Mexico included in this analysis appear in a single strongly supported clade, separated from *Erpobdella punctata* from Canada, that appears as the sister species of *E. melanostoma*. Based on this, there is no reason to consider Mexican specimens as a synonym or subspecies of *E. punctata*. We formally resurrect the specific epithet *mexicana* of Dugès to the Mexican specimens: *Erpobdella mexicana* (Dugès, 1876). Even though *E. mexicana* and *E. punctata* are very similar in the presence of one pair of labial eyespots, two annuli between gonopores and preatrial loops in the male reproductive system, *E. mexicana* is easily distinguishable based on the presence of a curve in each ejaculatory duct anterior to the respective cornua. According to our results, previous records of *E. punctata* from Mexico must be transferred to *E. mexicana*. However, records of Oka (1932) considered to be *Herpobdella punctata* by Caballero (1937) have to be re-evaluated; Oka argued that his *Herpobdella lineata* and *H. octoculata* each have three annuli between gonopores, which clearly is not the condition of *H. mexicana*. Therefore, those records should be considered as *Erpobdella ochoterenai*, the species that Caballero described five years earlier (Caballero, 1932).

The four samples of *Erpobdella ochoterenai* from different localities of Mexico appear in a single strongly supported clade. The sister relationship between *E. ochoterenai* (without preatrial loops in the male reproductive system), with *Erpobdella costata* (presenting preatrial loops), confirms the poor systematic value of this morphological character. Other species without preatrial loops include: *Erpobdella melanostoma. E. bucera* and *E. lineata,* none of which group together.

As noted before (Sawyer and Shelley, 1976; Klemm, 1982), *E. ochoterenai* is difficult to distinguish from *Erpobdella microstoma* from USA because both species show three annuli between gonopores and lack preatrial loops in the male reproductive system. López-Jiménez (1985) suggested that *Erpobdella ochoterenai* should be

a junior synonym of *E. microstoma*. Revision of Mexican specimens revealed that a mid-dorsal black line along the body occurs in almost all cases, even in fixed specimens. In some specimens, an additional pair of marginal lines along the body are visible. Moore´s original description of *Erpobdella microstoma* states "Not one of many examples of both young and old shows any pigment. This would indicate that during life they are red, the color of the blood showing through the integuments" (Moore, 1901). In a more recent account of USA leeches, Klemm (1982) argued that *E. microstoma* lacks black pigments. Despite no molecular data of *E. microstoma* being available to compare with mexican specimens, the use of the name *E. ochoterenai* for Mexican forms is strongly recommended.

Erpobdella triannulata is represented in this analysis by two samples that appear together in a clade with high bootstrap and Bremer values. *Erpobdella triannulata* is the member of the family that shows the most southern distribution and appears basal in the same clade with *E. costata* from Georgia and *E. ochoterenai* from Mexico. Bootstrap and Bremer support values are very low in basal branches of this clade, making any biogeographical interpretation premature. Also, geological history of Mexican territory has been very complex, producing extremely complicated biogeographic patterns (Marshall and Liebherr, 2000; Brooks, 2005). Additional samples from a wider geographical representation, especially those from the Southwestern United States, like those of the genus *Motobdella* (Davies et al., 1985; Govedich et al., 1998), are needed in order to clarify the biogeographic history of this group.

Based on the available data, it is impossible to distinguish if the large amount of genetic divergence among populations of *Erpobdella mexicana* and *E. ochoterenai* corresponds to intraspecific variation or if they are in fact cryptic species complexes; the ultimate determination of which may have substantial implications for ongoing efforts in DNA barcoding of the world's leech fauna (e.g., Siddall and Budinoff, 2005; DeSalle et al., 2005). Sampling of additional populations of these species is needed in order to clarify this question. Notably, sequence information is known only for single specimens of the taxa previously investigated by Siddall (2002) and by Trontelj and Sket (2000). Whether this degree of intraspecific variability in Erpobdelliformes is a general characteristic of the group would be revealed by more extensive sampling of multiple populations of other species in the genus. Species delimitation and identification on the basis of DNA-barcodes, typically relying on the CO-I locus, are predicated on there being a marked disparity between intraspecific and interspecific genetic variation. As such, barcode of life initiatives must be wary of conditions where that disparity is absent. A case already is known from leeches, where there is a lack of intraspecific genetic distance among species of *Theromyzon* (Siddall et al., 2005). Herein, we are seeing a case where apparently interspecific and intraspecific genetic distances are of similar magnitude for species of *Erpobdella* (Fig. 1). Notably, and unlike the DNA barcode-based delimitation of species of *Astrapes* (Lepidoptera) in Area Guanacaste, Costa Rica (Hebert et al., 2004), our evaluation of Mexican *Erpobdella* species has considered a much larger geographic range (> 500 km). It is necessary to explore the entire distribution range of these taxa in a continuous manner to ensure that resulting discontinuities are in fact delimiting cryptic species and are not artifacts of discontinuous sampling.

Because only species of Erpobdellidae are distributed naturally in the New World, preliminary comparisons of specimens from Amacuzac River in Morelos were done only with members of this family, but clear morphological differences were detected, like one pair of accessory copulatory pores in the ventral mid-line, one anterior and one posterior to the male and female gonopores. A salifid species, *Barbronia weberi* Blanchard, 1897 from India also presents accesory copulatory pores and is well known as a widespread invasive species in all continents (Moore, 1946; Mason, 1976; Pamplin and Rocha, 2000; Rutter and Klemm, 2001; Govedich et al., 2002). A detailed analysis of internal morphology revealed some differences between our specimens and *Barbronia weberi*. Our specimens present a pair of crop caeca and lack pharyngeal stylets, contrary to *B. weberi* that lacks crop caeca and presents pharyngeal stylets. These characteristics make our specimens identical to *Barbronia arcana* from Australia. The position of *Barbronia arcana* in the cladogram, as sister species of *Barbronia weberi,* confirms the morphological observations. The current distribution of *B. arcana* in Mexico is unknown, however, specimens were found in the northern Balsas River tributary. Balsas River drains into the Pacific Ocean (Tamayo and West, 1964). Based on known ecological characteristics of *B. weberi*, like rapid growth and the ability of adults and cocoons to be transported by aquatic plants (Govedich et al., 2003), we can expect that *B. arcana* could be dispersed in almost all the Balsas River. Obviously, this remains to be confirmed. This is the first record of *Barbronia arcana* outside Australia and the third record of the family Salifidae in the New world. The position of *B. arcana* in the cladogram corroborate the sister relationship between Erpobdellidae and Salifidae and the monophyly of the Suborder Erpobdelliformes. Additional samples from a wider taxonomic representation of Salifidae, are needed in order to establish the phylogenetic relationships of this group.

Geographic distributions of clades of erpobdelliform leeches reflect vicariance patterns seen for other non-blood-feeding leeches in the family Glossiphoniidae (Siddall et al., 2005). Specifically, there is a pair North American / Eurasian sister group relationships in the genus *Erpobdella* represented, on the one hand, by *E. dubia* and *E. obscura* sister to the Eurasian *E. octoculata* group, and on the other hand, the European *E. lineata* and *E. mestrovi* sister to the

North American remainder of the genus. Notably too, more basal lineages of Erpobdelliformes retain a distribution restricted to Gondwanan continents (with the exception of recent introductions of *B. weberi*). Taken together these patterns imply an origin of the genus *Erpobdella* following the opening of the Tethys (~175 Mya) with simultaneous isolation of North American and European taxa with the rifting of Laurasia and the opening of the North Atlantic (~100 Mya).

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