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Molecular evidence that *Langeronia macrocirra* and *Langeronia* cf. *parva* (Trematoda: Pleurogenidae) parasites of anurans from Mexico are conspecific

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Abstract

The genus *Langeronia* parasitizing the intestine of several species of anurans is distributed from North to Central America. We identified *Langeronia macrocirra* and *Langeronia* cf. *parva* from the same host and localities, and present here new data not applicable about their tegumental surface by scanning electron microscopy. We compared sequences of the rDNA ITS2 region and mtDNA *cox1* gene for the two morphotypes. ITS2 exhibited a high degree of conservation. Phylogenetic reconstruction using *cox1* revealed three clades (I, II, and III), which did not correspond to a previous identification or host. Little divergence was found within clades: sequences were identical in clade I, whereas clade II had 0.27% and clade III had 1.08%. Inter-clade divergence reached 8.69% (I vs. III). This pattern of genetic divergence indicated that both taxa probably belong to the same species, so we posit that the morphological changes could be correlated with development. Increasing sample size and geographical coverage will contribute to the taxonomy of the genus based on morphological and molecular evidence, and will open tracks toward the use of DNA barcodes to the genus in Mexico.

Keywords: *Digenean, parasites, morphology, scanning electron microscopy, anurans, cytochrome* c *oxidase I*

Introduction

The genus *Langeronia* includes six species distributed from North America to Central America, and occurs in the intestine of several anurans (*Lithobates*, *Rhinella*, and *Incilius*): *Langeronia macrocirra*, *Langeronia provitellaria*, *Langeronia parva*, *Langeronia jimenezi*, *Langeronia brenesi*, and *Langeronia burseyi*. Two species have been recorded in México: *L. macrocirra* parasitizing several species of *Lithobates* spp., *Rhinella marina*, and *Incilius valliceps* (Caballero and Bravo-Hollis 1949; Guillén-Hernandez et al. 2000; Bursey and Goldberg 2001; Paredes-Calderón et al. 2004; Espínola-Novelo and Guillén-Hernández 2008; Cabrera-Guzmán et al. 2010; Yáñez-Arenas and Guillén-Hernández 2010), and *L. jimenezi* parasitizing *Lithobates berlandieri* (Iruegas-Buentello and Salinas-López 1989; León-Règagnon et al. 2005). Little is known about the biology of this genus, and only the life cycle of *L. brenesi* is known (Goodman 1989). Morphological differentiation among species is based on a combination of characters such as body shape, pharynx, intestinal ceca, genital pore, and the distribution of vitellaria (Dailey and Goldberg 2000). The identification is difficult due to variability and a lack of distinctive characters to discriminate species (Ubelaker 1965). For this reason, some species have been misidentified, e.g. specimens originally determined to be *L. macrocirra* that were later transferred to *L. provitellaria* by Christian (1970). Morphology alone is not enough to distinguish species in several groups; therefore, new sources of characters are being explored for digeneans, such as the body surface using scanning electron microscopy (SEM) or DNA (León-Règagnon et al. 2001; Razo-Mendivil et al. 2008).

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Several molecular studies have been conducted using sequences of nuclear rDNA (internal transcribed spacer 2, ITS2) or mitochondrial DNA (cytochrome *c* oxidase subunit I, *cox1*) to determine relationships among species on parasites (Bell et al. 2001), species identification (Dallas et al. 2000; Bell and Sommerville 2002), discovery of genetically distinct but morphologically very similar species or species complex (McManus and Bowles 1996; Razo-Mendivil et al. 2010), as well as to complement taxonomic descriptions or re-descriptions (León-Règagnon et al. 2001; Razo-Mendivil et al. 2008).

A molecular study might be pertinent in the search for evidence if one is dealing with a single species and is detecting genetic variation levels. This is one of the objectives of a biodiversity research program that is currently extending around the world and is being developed in Mexico: The International Barcode of Life Initiative (http://www.barcodeoflife.org; http://www.mexbol.org/). This initiative consists of developing a DNA-based taxonomic system-based DNA barcode using partial sequences of the mitochondrial *cox1* gene, which provides strong species-level resolution (Hebert et al. 2003; Tautz et al. 2003).

Although several studies have employed partial sequences of *cox1* in digeneans (McManus and Bowles 1996; Bell et al. 2001; Razo-Mendivil et al. 2010), this fragment does not correspond to the specific region proposed for DNA barcodes (Folmer et al. 1994; Casiraghi et al. 2004); however, DNA barcodes have recently been explored systematically with the design of specific primers for digeneans (Moszczynska et al. 2009). For other parasite groups, successful amplification of the barcode region has been achieved (Casiraghi et al. 2004; Oceguera-Figueroa et al. 2005; Junker et al. 2010). No universal primers have been developed able to successfully amplify this region across every phylum and new efforts should focus on testing these new primers proposed by Moszczynska et al. (2009). In spite of this impediment, the antecedents regarding the performance of the other *cox1* fragment are valuable information (e.g. McManus and Bowles 1996), because this could represent an initial and complementary source for progress in biodiversity inventories through DNA barcodes of helminths.

As part of a research program to establish an inventory of the helminth fauna of amphibians and reptiles in Mexico, we analyzed specimens of *Langeronia* from several localities for morphological and molecular studies in order to contribute to the taxonomy of the genus.

Materials and methods

Samples and morphological study

From November 1998 to May 2006, *Lithobates vaillanti*, *L. Berlandieri,* and *R. marina* were collected by hand or using nets in several localities in Mexico (Table I). Hosts were killed with an overdose of anesthetic, and all organs were examined under a stereomicroscope. Digeneans were removed from the intestine of their hosts and placed in 0.65% w/v saline solution, and afterwards were killed by sudden immersion in hot 70% ethanol to preserve morphological traits for further identification. These were stained with Meyer's paracarmine and or Gomori's trichrome, cleared in methyl salicylate, and mounted in Canada balsam. Measurements are given in millimeters unless otherwise stated. Minimum and maximum values are given followed by the arithmetic mean and SD in parentheses (data not shown). For SEM, specimens were stored in 4% formalin, dehydrated in series of gradual alcohol baths, and critical-point dried. Specimens were coated with a gold–palladium mixture and examined in a Hitachi S-2460N, Tokyo, Japan at 15 kV, scanning electron microscope. For taxonomic determination at the species level, we used original descriptions and specialized literature.

Voucher specimens were deposited at the Coleccion Nacional de Helmintos, Instituto de Biología, UNAM, Mexico City (CNHE), with the following accession numbers: *L. macrocirra* from *L. vaillanti*: Laguna Escondida, Veracruz (4874, 4891), San José Independencia, Oaxaca (4876), Chapultepec (4877), Río Pizote, Guanacaste, Costa Rica (7226); from *L. berlandieri*: La Selva Biological Station, Costa Rica (7227), El Petén (7228); and from *R. marina*: Lago de Catemaco, Veracruz (4875, 4892), Salto de Eyipantla, Veracruz (7229); Coquimatlán (4883, 4884, 4895, 4896); and *Pseudosonsinotrema chabaudi* Caballero and Caballero 1969 from *L. vaillanti*: Río Pizote, Guanacaste, Costa Rica (7230). Specimens of genus *Langeronia* from Colección de Helmintos de Costa Rica, Universidad de Costa Rica (CHCR), US National Parasite Collection (USNPC, Beltsville, MD, USA) and CNHE were also examined during this study for comparison: *L. macrocirra* (CNHE 1127, 1525, 1526, 1527, 3307, 3631, 4092, 4093, 4891, 13885; CHCR 200-19, 200-19bis, USNPC 89185), *L. provitellaria* (USNPC 47569, 47570), *L. parva* (USNPC 70557, 70558), *L. brenesi* (USNPC 76941), and *L. burseyi* (USNPC 89628).

DNA extraction, PCR amplification, and sequencing

For molecular work, specimens were washed with saline solution and preserved in 100% ethanol. Genomic DNA was extracted individually from the adult worms following a standard phenol/chloroform extraction (Hillis et al. 1996; Palumbi 1996) and the DNeasy

Table I. Host, locality, geographical coordinates, and GenBank accession numbers for *cox1* and ITS2 sequences of analyzed samples.

Host	Locality	Geographical coordinates	GenBank accession numbers	
			ITS2	*cox1*
L. macrocirra				
L. vaillanti	Laguna Escondida, Veracruz	18°35′30.2″N, 95°05′15.6″W	HM853578	HM853564
	Rio Pizote, Guanacaste, Costa Rica	10°56′24″N, 85°24′46″W	HM853580	HM853568
L. berlandieri	La Selva Biological Station, Costa Rica	10°26′15.03″N, 84°0′1.18″W	–	HM853569
	El Petén: in front of Cerro Cahuí, Guatemala	16°59.82′N, 89°42.30′W	–	HM992938
R. marina	Lago de Catemaco, Veracruz	18°25′09.4″N, 95°06′51.5″W	HM853581	HM853565
	Coquimatlán, Colima	19°09′92″N, 103°49′56″W	HM853579, HM853582	HM853566
	Salto de Eyipantla, Veracruz	22°18′ N, 95°12′W	–	HM853567
L. cf *parva*				
L. vaillanti	Laguna Escondida, Veracruz	18°35′N, 95°06′W	HM853571, HM853577	HM853557
	San José Independencia, Oaxaca	18°15′N, 96°13′W	HM853573, HM853575, HM853576	HM853559, HM853561, HM853563
	Chapultepec, Oaxaca	18°07′08″N, 96°50′2″W	HM853574	HM853560 HM853562
Lithobates sp. Colima⋆	Coquimatlán, Colima	19°09′92″N, 103°49′56″W	HM853572	HM853558
P. chabaudi†				
L. vaillanti	Rio Pizote, Guanacaste, Costa Rica	10°56′24″N, 85°24′46″W	HM853570	HM853556

⋆ This species is not yet described formally, but is recognized as *Lithobates* sp. Colima by Zaldívar-Riverón et al. (2004); † Caballero and Caballero (1969) erected *Brenesia* with *B. chabaudi* from a species of *Lithobathes*; this species is typical of intestine of frogs. This genus has been subject to taxonomic controversy (Sullivan 1971). According to Lotz and Font (2008), *Brenesia* is a synonym of *Pseudosonsinotrema* within Pleurogenidae.

Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions.

ITS2 and *cox1* (partial sequence) were amplified by PCR. All PCRs were performed in a final volume of 25 µl (2.5 µl of 10x PCR buffer, 2.0 µl of 10 mM dNTP mixture [200 mM each], 1.25 mM $MgCl_2$ [50 mM], 1.0 µl each primer [10 pmol/µl], 1–2 µl template DNA, 0.125 µl Taq DNA polymerase [5 units; Biogenica, Mexico city, Mexico], and the remaining volume of sterilized distilled water). Primers ITS-F (5′-TGTGTCGATGAAGAACGC AG-3′) and ITS2-RIXO (5′-TTCTATGCTTAAAT-TCAGGG-3′) were used to amplify ITS2 region (Gasser and Hoste 1995). Primers JB3 (5′-TTTTT-TGGGCATCCTGAG GTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′) were used to amplify partial fragments of *cox1* (Morgan and Blair 1998). ITS2 PCR conditions were: 1 min at 94°C, 35 cycles of 30 s at 92°C, 30 s at 50–55°C, 1 min 30 s at 72°C, and a final 5-min elongation period at 72°C. *cox1* PCR conditions were: 1 min at 94°C, 30 cycles of 1 min at 92°C, 1 min 30 s or 2 min at 47–50°C, 3 min 30 s at 72°C, and a final 4-min elongation period at 72°C. PCR products were purified using the QIAquick (Qiagen) purification kit and were sequenced with a Dye Terminator Cycle Sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA) on an ABI PRISM 3100 Genetic Analyzer.

Sequence alignment, genetic distances, and phylogenetic reconstruction

Sequences were aligned in the program Clustal W (Thompson et al. 1994) and corrected manually with BioEdit v5.0.6 (Hall 1999). Uncorrected pairwise distance measures were calculated in PAUP⋆ v4b10 (Swofford 2002). The phylogenetic relationships among *cox1* sequences were analyzed using the maximum parsimony optimality criterion in PAUP⋆. Heuristic searches were performed with the tree bisection–reconnection branch-swapping option, and all characters were unweighted and unordered. Node support was evaluated using the non-parametric bootstrap method and 1000 pseudo-replicates (Felsenstein 1985). Sequences were deposited in GenBank (Table I).

Results

Morphological analysis

We found two species (*L. macrocirra* and *L.* cf. *parva*; Figure 1) differing in body size dimensions (data not shown), e.g. distinct pre-pharynx, esophagus length, and the position of acetabulum. By standardizing the technique of fixation (i.e. not flattened), the position of the genital pore and presence of pre-pharynx do not remain constant (personal observation). The body shape dimensions as well as the shape and distribution of vitellaria could be related to the degree of development of the fluke, rather than the setting process of fixation. A "tuck" was observed in the morphotypes close to the genital pore (Figure 1), and is not consistently present; it seems to be a product of the ventral adhesion suckers between individuals during cross-fertilization (*in vivo* observation). By examining the surface morphology of adults (Figure 1), we found that the body tegument surface has spines that are more abundant in the middle body, and the quantity and size of the spines markedly diminish toward the posterior end; spines have a see-saw shape; two types of papillae exist around the oral sucker and the acetabulum (type I, ciliated dome-shaped papillae and type II, non-ciliated dome-shaped papillae), and small ciliated papillae are found among the spines; the genital pore was located ventrally about mid-length of the left cecum; and eggs were operculated.

ITS2 sequences

The alignment was 235 bp in length (length range: 229–233 bp) and comprised 13 sequences. ITS2 sequences were highly similar for all samples regardless of previous identification, host, or geographical origin (data not shown). The *Langeronia* sequences exhibited 17.09% maximum nucleotide divergence with respect to the outgroup, *P. chabaudi*. Identical sequences were obtained (e.g. *L.* cf. *parva* from *L. vaillanti* from Laguna Escondida, and *L. macrocirra* from *R. marina* from Lago de Catemaco), while populations differed by a 0.87% of uncorrected distance (e.g. *L. macrocirra* from *L. vaillanti* from Guanacaste vs. *L. macrocirra* from Lago de Catemaco). This locus is highly conserved and thus does not provide sufficient information at this taxonomic level for species delimitation.

Cox1 *sequences and phylogenetic relationships*

A total of 368 bp of *cox1* were sequenced from 15 individuals and aligned unambiguously. The *cox1* sequences differed by 21.19–22.01% with the outgroup, *P. chabaudi* (Table II). Maximum parsimony cladistic analyses resulted in seven equally parsimonious trees (length = 121 steps, consistency index = 0.9339, retention index = 0.9292, rescaled consistency index = 0.8678). The strict consensus tree revealed three clades (Figure 2), which are not consistent with the previous morphological identification or host infected: clade I with identical sequences from Colima, Mexico (on the Pacific slope, both morphotypes); clade II with sequences divergent by 0.27% from Costa Rica and Guatemala; and, finally, clade III with sequences divergent by 1.08% consisted of samples from Veracruz and Oaxaca (both morphotypes). Among clades, the divergence is 6.79–7.33% (II vs. III), 7.88–8.15% (I vs. II), and 8.15–8.69% (I vs. III). Within the same

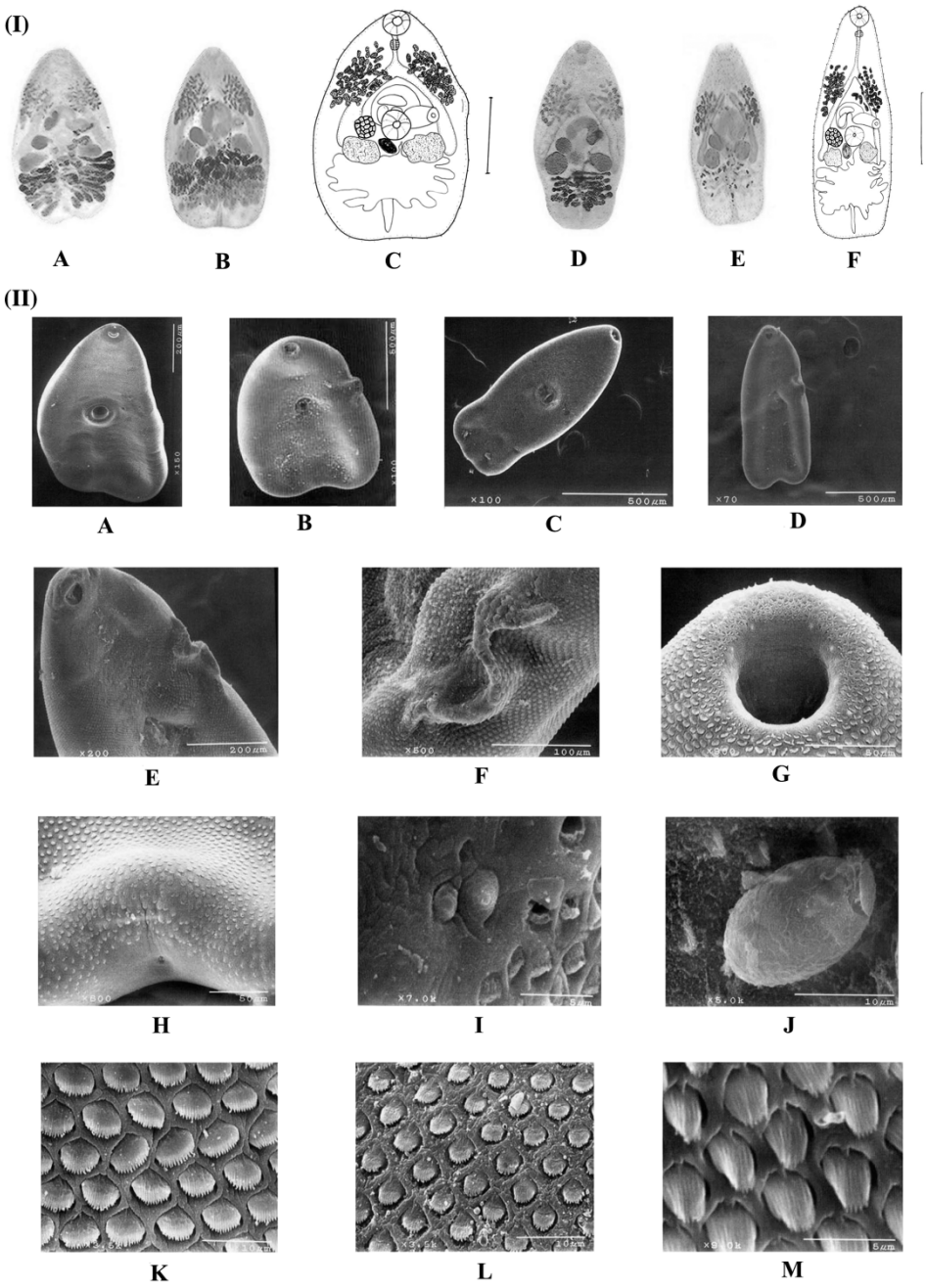

Figure 1. Morphological comparison of two nominal species of identified *Langeronia* and SEM photomicrographs. (I) General morphology: *L. macrocirra* (A–C) and *L.* cf. *parva* (D–F). (A) Holotype, *Lithobates* sp. (locality unknown, CNHE 13885), Mexico. (B) *R. marina* Lago de Catemaco, Veracruz (CNHE 4875). (C) *R. marina*, Lago de Catemaco, Veracruz (CNHE 4875). (D) Paratype, *Lithobates pipiens*, Alburg, Vermont, USA (USNPC 70558) and *L. vaillanti*, Laguna Escondida, Veracruz (CNHE 4874). (E) *L. vaillanti*, Laguna Escondida, Veracruz (CNHE 4874). (F) *L. vaillanti*, Laguna Escondida, Veracruz (CNHE 4874). Reference bar: $C = 0.5$ mm and $F = 0.3$ mm. (II) SEM photomicrographs: *L. macrocirra* from Lago de Catemaco, Veracruz, *R. marina*: (A, B, F–I, K, and L). *L.* cf. *parva* from Laguna Escondida, Veracruz, *L. vaillanti* (C–E, and J).Whole worm without tuck (A and C) and with tuck (B and E). Details of the tuck (E and F). Ciliated dome-shaped papillae (type I) on ventral surface anterior part of the oral sucker (G). Excretory pore (H). Small non-ciliated dome-shaped papillae (type II) close to a papillae type I on ventral surface around the acetabulum (I). Egg rough elliptical and operculate (J). Tegumental spines allocated near to the acetabulum (K). Spines allocated near to the posterior end (L). Small papillae ciliated among spines (M).

Table II. Pairwise genetic distances (%) among *cox1* sequences of analyzed specimens.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	PC	–														
2	LP2	21.19	–													
3	LM3	21.19	0	–												
4	LM7	22.01	8.15	8.15	–											
5	LM6	22.01	7.88	7.88	0.27	–										
6	LM5	22.01	7.88	7.88	0.27	0	–									
7	LM1	21.46	8.15	8.15	0.73	0.73	0.73	–								
8	LP1	21.19	8.15	8.15	0.70	0.70	0.70	0.27	–							
9	LM4	21.19	8.15	8.15	0.70	0.70	0.70	0.27	0	–						
10	LP7	21.46	8.42	8.42	0.67	0.67	0.67	0.54	0.27	0.27	–					
11	LM2	21.46	8.42	8.42	0.70	0.70	0.70	0.81	0.54	0.54	0.27	–				
12	LP4	21.46	8.42	8.42	0.70	0.70	0.70	0.81	0.54	0.54	0.27	0	–			
13	LP6	21.46	8.42	8.42	0.70	0.70	0.70	0.81	0.54	0.54	0.27	0	0	–		
14	LP5	21.73	8.69	8.69	0.73	0.73	0.73	1.08	0.81	0.81	0.54	0.27	0.27	0.27	–	
15	LP3	21.46	8.42	8.42	0.70	0.70	0.70	0.81	0.54	0.54	0.27	0	0	0	0.27	–

Notes: Distances are expressed as a percentage of nucleotide differences. LM1, *L. macrocirra*; LP, *L.* cf. *parva*; PC, *P. chabaudi*.

locality, sequences divergence ranged from 0 to 0.27% (e.g. from Colima or Oaxaca, respectively; see Table II).

Discussion, conclusions, and new prospects

The taxonomy of the genus *Langeronia* has been controversial due to morphological variation (Ubelaker 1965; Brenes et al. 1960). This variation has been observed in specimens of *L. macrocirra* from Mexico (e.g. Paredes-Calderón 2000). Herein, we corroborate the variability of some diagnostic characters, e.g. the presence and absence of prepharynx, the position of genital pore, and the shape and distribution of vitellaria. Variability was observed within the same morphotype and this could be partially owed to the process of fixation of the material (flattened or not) or a case of polymorphism. There are several studies exploring the tegumental surface in adult Pleurogenidae (Podyznaya 1986; Ferrer et al. 1996; Bogéa and Caira 2001), and observed tegumentary papillae are distributed around the oral sucker, acetabulum, and between the tegumentary spines. The same types are observed around the oral sucker, acetabulum, and among spines in specimens of *Langeronia*.

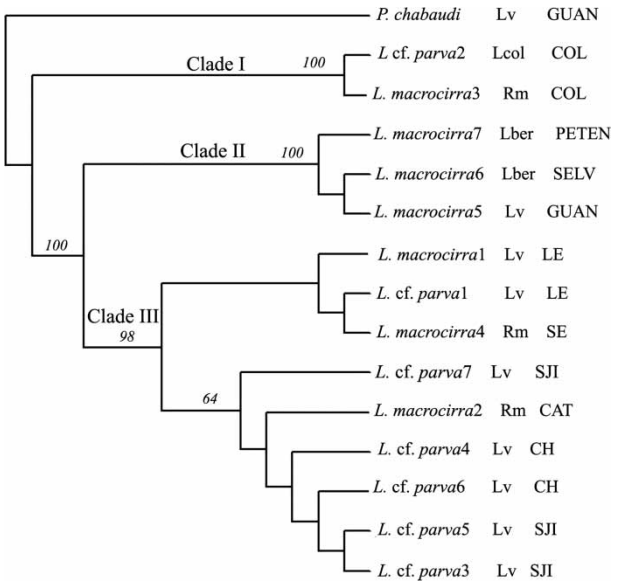

Figure 2. Strict consensus tree of seven equally parsimonious trees obtained from maximum parsimony analysis using partial *cox1* gene sequences. Bootstrap percentages are shown above internal nodes. Geographical origin is designated as follows: Colima: Coquimatlán (COL); Veracruz: Lago de Catemaco (CAT), Laguna Escondida (LE), Salto de Eyipantla (SE); Oaxaca: San José Independencia (SJI), Chapultepec (CH); Costa Rica: La Selva Biological Station (SELV), Rio Pizote, Guanacaste (GUAN); Guatemala: El Petén (PETEN). Hosts: *L. vaillanti* (Lv), *L. berlandieri* (Lber), *Lithobates* sp. Colima (Lcol), *R. marina* (Rm).

Defining species is not simple, except for organisms with clear morphological differentiation. This difficulty arises when certain groups exhibit a wide variability in morphology or phenotypic plasticity, such as in the case of *Langeronia.* Variability has been observed in specimens collected from different host species and can often be attributed to phenotypic plasticity and could be correlated with the degree of host specificity, or sometimes be the product of rate development (Brooks 2003).

Initially, the ITS region was used to distinguish species of digeneans when pairs of congeners showed divergence below 2%. Later on, *cox1* was shown to be an accurate marker in studies of molecular taxonomy in parasites (Rollinson et al. 1986; Morgan and Blair 1998). The detection of cryptic species using mtDNA showed up to 21% divergence in congeneric species (Bowles et al. 1993; Morgan and Blair 1998; Bell et al. 2001; Razo-Mendivil et al. 2010), with the mean pairwise divergence between congeners of the barcode region at 19% (3.9–25%) (Moszczynska et al. 2009). Our ITS2 data showed that both morphotypes are less than 1% divergent. Compared with previous studies (e.g. Vilas et al. 2005), the observed genetic differences can be attributed to intraspecific variation of a single species of the genus, which in this case

would correspond to *L. macrocirra*. However, the rate of change in a region of DNA may vary from one group to another, and it is likely that the ITS2 in this group does not provide information at the species level. Therefore, it was necessary to obtain information from a mitochondrial gene that harbors a higher evolutionary rate.

The analysis of *cox1* in *Langeronia* showed three clades (Figure 2), which are not consistent with the morphological identification. Nevertheless, there is a high level of divergence among the three clades, e.g. 8.15–8.69% in clade I vs. clade III. If the morphotypes of *Langeronia* are sympatric, parasitizing the same host species and individual at the same time, a plausible explanation for the morphological variability is related with the rate of development (heterochrony), which could explain that the changes in age generate changes in morphology, and is thought to be a source of evolutionary innovation (Brooks 2003).

The presence of three genetic and geographical clades of *Langeronia* that contradicts the morphological evidence in this preliminary analysis poses a new challenge in the study of the genus. Future work should explore the complete DNA barcode region (e.g. Folmer et al. 1994; Tautz et al. 2003; Casiraghi et al. 2004; Moszczynska et al. 2009), and perhaps other markers, as well as extend to the study of life cycles and detailed ultrastructural examination in order to find robust evidence for the disentanglement of the taxonomy of the group.

There are several reasons to extend DNA barcoding to parasites, which can complement biodiversity studies (Moszczynska et al. 2009): facilitates the discovery and identification of new species (undescribed or unrecognized), the potential detection of parasites of biomedical and agricultural importance, biodiversity inventory initiatives associated with conservation, identification using developmental stages that would not be possible using only morphology (from several intermediate hosts or their free-living forms), the understanding of complex life histories, and the identification of cryptic and complex species will enhance and accelerate taxonomic efforts (Hebert et al. 2003; Casiraghi et al. 2004; Oceguera-Figueroa et al. 2005; Moszczynska et al. 2009; Junker et al. 2010). In general, we consider DNA taxonomy and DNA barcoding to be a good initiative complementing the traditional taxonomic practice of the genus in Mexico.

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