A new cryptic species of *Leberis* Smirnov, 1989 (Crustacea, Cladocera, Chydoridae) from the Mexican semi-desert region, highlighted by DNA barcoding

Una nueva especie críptica de *Leberis* Smirnov, 1989 (Crustacea, Cladocera, Chydoridae) procedente de una región semidesértica mexicana, evidenciada por los códigos de barras del ADN

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ABSTRACT

DNA barcoding, based in the sequence of a gene from the mitochondria, the Citochrome C oxidase (*CO1*), has been proved to be an excellent tool to identify many animal groups from invertebrates to vertebrates. After barcode several Cladocera, we gathered evidence about the existence of two *Leberis* species in the north of Mexico. Minimal *CO1* divergence between both taxa was 14.3%, due to the GC% in the third codon position. A detailed morphological analyses uncovered one of the species as *L. davidi*, dwelling from South America to Mexico and the other as *Leberis chihuahuensis* new species, apparently restricted to semi-desert temporary pools. The parthenogenetic female of the latter is characterized by a small size, blunt rostrum, long and curved spine in the first exopodal segment of the second antenna, absence of accessory seta and sensilla in the first thoracic limb, and a brush-shaped seta in the fourth limb, among other characters. Males have a long and narrow postabdomen and a series of similar sized spinules in the posterior margin of the valves. By the first time a cladoceran is described from both views, the morphology and *CO1* sequence.

Keywords: Anomopoda, Branchiopoda, taxonomy, new species.

RESUMEN

Recientemente una técnica molecular denominada códigos de barras, basada en secuenciar una fracción del gen mitocondrial que codifica para la citocromo oxidasa C (conocido también como *CO1*) ha demostrado ser una excelente herramienta para identificar especies animales, desde invertebrados hasta vertebrados. Al aplicar esta técnica a cladóceros de agua dulce de diversos lugares, se obtuvo evidencia de la presencia de dos especies del género *Leberis* en el norte de México. La mínima divergencia en el *CO1* entre ambos taxa fue de 14.3%, debida principalmente al contenido de Guanina-Citosina en la posición del tercer codon. El análisis detallado de las estructuras anatómicas de ambas especies permitió identificar a una de ellas como *L. davidi,* distribuida desde Sudamérica hasta México y la otra corresponde a *L. chihuahuensis* sp. nov., aparentemente restringida a charcos temporales de regiones semidesérticas. En esta última, la hembra partenogenética se caracteriza por su talla pequeña, el rostro redondeado, la forma y longitud de la espina del primer segmento exopodal de la segunda antena, la ausencia de setas accesorias y sensilas en el lóbulo distal externo del primer apéndice torácico y la presencia de una seta con forma de brocha en el cuarto apéndice torácico, entre otras características. El macho posee un postabdomen largo y angosto, y una

serie de espinas de igual tamaño en el margen posterior de las valvas. En este trabajo se conjunta por primera vez la descripción morfológica detallada con la secuencia del *CO1* para describir una nueva especie de este grupo.

Palabras clave: Anomopoda, Branchiopoda, taxonomía, nueva especie.

INTRODUCTION

Among cladocera, *Leberis* Sinev, 2005 belongs to the Chydoridae. This is one of the most difficult anomopod families from a taxonomical and phylogenetic point of view. Two of its subfamilies (Chydorinae and Aloninae) have been continuously in a state of change during the last two de1cades, due to discovery of new species or genera, and re-allocation of taxa (Ciros-Pérez & Elías-Gutiérrez, 1997; Elías-Gutiérrez & Suarez-Morales, 1999; Kotov *et al.*, 2003; Sinev *et al.*, 2005; Smirnov *et al.*, 2006; Rowe *et al.*, 2007).

Currently, confusion within Aloninae is one of the major problems in cladoceran systematics; further, several authors have stated that the taxonomy of this subfamily is still far from stable, and many re-definitions remain to be made (Kotov, 2004).

Recently, Sinev *et al.* (2005) translocated the *diaphana*-like *Alona* to the genus *Leberis* Smirnov, 1989. This genus was mainly based on the presence of a dorsal keel and a blunt rostrum, as well as on the number of setae on exopodites II-V and gnathobase II-IV. This genus was earlier proposed by Smirnov (1989) for an *Alona*-like species from Australia.

Sinev *et al.* (2005) studied in detail *Leberis davidi* Richard, 1895 from Haiti and common in central-southeast Mexico, from the lowlands near the Gulf of Mexico shore to the highlands in Chiapas, with the northernmost records on the Central Plateau (at more than 2300 m asl). The species has been found also from Caribbean islands (Cuba and Jamaica) and South America (Argentina and Brazil) as well (Sinev *et al.,* 2005).

All previous taxonomic studies in *Aloninae* have been exclusively morphologically based. Recently, molecular tools have been proposed to improve the taxonomical resolution and species recognition. One of them is the barcoding, based on the sequence of a fragment of the mitochondrial DNA, named *CO1* (Hebert *et al.,* 2003). Barcodes have been extensively used during the last three years to identify vertebrates and invertebrates, including crustaceans (Barrett & Hebert, 2005; Ward *et al.*, 2005; Hajibabaei *et al.*, 2006; Costa *et al.*, 2007; Kerr *et al.*, 2007; Bucklin *et al.*, 2007, *Rowe et al.*, 2007). Nevertheless, some controversies are present (Will & Rubinoff, 2004; Prendini, 2005; Whitworth *et al.*, 2007).

In the framework of this new taxonomic approach, herein we describe a new species of *Leberis* found in a northern Mexican semi-desert region and compare it with close relatives from a morphological point of view, but also comparing their *COI* sequence data.

MATERIAL AND METHODS

Samples were collected with a 50 µm plankton net attached to a handle and fixed with 4% formaldehyde and 96% ethanol (for DNA analyses).

Studied material includes *Leberis davidi* related material from different localities in Mexico (see below). Specimens were examined and illustrated using differential interference contrast microscope or phase contrast. Complete or micro-dissected specimens were drawn using a camera lucida, to complete the analysis of the appendages. Measurements were realized with an eyepiece micrometer.

All original samples are deposited in the zooplankton collection at El Colegio de la Frontera Sur, Chetumal Unit.

DNA barcoding for three species (the new species of *Leberis*, and their relatives *L. davidi* and *Alona glabra* Sars, 1901) was carried out at the Canadian Centre for DNA Barcoding (University of Guelph), using standard protocols (Hajibabaei *et al.*, 2005). DNA was extracted from whole body homogenates using a mix of Proteinase K with invertebrate lysis buffer and digested overnight at 56ºC. Genomic DNA was subsequently extracted using a membrane-based approach on the Biomek NX$^{©}$ liquid handling station and a AcroPrep 96.1 ml filter plate with 2.0 µm PALL glass fiber media. Approximately 600-658 bp were amplified from the *COI* using LCO1490 and HCO2198 primers (Folmer *et al.*, 2007). The 12.5 µl PCR reaction mixes included 6.25 µl of 10% trehalose stabilizer, 2 µl of ultrapure water, 1.25 µl of 10X PCR buffer, 0.625 of MgC_{l2} (50 mM), 0.125 µl of each primer (0.01 mM), 0.0625 µl of each dNTP (0.05 mM), 0.625 µl of Taq polymerase (New England Biolabs or Invitrogen), and 2.0 µl of DNA template. PCR products were visualized on pre-cast agarose gels (E-Gels$^{©}$, Invitrogen) and the most intense products were selected for sequencing. Products were labelled by using the BigDye$^{©}$ Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) as described in Hajibabaei *et al.* (2005) and sequenced bidirectionally using an ABI 3730 capillary sequencer following the manufacturer's instructions.

Sequence data, electropherograms, trace files, primer details, photographs and collection localities for specimens are available within the project Leberis of Mexico on the Barcode of

Life Data System (http://www.barcodinglife.org) (Ratnasingham & Hebert, 2007) and the GenBank database.

Sequences were aligned using SEQSCAPE v.2.1.1 software (Applied Biosystems, Inc.). Sequence divergences were calculated using the Kimura two parameter (K2P) distance model (Kimura, 1980). Neighbor-joining (NJ) trees of K2P distances were created to provide a graphic representation of the patterning of divergence between species (Saitou & Nei, 1987). A simplified tree of all the species was elaborated with the MEGA 3 software (Kumar, *et al.*, 2004).

RESULTS

This genus was recently proposed by Sinev *et al.* (2005), who gave a detailed diagnosis of it and its relationship with close relatives as *Celsinotum* and other Aloninae. Its main features are the presence of a dorsal keel or ridge on the valves, the rostrum short and blunt, postabdomen ornamented with groups of small denticles, and the exopodites of limbs III-V bear 6, 6 and 4 setae respectively.

Order Anomopoda Sars, 1865

Family Chydoridae Stebbing, 1902

Subfamily Aloninae Dybowski & Grochowski, 1894

Genus *Leberis* Smirnov, 1989

Leberis chihuahuensis sp. nov.

Holotype: One adult parthenogenetic female in ethanol 70% and glycerol, access number ECO-CH-Z-03551, length 0.42 mm, height 0.35 mm.

Allotype: One adult male in ethanol 70% and glycerol, ECO-CH-Z-03562.

Paratypes: One ephippial, four parthenogenetic females (three of them dissected) (ECO-CH-Z-03561, 03564-03566, 03569), one immature and two mature males (one dissected) (ECO-CH-Z-03563, 03567-03568), all mounted in a mixture of glycerin-formaldehide, sealed with Entellan mounting medium. Five parthenogenetic females preserved in 96% ethanol and glycerol (ECO-CH-Z 03552). All material deposited in Ecosur, Chetumal Unit.

Two paratypes in 96% ethanol and glycerol and one paratype in one slide (Access Number CNRC25074) deposited at Instituto de Biología (Universidad Nacional Autónoma de México, UNAM).

COI Reference Sequences: BOLD (www.boldsystems.org) under the project Leberis of Mexico, ZPLMX156-06, ZPLMX348-06-ZPLMX350-06, GenBank access numbers to the same sequences EU350593-EU350596.

Additional material used for comparison: *Leberis davidi* (Richard, 1895) from Mexico. Numerous females from Papasquiaro pond (Durango) (24°30'53.6" N, 104°39'51.8"W) collected 04/Oct/04, access number ECO-CH-Z 03555-03556; two adult females from a small pond near Pojoj (Chiapas) (16°05'50"N, 91°40'05"W), collected 16/Apr/00, access numbers ECO-CH-Z 03553-03554; one mature male and three parthenogenetic females from km 28 Jilotepec-Ixtlahuaca (State of Mexico) (19°49'13" N, 99°42'22"), access numbers CI1044, 1122 and 1124, collected 28/Jan/94; one female collected from km 44 highway Toluca-Atlacomulco (State of Mexico) (19°39'51"N, 99°47'52") collected 11/Oct/93.

COI Reference Sequences for *Leberis davidi:* BOLD (www.boldsystems.org) under the project Cladocera of Mexico, ZPLMX104-06, ZPLMX351-06,ZPLMX353-06-ZPLMX355-06, GenBank access numbers to the same sequences EU350597-EU350601, Specimens with access numbers ECO-CH-Z-03556-03557 at El Colegio de la Frontera Sur.

Alona glabra (for genus level comparison of the sequences). Two females from the same type locality for *L. davidi*, and four from Xacatzun, Guatemala (15° 53' 36.4" N; 90° 14' 49.8" W). *COI* sequences under the project Cladocera of Mexico in BOLD (www.boldsystems.org), access numbers ZPLMX387-06, ZPLMX389-06, ZPLMX617-06 to ZPLMX620-06. GenBank access numbers to the same sequences EU350587-EU350592. Specimens with access numbers ECO-CH-Z-003393-03394 at El Colegio de la Frontera Sur, Chetumal.

Diagnosis. Parthenogenetic female. Smallest *Leberis* known, parthenogentic females around 0.5 mm (0.444 ± 0.004 mm), body ovoid (body height/ body length = 0.62-0.82), dorsal keel developed. Valves with 51-55 ventral setae. Head and valves finely sculptured, ornamentation only noticeable in dry specimens. Rostrum short and blunt. Ventral margin of valves with 51-56 setae, posterior margin with inner spinules forming a continuous, non clustered row. Postabdomen with distal dorsal margin strongly sclerotized, margin covered with 15-20 groups of small marginal denticles and 9-12 lateral groups of setules. Postabdominal claws of moderate length. Length of basal spine exceeding width of claw base. Spine on first exopod segment of antenna exceeding in length the second segment, strongly curved distally. Outer distal lobe (ODL) with single seta, no sensilla and no accessory seta, inner distal lobe (IDL) with two setae. Last seta on endopodite IV with well developed characteristic brush-shaped seta. Epipodites with no finger-like projections. Male. Body more elongated than female, with narrower postabdomen. Sperm duct openings ventral, above base of postabdominal claws. Ventro-distal angle with blunt projection, preceding the genital opening. Postabdominal claws less elongated than in females, with thin basal spine. Antennula with nine terminal aestheteascs and one lateral aesthetasc. First thoracic limb with brush seta forming a pad near base of U-shaped hook

Description. Parthenogenetic female. Juvenile individuals with body relatively more ovoid in lateral view. Body of adult females inflated (Fig. 1.1), less ovoid than *L. davidi* (see Sinev *et al.,* 2005), maximum height at mid-body. Length of adult females ranging from 0.460 to 0.490mm, height from 0.310 to 0.370 mm. Dorsal margin evenly curved from head to valves. Valves round, broadly separated in fixed specimens, with posterior margin slightly convex; dorsal and ventral angles more or less expressed in an ample curve. Ventral margin convex, sometimes wavy due to depressions on proximal third, with 51 to 55 marginal unilaterally feathered setae, shorter at anterior margin, longer on last third, shortening again towards the postero-ventral margin. The last 6-7 setae before the angle naked, pointed, spine-like, followed by a single row of spinules, starting marginally, continued along inner margin to the dorsum, not forming groups (Fig. 1.2). Between each pair of setae 2- 4 small submarginal denticles present. Surface of valves with fine longitudinal ridges, only visible in dried specimens or under high magnification in the stereomicroscope. Body laterally compressed in anterior view, with a dorsal keel.
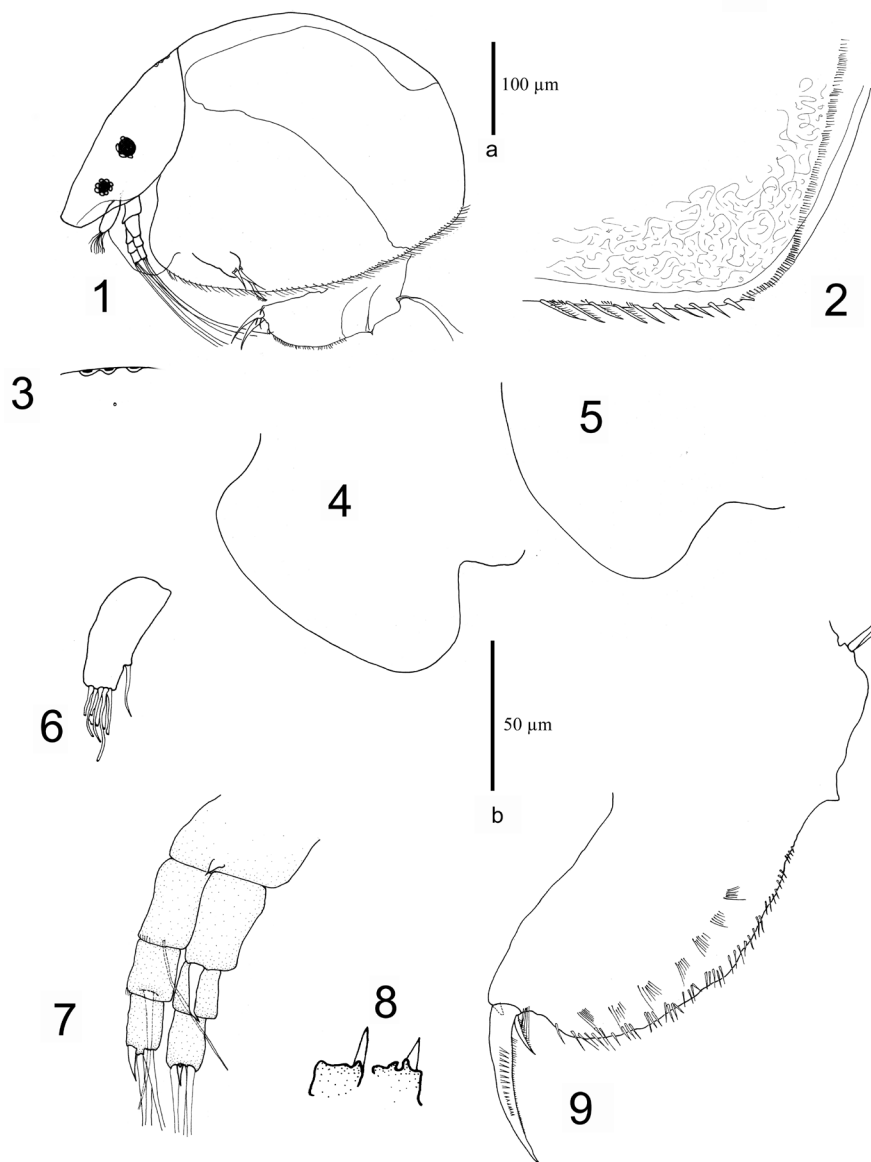

Figure 1. *L. chihuahuensis* sp. nov., parthenogenetic female. 1.1 Habitus; 1.2 Armature of postero-ventral part of valve; 1.3 Lateral view of cephalic pores; 1.4-1.5 Labrum variation; 1.6 Antennule, lateral; 1.7 Antenna, lateral; 1.8 Terminal spines of exopod and endopod, with hillocks; 1.9 Postabdomen, lateral view. Scale bar "a" is for Fig. 2.1; scale bar "b" is for all other figures.

Head small, not keeled with a broad convex curve along posterior margin, continuous with valves. Rostrum developed, blunt at tip. Eye larger than ocellus, both surrounded by hyaline lenses (Fig. 1.1). Distance from tip of rostrum to ocellus notoriously greater than between ocellus and eye.

Head shield elongated, with posterior margin evenly round. Three major head pores connected (Fig. 1.3). Post pore (PP) distance to the margin of head shield = 2.3-2.6 inter pore (IP) distance. Two minute isolated lateral minute pores, one in each side, at 1.6 IP distance from central major head pore.

Labrum ample, varying from a wide oval shape to a narrower ventral posterior margin. Apex always blunt with an ample curve. No setulation on margins and no other special feature as teeth or wavy margins (Figs. 1.4, 1.5).

Antennule (Fig. 1.6) elongated, shorter than rostrum, with smooth surface, with no ridges or transverse rows. Posterior sensory seta inserted on last third, about the half of antennule length, projecting away from tip. Nine unequally long asthetascs, three of them markedly longer than the others. All aesthetascs projected beyond tip of rostrum.

Antenna short (Fig. 1.7), with 1-0-1/0-0-1 spines, 0-0-3/1-1-3 setae. Spine on first exopodal segment robust, heavily curved on last fourth, slightly longer than second segment. Both distal segments with hillocks at spine base (Fig. 1.8). Seta on basal segment of the endopod thin, short, slightly longer than apical segment. Apical setae long, unilaterally setulated. Coxa with a small spine between the insertion of antennal branches. No setae were observed emerging from here. Exopod and endopod segments with minute spinulae near distal margin.

Postabdomen (Fig. 1.9) broadly curved, tapering distally. Ventral margin straight, with no ridges or setules. Dorsal margin convex, with long postanal side, 2.4-2.8 times longer than preanal. Distal margin slightly convex, with the anterior-ventral corner strongly chitinized and a dark brown coloration, just in the anterior margin of the postabdominal claw insertion. Both angles, preanal and postanal, well expressed.

Postanal part with 7-8 clusters of lateral setules, decreasing in size proximally, both in each cluster and the whole groupings. Distalmost setule in each group longer. Ventral margin with 7-9 clusters of marginal denticles. Sometimes members of each group with different length, but no clearly in a definite position. Groupings become more diffuse proximally, close to the anus with spinules diminishing in size.

Postabdominal claw regular in size, longer than preanal portion of postabdomen with one continuous pecten along dorsal margin and a row of lateral spinules. Basal spine with a row of setules in dorsal margin, 3.5 to 4 times shorter than claw. A bunch of long setules at claw base.

Mandible asymmetric, elongate, the last third incurved. Masticatory surface with two rows of small spines, and six major teeth (Fig. 2.1)

Trunk limb I (Fig. 2.2, 2.3), epipodite ovoid. Limb corm almost rectangular in lateral view, big, with 6-8 rows of setules along the anterior margin. Accessory seta and sensilla absent, ODL large, bottle-shaped, bear only one long bi-segmented seta with unilaterally setulated distal segment. IDL with clusters of setules, and two bisegmented setae of different length with short but thick setules in the distal segment (Fig. 2.3). Four clusters of setules on ventral face of IDL. Endite 3 with four setae subequal in length (a-d), one of them lies anteriorly to others (d), a small lobe-like receptor near its base. On endite 2 three bi-segmented setae (e-g), two longer ones (f-g) unequal in length, with strong setules along one margin. Third seta (e) short, bilaterally setulated. Endite 1 with three setae (h-i), the first one bilaterally setulated, the other two bi-segmented. First seta (j) naked. Fascicles of thin setules on inner face of limb, plus bunches of longer and robuster setules at ventral margin of limb. Two ejector hooks of similar size.

Trunk limb II (Fig. 2.4), exopodite sub-rectangular, relatively large, with two lobes in the tip, each bearing a bunch of setules. Inner portion of limb (endopodite) with eight 'scrapers' (1-8) decreasing progressively in length basad, except 3 and 4 almost of the same size, with a small receptor in the middle of them (see Fig. 2.4, insert). The distalmost six scrapers (1-6) with thin setules distally and naked basal segments. The two proximal scrapers with comb-like appearance, and thicker setules. A series of small blunt projections posteriorly to distal scrapers. Portion of gnathobase II bordering the endopodite with inflated bulbous projection, densely setulated, followed by a small triangle-shaped receptor (R in Fig. 2.4). Distal armature of gnathobase with three elements, the first bi-segmented with a setulated tip (arrow in Fig. 2.4). Filter plate II with seven setae, the two members closest to endopodite shorter, with inflated basal portion and special dense setulation, the third element longer, with the fourth being the longest and remaining three slightly decreasing in size.

Trunk limb III (Fig. 2.5, 2.6), exopodite rectangular, with two lateral setae (5-6), basalmost (6) longer, and three distal setae (1-3) of subequal size, all of them bi-setulated, seta 4 massive, the longest and bi-setulated. Distal endite (Sensu Kotov, 2000) (corm of external endite sensu Dumont & Silva Briano, 1998) sub-rectangular with three setae (a-c), distalmost one (a) and medium (b) stout, with setules in one side, basalmost seta (c) with a bunch of setae in the distal end. A small sensilla between setae a-b. Basal endite larger than distal, with four setae (d-g), a lamellar sensillum near seta g. On posterior limb face (Fig. 2.6), four soft setae (1-4), all armed with sparse, fine setules. Distal armature of gnathobase with long, curved seta (h), bearing long setules basally and distally short setules in the opposite side, and a lamellar sensilla. Filter plate III formed by seven bilaterally
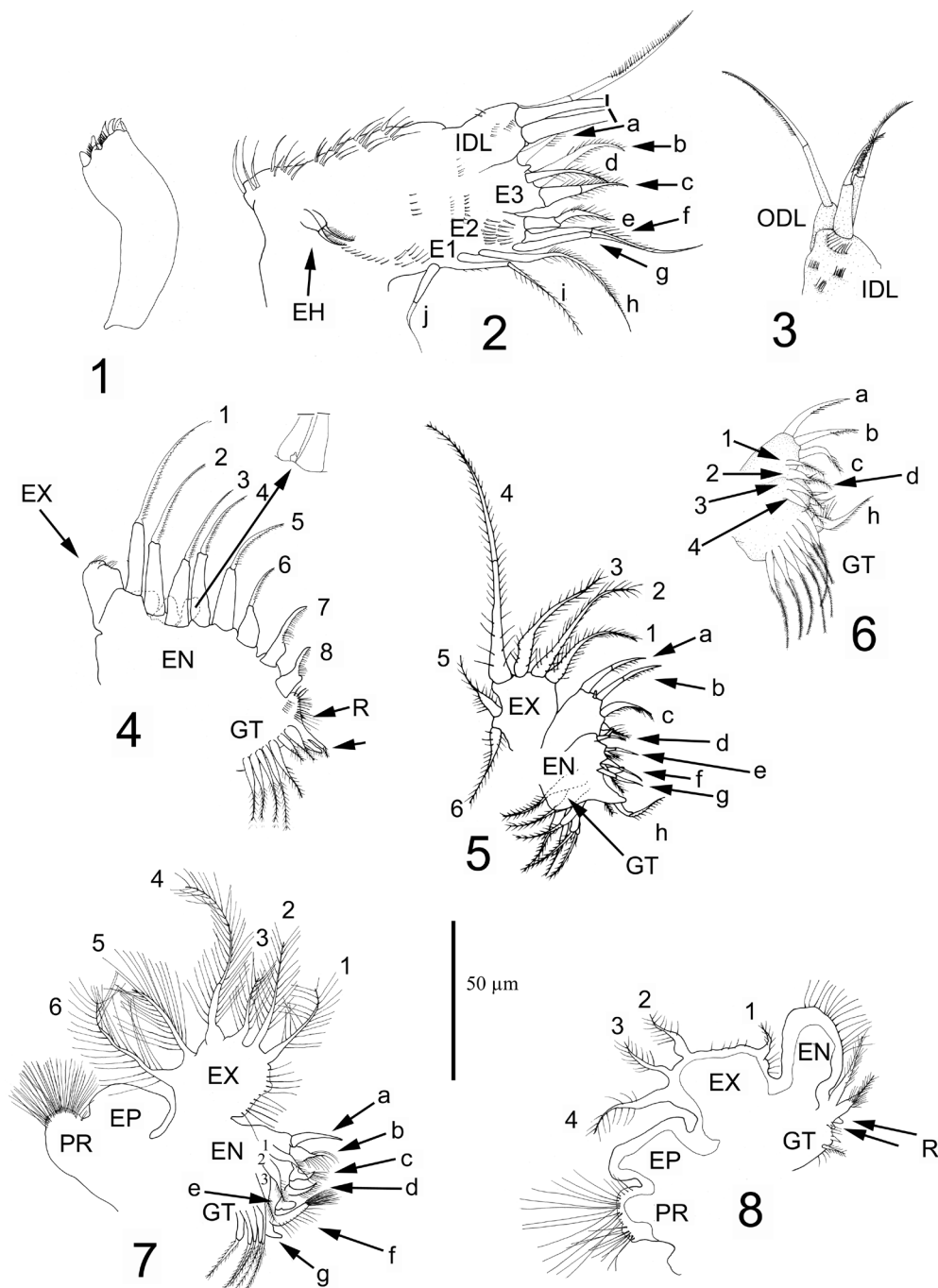
Figure 2. *L. chihuahuensis* sp. nov. thoracic appendages of parthenogenetic female. 2.1 Mandible; 2.2 Limb I; 2.3 Distal portion of limb I; 2.4 Limb II and its gnathobase; 2.5 Limb III; 2.6 Endopod of limb III, posterior face; 2.7 Limb IV; 2.8 Limb V 7. E1-E3 = Endite 1-3; EH = Ejector Hooks; EN = Endopodite; EP = Epipodite; EX = Ecopodite; GT= Ganthobase; IDL = Internal Distal Lobe; PR = Pre-epipodite; R = Receptor. Setae numbers are explained in text.

setulated setae, with inflated basal segments arising from inner surface of gnathobase.

Trunk limb IV (Fig. 2.7), pre-epipodite globular with long setules and ovoid epipodite. Exopodite wide, sub-quadrangular, with

six setae (1-6), bi-setulated. Setae 1, 3 shorter, 2 slightly longer and 4 the longest, bisegmented. Setae 5 and 6 similar to 2 in length.

Distal portion of endopodite with four marginal setae (a-d). Distalmost seta (a) stout, spine-like, naked. Setae b-d with
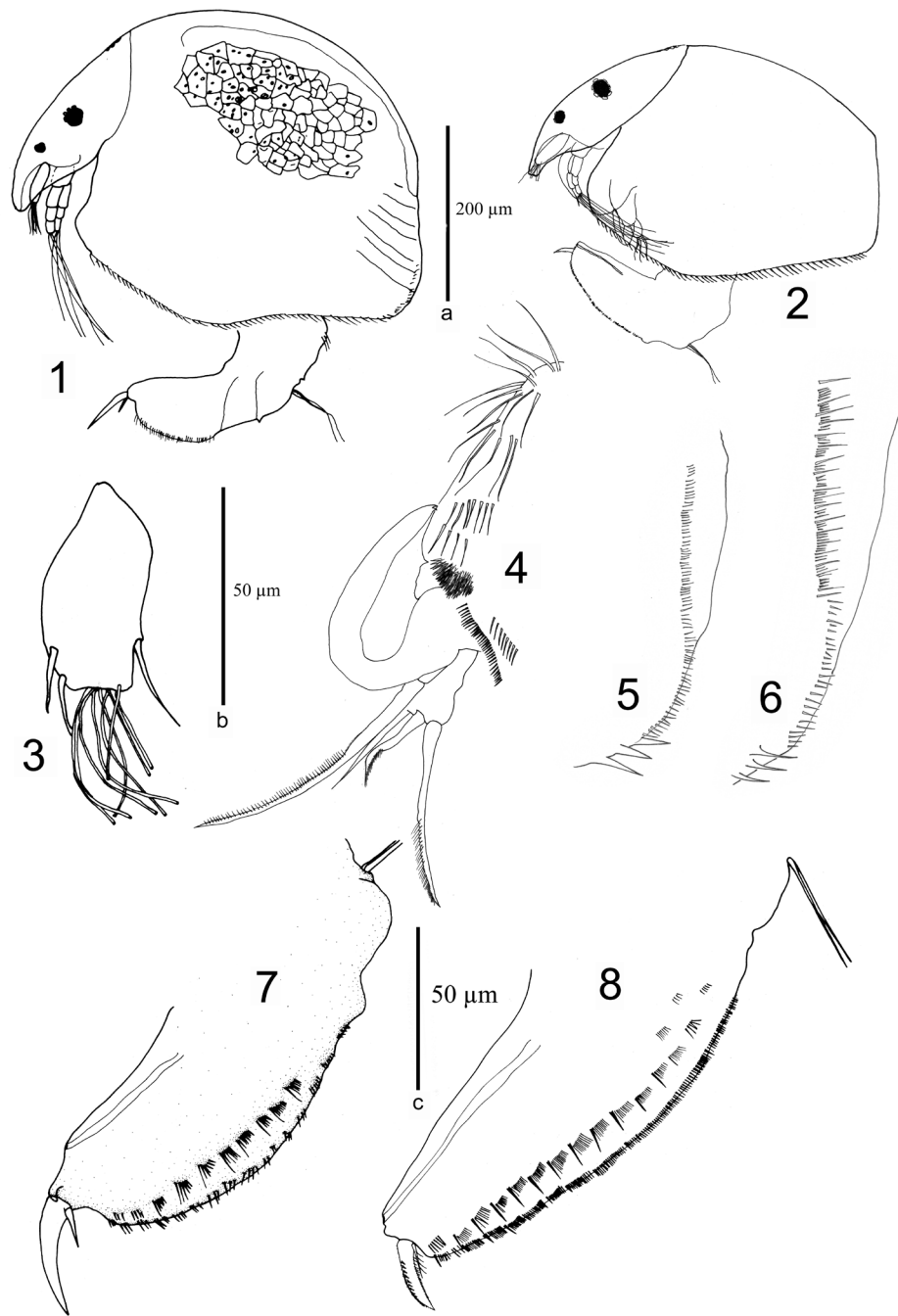
Figure 3. *L. chihuahuensis,* sexual forms. 3.1 Ephippial female; 3.2-3.8 Male 3.2 Habitus, lateral; 3.3 Antennule; 3.4 Limb I, with copulatory hook; 3.5 Armature of postero-ventral margin; 3.6 Armature of postero-ventral margin of male *L. davidi* for comparison (character not described by Sinev *et al.*,, 2005); 3.7 Postabdomen of immature male; 3.8 Postabdomen of mature male, lateral.

inflated basal segments and slender, unilaterally setulated distal segments, in seta b basal segment more inflated than the others, setulated as well. On posterior limb face, three soft setae (1-3). Distal armature of gnathobase with a big, lamelar sensillum (e), a peculiar stout brush-shaped seta (f) with setules along one side, and a receptor on anterior limb face (g). Filter plate with five setulated setae, subequal in size.

Trunk limb V (Fig. 2.8), pre-epipodite bilobed, each lobe with long setules, and epipodite ovoid with distal end projected as a lobe. Exopodite subovoid, with four lateral, densely setulated setae (1-4), the one (1) shorter than the others. Inner limb portion elongated, as a flat lobe, with setulated inner margin, followed at inner face by two setulated setae of different length. Gnathobase V with one hyaline setulated seta proximally. Distally two small

hillock-like receptors (R in Fig. 2.8) followed by a leaf-like hyaline projection in "filter plate" V.

Ephippial female. Similar to parthenogenetic female, with similar body proportions (body height/ body length = 0.69), bigger in length (0.530 mm), wall of carapace additionally chitinized, with low, thick dorsal keel. Ephippium slightly pigmented, brownish, covered by polygonal sculpture, fine dots visible under optical microscope on the polygons (Fig. 3.1).

Male. General body shape of both instar I males studiedsimilar to that of juvenile females of same instar (length = 0.370 mm). Adult male with more elongated body (length 0.43-0.48 mm) than female due to more straight dorsal margin, resulting in slightly less body height (body height/ body length = 0.6-0.7) (Fig. 3.2). Valves flattened, postero-ventral corner with 3-4 naked setae, followed by a marginal row of spinules of similar size (in *L. davidi* these spinules are notoriously different in size and grouped) (see Figs. 3.5 and 3.6). Rostrum blunt and head pores as in female. Antennule elongated, with one lateral aesthetasc close to male seta (Fig. 3.3).

Postabdomen of instar II (Fig. 3.7), similar to that of female (Postanal/Preanal = 2.7), with more convex ventral margin and sperm ducts opening far from distal end, with a notch after them. Postabdomen of juvenile instars armed with same marginal denticles and lateral setae as females. Postabdominal claw similar to female, with long basal spine. In adult males the postanal region is more elongated and narrow (Postanal/Preanal = 3.0-3.1) (Fig. 3.8), openings of sperm ducts located distally, preceded ventrally by special short, blunt projection. Armature of the postabdomen thinner but more dense than in females forming a series of groups (with first member thicker) of marginal spinules. Lateral setules forming 11-14 groups. Postabdominal claws shorter and more robust than in females, with a very thin basal spine, surrounded in both margins by setules, forming a comb in concave side.

Trunk limb I (Fig. 3.4) with copulatory hook elongated, U-shaped, with two crests distally. ODL with setulated seta. IDL with male seta, and two other setae of different size. A portion of limb near hook inflated, and supplied with copulatory brush consisting of a pad and a row of relatively short, robust setules. Margin with rows of setae as in female.

Barcode. A 629-654 nucleotide sequence of the section of *COI* gene used for barcoding by the BOLD informatics database (Ratnasingham and Hebert, 2007) was obtained from four topotypes. Average sequence divergence was 1.16 ± 0.2 between them. The following sequence is for specimen ZPLMX156-06

TCTTGTTTGGGGCTTGGGCAGGTATAGTAGGGACTGCTTTGAGACT
TTTAATTCGAATTGAGTTGGGACAAGCTGGGTCTCTTATTGGTGACGATC
AGATTTATAATGTTATTGTAACTGCTCATGCTTTTGTTATGATTTTTTTTATA
GTAATGCCAATTTTAATTGGGGGGTTTGGGAATTGATTAGTGCCTTTGATG
TTGGGGAGCCCCAGATATAGCATTTCCTCGAATAAATAATTTGAGTTTTTG
GTTTTTACCTCCCTCGTTAACTTTACTTTTGGTTGGGGGGGCAGTAGAAA
GTGGGGCTGGTACCGGTTGAACGGTTTATCCTCCTTTATCGGCTGGTATTGC
TCATGCTGGAGCCTCTGTTGATTTAAGCATTTTTTCTTTACATTTAGCGGGG
ATCTCTTCAATTTTAGGGGCAGTTAATTTTATTACAACAGTAATTAATATACG
ATCTTATGGAATAACTTTAGATCGAATCCCTCTTTTTGTTTGGGCTGTGGCA
ATTACTGCTTTATTGCTTTTATTAAGTCTACCTGTTTTGGCAGGAGCTATTAC
TATACTTTTAACTGATCGTAATCTAAACACATCTTTTTTTGATCCTGCTGGCG
GGGGAGATCCCATTTTATACCAACATCTATT

For comparison, the sequence for *L. davidi* (ZPLMX 104-06) is also provided:

TACATTGTACTTTCTATTTGGTGCTTGAGCGGGAATGGTAGGAACT
GCTTTAAGATTGTTAATTCGTANTGAGTTAGGGCAAGCAGGCTCTCTTAT
TGGTGATGATCAAATTTATAATGTTATTGTGACTGCCCATGCATTTGTTATA
ATTTTTTTTATGGTAATGCCAATTTTAATTGGGGGATTCGGTAATTGACTTG
TACCTTTAATGCTAGGGGCCCCTGATATGGCATTTCCTCGAATAAATAAT
CTCAGTTTTTGATTTCTACCTCCATCATTGACTTTACTCTTAATTGGGGG
GGCTGTGGAAAGAGGAGCAGGGACGGGTTGAACAGTTTATCCTCCATTA
TCAGCTGGAATTGCCCATGCAGGAGCTTCTGTTGATTTAAGTATTTTTTCT
TTACATTTAGCTGGTATTTCTTCTATTTTAGGAGCTGTTAATTTTATTACGACA
GTGATTAATATACGATCTTATGGAATAACTTTGGATCGAATTCCTTTGTTTGT
CTGAGCTGTAGCAATTACTGCTCTGTTACTCTTGTTAAGTTTACCTGTTTTAG
CAGGAGCTATTACTATACTTTTGACTGATCGGAATTTGAACACGTCTTTTTT
TGATCCTGCTGGTGGGGGAGATCCTATTTTATATCAACATTTATT

In Figure 4 we show an ID tree with the two *Leberis* compared in this study. As an outgroup, we used *Alona glabra*. Divergence between *L. davidi* and *L. chihuahuensis* sp. nov. is at least 14.3% whereas divergence with *Alona glabra* ranged from 19.3% to 20.8%.

Main variation in the sequences of both species was in the GC% of the third codon position. In *L. chihuahuensis* the content varied from 18 to 23% while in *L. davidi* it was 17.6-18.7%. Main total variation was given by the G% with values 20.4-21.3% in *L. davidi* contrasting with 22.4-22.7% found in *L. chihuahuensis* sp. nov.

Type locality. Small puddle located at Km 217 on the Highway Jiménez-Torreón (Federal Hwy 45) (27°03'24.5" N, 104°47'31.5" W), a semi-desert area south of the Chihuahan Desert area. All material was collected by Manuel Elías-Gutiérrez, Tania Garfias-Espejo and José Angel Cohuo Colli in October 4, 2004.

Etymology. The species epithet honours the biggest state of Mexico (Chihuahua) where published works on freshwater biology are very scarce.

DISCUSSION

Morphology and distribution. The first report of *diaphanus*-like specimens in the Americas was by Elías-Gutiérrez (1996) on the Central Plateau. He considered, as did Frey (1991), that this species could be one of the few chydoridae with a true cosmopolitan dis-

tribution. Later on, analyses by Sinev *et al.* (2005) of *L. davidi* from America and *L. diaphanus* from Australia demonstrated that diffe-rences between these two species are subtle (body length, shape of labrum, some ratios as PP/IP, some setae lengths and modifica-tions of epipodites) but consistent. The morphological differences between *L. davidi* and *L. chihuahuensis* sp. nov. are stronger than those found between *L. davidi* and *L. diaphanus* (see Table 1).

Both *L. chihuahuensis* and *L. diaphanus* share some fea-tures, such as their small size, the lower number of marginal setae on the valves and the proportion between the setae of

exopodites III and IV (see Sinev *et al.*, 2005). Nevertheless, features such as the absence of sensilla and accessory seta on IDL, the morphology of males, and the disjunct geographical localities of both species, among other features, allowed us to consider these specimens as representatives of separate taxa. The other well-described species, *L. aenigmatosus*, from Australia (Smirnov, 1989), is clearly the largest member of this genus (up to 1.1 mm) and has many differences in the structure of the thoracic limbs, e.g. a different number of setae on limb II (Sinev *et al.*, 2005).
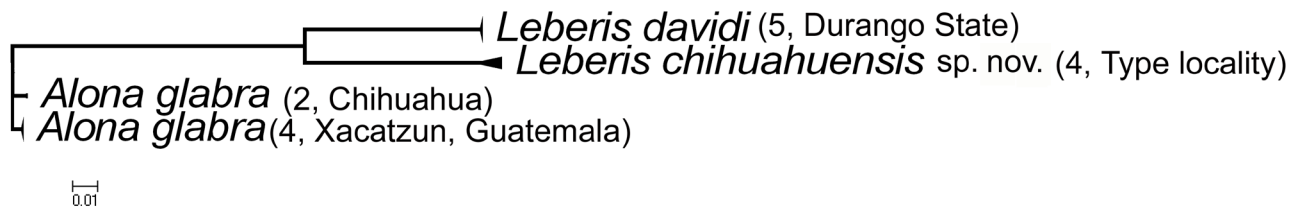

Figure 4. Simplified NJ ID tree of K2P distances of the species compared in this study. Number of specimens barcoded and locality between brackets.

Table 1. Main morphological differences between the two species of *Leberis* found in Mexico.

Character	L. davidi	L. chihuahuensis
Size of Female	up to 0.69 mm	up to 0.53 mm
Ventral margin of valves	with 84-95 setae	with 51-55 setae
Posterior margin of valves	With internal spinules short not forming groups	With longer internal spinules forming groups
Headpores	1.5-2.1 PP/IP	2.3-2.6 PP/IP
Margin of labrum	wavy	convex
Spine in basal segment of exopod	evenly curved, shorter than second segment	last third very curved, slightly longer than second segment
ODL	with sensilla and short accessory seta	without sensilla and accessory seta
Exopodite III	setae 1 and 2 of same length	seta 2 longer than seta 1
Limb IV	Exopodite with seta 2 longer than seta 1	Exopodite with setae 1 and 2 of same length
	No brush shaped seta in endopodite	Endopodite with brush-shaped seta
Male		
Posterior margin of valves	With internal spinules short not forming clear groups	With longer internal spinules very long, forming groups with first member much longer than the others
Antennule	With two lateral aesthetascs	With one lateral aesthetasc
Postabdomen	Postanal/Preanal=3.5	Postanal/Preanal=3.0-3.1
	Groups clearly formed all along postanal region	Groups formed but they are not so clear near the anus
Claw	Long, not too robust	Short and robust
	Basal spine thick and large	Basal spine relatively short and thin

We did not consider the epipodite projections in our data, because this could be a variable character. Kotov *et al.* (2004) found variable epipodites in *Macrothrix elegans* Sars, 1901, mostly on limb V. They speculated about oxygen concentration and epipodite size relationship, but this phenomenon has not been studied yet. We actually do not know if the finger-like projections of the epipodites described by Sinev *et al.* (2005) for *L. davidi* could be a variable character. These projections were not found in *L. chihuahuensis*. In terms of geographical distribution, it was thought that *L. davidi* was the only *diaphanus*-like representative in the New World, and the question about *L. diaphanus* and its endemicity to Australia remains open. With evidence gathered from the American *Leberis* at this moment represented by two species, it is likely that records from Asia represent a complex of species, probably more diverse than the American forms.

In the Americas, *L. davidi* is distributed from Argentina to Mexico, including the Caribbean islands (Sinev *et al.*, 2005) whereas *L. chihuahuensis* is currently known from a single locality in a semi-desert region. These contrasting distributional patterns could be a result of the lack of surveys on the genus in North America. It has been considered to be typically tropical. The two main biogeographical regions of the Continent (Nearctic and Neotropical) meet in Mexico, and thus, a mix of species from both origins has been recognized among the Cladocera (Elías-Gutiérrez *et al.,* 2006). It is possible that *L. davidi* represents the southern form of this genus, and *L. chihuahuensis* could be the northern one. However this hypothesis will require more detailed studies on the distribution and taxonomy of the Chydoridae in the north, where semi-desert regions remain practically unknown for this kind of aquatic fauna. An example of the lack of knowledge among the Anomopoda is the recent discovery of two new families, the Dumontidae, in a semidesert area of Oregon, USA (Santos-Flores & Dodson, 2003), and the Gondwanotrichidae from the southern coastal zone of Western Australia (Van Damme *et al.*, 2007a, b).

Barcoding data. As stated in the introduction, validity of the barcodes to identify species or even genera has been demonstrated for many groups as birds, insects and fish. In the crustaceans, Bucklin *et al.* (2007) demonstrated their value for species recognition in euphasiids and Costa *et al.* (2007) among groups of the entire class.

In our case, barcodes detected more than 14% divergence between two different *Leberis* from populations in the north of Mexico, thus highlighting the presence of two species. These molecular differences confirmed the morphological analyses. We did not find noticeable differences among the specimens of *L. davidi* from this and the other localities of Mexico, less than 300 km away, but the topotype material from Haiti should be barcoded.

Furthermore, the clear divergences of both species of *Leberis* with respect to *Alona glabra* confirms Sinev's *et al.* (2005) decision, based on morphology, to consider it as a separate genus. In accordance with Costa *et al.* (2005), divergences close to 20% are found between genera of Crustacea. More than 20% divergence within species for *CO1* in other groups such as fish raised questions about the integrity of some genera, such as *Coryphopterus*, a gobiid (Victor, 2007).

Within the Aloninae, this paper contributes to a better understanding of the taxonomy of the *diaphanus*-like group, where the status of several species remains to be clarified (Frey, 1991; Sinev *et al.*, 2005). It is clear that recognition of species in this subfamily, particularly in reference to all *Alona*-related genera is difficult, and requires the examination of material from different continents. Barcodes can contribute in a definite way to clarify the status of most of these Cladocera species.

Integral studies, involving molecular data with a standardized gene as the *CO1*, and a detailed account of the morphological characters represent a complete, upgraded approach to explore the taxonomy of different groups, as in the example presented here. In the near future this new standard will allow us to establish with more certainty the relationships among the species and even genera within the most complex groups such as the Cladocera, a particular group that remains poorly understood, in which new discoveries are common, mostly from the tropical regions.

However, discussions about relationships within the subfamily will remain and the *COI* by itself only will allow species identification and help to establish within-genera relationships.

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