Morphological, ecological, reproductive and molecular evidence for *Leptodiaptomus garciai* (Osorio-Tafall 1942) as a valid endemic species

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The aim of this work is to elucidate the biological and taxonomic status of a copepod from a hyposaline lake originally described as Diaptomus garciai but later synonymized with the freshwater Leptodiaptomus novamexicanus. We compared parapatric populations of L. novamexicanus s. l., from the deep Lake Alchichica (hyposaline and oligotrophic) and from temporary ponds (freshwater and hypereutrophic), using morphological analyses, molecular markers, ecophysiological tests (salinity tolerance) and inter- and intrapopulation breeding trials. Morphological analysis showed slight but consistent differences among the freshwater (specific conductivity at $25°C$ $K_{25}=$: $440\ \mu S\ cm^{-1}$) and hyposaline ($K_{25} = 13\ 300\ \mu S\ cm^{-1}$) populations. Fitness of copepods from Lake Alchichica was dramatically reduced at salinities lower than $4.5\ g\ L^{-1}$ ($K_{25} = 8100\ \mu S\ cm^{-1}$). The opposite was true for the freshwater populations which were not able to survive, grow or reproduce at salinities higher than $2.5\ g\ L^{-1}$ ($K_{25} = 4680\ \mu S\ cm^{-1}$). Interbreeding was not observed between individuals from freshwater and brackish populations. Additionally, there was an important divergence in nucleotide sequence variation (mitochondrial gene cytochrome oxidase subunit I) between L. garciai and the freshwater populations (KP2: $4.4-8.4\%$). Based on ecological specialization, reproductive isolation, morphological and molecular divergence, we conclude that L. garciai is a different biological species from L. novamexicanus and a valid taxon.

INTRODUCTION

In the last three decades, exhaustive comparative analyses based on molecular markers, interbreeding trials and/or detailed morphometry have revealed that many copepod species traditionally seen as cosmopolitan and/or with wide physiological tolerances are in fact assemblages of cryptic species of regionally restricted taxa (Carrillo *et al.*, 1974; Frost, 1974; Reid, 1998; Lee, 2000). Morphological conservatism contributes to difficulties in recognizing distinct biological species for this group. Moreover, species boundaries are often difficult to define because of the lack of data linking genetic and phenetic (morphology, ecology etc.) diversity with patterns of reproductive compatibility (Lee, 2000).

The calanoid copepod *Leptodiaptomus novamexicanus* s. l. (Herrick, 1895) is a North American species, spanning a broad geographic range from temperate British Columbia (Wilson and Yeatman, 1959) to the tropical Yucatan Peninsula (Suárez-Morales *et al.*, 1996). This copepod has been the centre of attention in several

ecological studies because of its dominance as a primary grazer in lakes throughout the region (e.g., Brett *et al.*, 1994). *Leptodiaptomus novamexicanus* s. l. has been recorded in water bodies that vary from permanent deep oligotrophic lakes to temporary eutrophic ponds, which in general are freshwater systems with pH values from neutral to relatively acidic, and alkalinity and hardness from low to medium (see Grimaldo-Ortega *et al.*, 1998). In addition, this copepod has also been recorded from a single brackish lake, Lake Alchichica in the State of Puebla, Central México, where it is the only calanoid copepod, and the dominant zooplankon species (Lugo *et al.*, 1999). Interestingly, this population of calanoid copepods from Lake Alchichica was originally described by Osorio-Tafall (Osorio-Tafall, 1942) as *Diaptomus garciai*, though recognizing its closeness with *L. novamexicanus*. *Diaptomus garciai* was described and reported only in Lake Alchichica before being synonymized by Wilson and Yeatman (Wilson and Yeatman, 1959) under *Diaptomus (Leptodiaptomus) novamexicanus* (Herrick, 1895). Unfortunately, Wilson and Yeatman did not provide any explanation, and all material from Osorio-Tafall, including the types deposited in the Polytechnic Institute of Mexico (IPN), has been lost (Suárez-Morales *et al.*, 2000).

In light of the recent discoveries regarding cryptic species, we suggest that the capacity of *L. novamexicanus* s. l. to colonize a wide range of aquatic systems including freshwater as well as brackish environments is at least doubtful. We hypothesize that this copepod in fact consists of a complex of cryptic species, with the population inhabiting hyposaline Lake Alchichica being a distinct biological species. We tested our hypothesis by gathering additional evidence to the original observations by Osorio-Tafall (Osorio-Tafall, 1942) from different sources. First, we carried out a detailed morphological analysis of Lake Alchichica copepods and three additional populations of *L. novamexicanus* s. l. from Central Mexico and California (USA). Second, we used DNA barcodes to measure the genetic divergence between populations at the mitochondrial gene cytochrome oxidase subunit I (COI). Third, we measured the biological performance of *L. novamexicanus* s. l. populations from two contrasting environments (Alchichica: oligotrophic, hyposaline lake vs. Ixtlahuaca: eutrophic, freshwater pond) across a salinity gradient to evaluate their tolerance to salinity. Fourth, we performed interbreeding trials to determine whether sexual recognition existed between both populations and measured the reproductive success of mating events to evaluate potential gene flow. Finally, we integrated our results to elucidate the biological and taxonomic status of the Lake Alchichica population.

METHODS

Collection localities

Organisms were collected in four water bodies located in Central Mexico. Lake Alchichica is located at 19°24'N, 97°24'W; 2300 m above sea level. It is a perennial, oligotrophic, saline crater lake (TDS = 8.3–9.0 g L^{-1}; pH 8.7–9.2; specific conductivity at 25°C, K_{25} = 12676–13727 μS cm^{-1}) in a semi-desert region, with a maximum depth of 62 m and a mean depth of 40.9 m (see Adame *et al.*, 2007 for further details). Ixtlahuaca is located at 19°29'05"N, 99°44'07"W, 2200 m above sea level. It is a eutrophic, temporary, freshwater pond (TDS = 0.1 g L^{-1}; pH 7.4; K_{25} = 204.7–220.9 μS cm^{-1}). For measuring COI divergence (barcodes), additional material was obtained from the freshwater ponds Invernadero (19°15'0"N 99°37'0"W) and Borregos (19° 49'21"N 99°42'22"W).

Adult males and females of *L. novamexicanus* s. l. were collected with horizontal hauls with a conical zooplankton net (100 μm mesh size) from all localities. One fraction of the material was fixed in 100% ethanol for morphological examination and to perform the DNA barcodes. The remaining material was kept alive and transported to the laboratory for culturing.

Morphological analyses

Morphological analysis was carried out with adult individuals from both sexes collected from Alchichica, Ixtlahuaca and Borregos. Since no type material of *L. novamexicanus* (Herrick, 1895) is available, we tried to obtain animals nearby the type locality. Herrick (Herrick, 1895) specifies as the type locality "the tank of the city works in Albuquerque, New Mexico". Unfortunately, no recent record of this species is available in the literature, and we were not able to collect material from this area, because it does not exist anymore. However, we got additional organisms from Castle Lake, California (Collected in 2002, kindly donated by C. Goldman and S. Park, UCLA) in order to compare *L. novamexicanus* s. l. populations from Central Mexico with at least another one occurring as near as possible to the type locality. A detailed morphological analysis was performed following standard procedures in calanoid taxonomy (e.g. Chiambeng and Dumont, 2002; Elías-Gutiérrez *et al.*, 1999; Suárez-Morales *et al.*, 2000) with a stronger focus on structures related to sexual recognition and mating processes. Observations and drawings were carried out at high magnifications (mainly at 1000x) with a drawing tube RV1 attached to a Zeiss III RS compound microscope. Scanning electron microscopy (SEM) was

performed on several complete and dissected specimens from Alchichica, Castle Lake and Ixtlahuaca with a JEOL JSM6360LV microscope.

DNA barcodes

DNA barcoding from ethanol fixed samples from four localities (Alchichica, Ixtlahuaca, Borregos and Invernadero) was carried out at the Canadian Centre for DNA Barcoding, using standard protocols (Hajibabaei *et al.*, 2006). DNA was extracted from whole body homogenates using a mix of Proteinase K with invertebrate lysis buffer and digested overnight at 56°C. 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 fibre media (Ivanova *et al.*, 2006). Approximately 600–658 bp were amplified from the COI using LCO1490 and HCO2198 primers (Folmer *et al.*, 1994). The 12.5 μL PCR reaction mixture included 6.25 μL of 10% trehalose stabilizer, 2 μL of ultrapure water, 1.25 μL of 10× PCR buffer, 0.625 of MgCl$_2$ (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.*, (2006) and sequenced bidirectionally using an ABI 3730 capillary sequencer following manufacturer's instructions. 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). Neighbour-joining (NJ) trees of K2P distances [provided in Barcode of Life Database (BOLD)] were created to provide a graphic representation of the patterning of divergence between species (Saitou and Nei, 1987). A simplified tree of all the species was elaborated with the compress/expand feature of a subtree provided in the MEGA 3 software (Kumar *et al.*, 2004). A maximum parsimony analyses in PAUP with bootstrapping of ∼1000 replicates was also performed.

Biological performance along salinity gradients

Experimental conditions and organisms

Cultures were established with organisms from Alchichica and Ixtlahuaca. A total of 300 individuals from each locality were used to start the cultures. Egg-carrying females and adult males morphologically identified as *L. novamexicanus* s. l. were cultured in 2 L containers. Culture media for Alchichica and Ixtlahuaca populations were prepared by filtering Lake Alchichica water (0.2 μm MF-Millipore filters) and EPA medium (Weber, 1993; 0.1 g L^{-1}; K_{25} = 440 μS cm^{-1}), respectively. The latter was considered as the freshwater treatment (hereafter called as 0 g L^{-1} treatment). Culture medium was renewed completely once a week. Organisms were maintained in a temperature-controlled room (18 ± 1°C) with dim light, and fed every 3 days on a mixture of the chlorophytes *Nannochloris atomus*, *Chlorella* sp. and *Scenedesmus* sp. (1:1:1 ratio; ∼20 mg C L^{-1}) at the corresponding field salinity for each copepod population. Algae used in this study were cultured with EPA medium (*Chlorella* sp. and *Scenedesmus* sp.) or artificial saline water at 9 g L^{-1} (*N. atomus*) prepared with commercial sea salts (Instant Ocean, Aquarium Systems) dissolved in autoclaved, deionized water, and fertilized with f/2 medium (Andersen, 2005). Copepods were kept in the laboratory for at least 2 months before being used in the experiments.

Effect of salinity on survivorship, somatic growth and reproduction

Four salinity treatments were set up: 0, 2.5, 4.5 and 9.0 g L^{-1}, which were prepared by mixing EPA medium and Lake Alchichica water (see details above); the two extreme salinities were considered as controls, 0 g L^{-1} (i.e., EPA medium) for the Ixtlahuaca population, and 9.0 g L^{-1} for Alchichica population. At each salinity level, at least 45 copepodids (CIII) from each population were individually placed in wells (Evergreen^{TM} polystyrene 6-well plates) containing 8 mL of medium and fed as previously explained throughout the experiment. When possible, organisms were acclimated at least for 3 days before the experiment; if they did not survive the acclimation period, copepods were taken directly from the stock cultures. Animals were examined daily under a stereomicroscope to record survival, and then were transferred to containers with fresh medium and food. Experiments were terminated on the seventh day. We tested the effect of salinity, time and their interaction on the survivorship of the copepodids by analysis of variance for repeated measures (ANOVAR; von Ende, 1993). The probability values (*P*) of the main effects and their interaction were obtained by the Greenhouse-Geisser adjustment (von Ende, 1993). All statistical analyses were performed with SPSS for Windows, release 12.0.0 (SPSS Inc., 2003).

The effect of salinity in somatic growth was measured in two phases: (i) as percentage of CIII copepodids

moulting to CIV to measure the immediate effect of salinity and (ii) as a percentage of CIV copepodids reaching the adult stage to evaluate the mid-term effect of salinity on development. The salinity levels tested, as well as the medium renewal, food, light, temperature and general conditions, were the same as in the survivorship experiment. At least 45 CIII copepodids were selected from the two populations for each salinity treatment. Survivorship and moulting events were recorded daily. The experiment was run for 14 days, enough time to allow most copepodids in the control vessels to develop into the adult stage. Collected data were used to calculate the percentage of copepodids moulting from CIII to CIV, and the percentage of CIV copepodids becoming adults. Data were arc-sin transformed prior to performing statistical analyses. Depending on the homogeneity of variances (Levene test; $P > 0.05$), the results were analysed by ANOVA or by Kruskal–Wallis test (Dytham, 2003) to determine the effect ($P \leq 0.05$) of salinity. If significant differences were found, a *post hoc* Student–Newman–Keuls test (Dytham, 2003) was carried out.

Mating trials

With the aim of assessing the effect of salinity on intrapopulation reproduction, mating trials with each population were also performed at 0, 2.5, 4.5 and 9 g L^{-1}. Each mating trial was made up of one adult female plus two males, selected from the pre-experimental cultures, in order to increase the possibility of a successful mating. At each salinity level, there were at least 5 replicates; each experimental trio was placed in a separate 50 mL flask under the general experimental conditions of temperature, light and food (see above). Animals were examined daily for 7 days during the replacement of the culture medium. We recorded the percentage of copulated females (females carrying a spermatophore), percentage of fertilized females (females with egg sac), clutch size (number of eggs per female) and percentage of hatching (number of hatched larvae per sac). Data collected were analysed by ANOVA or Kruskal–Wallis test depending on the data distribution (see description here above) to look for significant differences on clutch size and percentage of hatching due to salinity treatments. Student-Newman-Keuls test were performed as in the previous experiments.

Subsequently, we performed interpopulation reproductive trials at 2.5 g L^{-1}, as this was the salinity in which copepods from Ixtlahuaca Pond and Lake Alchichica could survive simultaneously for at least some days (see Results). Intrapopulation trials were used as controls. We arranged two combinations: (i) Alchichica females ×

Ixtlahuaca males and (ii) Alchichica males × Ixtlahuaca females. General experimental conditions, procedures, and recorded variables were as previously described for intrapopulation trials. Results were compared with the intrapopulation mating data at salinity controls (0 and 9.0 g L^{-1}, for Ixtlahuaca and Alchichica, respectively).

RESULTS

Morphology

The four analysed copepod populations (Alchichica, Ixtlahuaca, Borregos and Castle Lake) correspond well with the general description of *L. novamexicanus* provided by Herrick (Herrick, 1895; see also e.g. Marsh, 1907; Wilson and Yeatman, 1959). However, some morphological variability was found. Adults from Ixtlahuaca and Borregos were larger than those from Alchichica and Castle Lake (ANOVA, $P \leq 0.05$), although sexual dimorphism in size within each population was not statistically significant (Table I). Females from the four populations share the general structure of body shape, antennae, mouthparts and thoracic limbs, including the fifth pair (see detailed description hereafter). The same was true for the urosome, except for a process at the right posterior margin of the genital segment (Fig. 1A, C, and E). For Lake Alchichica animals, this process was about one-third as long as the length of the second urosomite (Fig. 1A), whereas for the copepods from the rest populations this was at least two-third as long as the next segment (Fig. 1C and E). For males, most of the structures were similar among populations, except for the length of the lateral process on the antepenultimate segment 19 of the antennule which was less than half of the length of the penultimate segment 20 in Lake Alchichica copepods (Fig. 1B), whereas in the

Table I: Body size in adults from four populations of Leptodiaptomus novamexicanus s. l. from Central Mexico (Alchichica, Ixtlahuaca and Borregos) and California, USA (Castle Lake)

	♀			♂		
	Range	Mean ± SE	n	Range	Mean ± SE	n
Alchichica	0.87–1.02	0.96 ± 0.04[a]	20	0.84–1.03	0.91 ± 0.04[a]	20
Ixtlahuaca	0.98–1.12	1.06 ± 0.03[b]	20	0.87–1.12	0.98 ± 0.06[b]	20
Borregos	1.18–1.2	1.19 ± 0.0[b]	20	1.10–1.18	1.15 ± 0.03[b]	20
Castle	0.89–1.07	0.96 ± 0.05[a]	15	0.83–0.98	0.89 ± 0.03[a]	17

Homogeneous groups (ANOVA, $P \leq 0.05$; Tukey's test) are indicated by same letters.
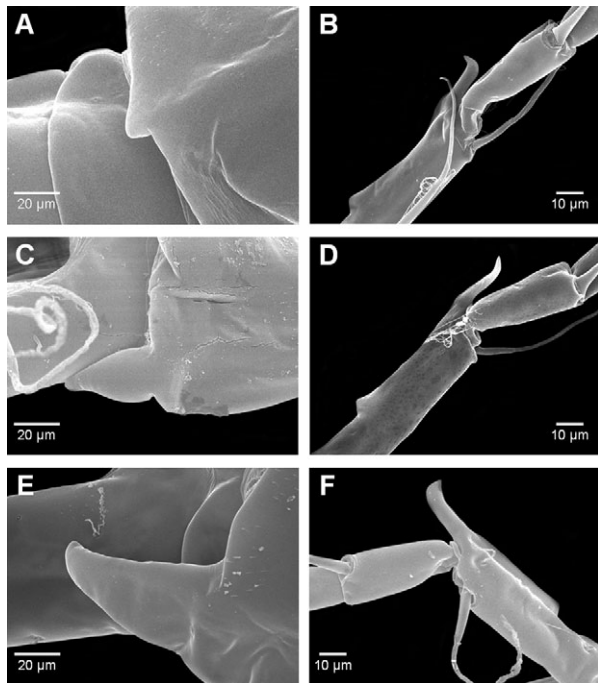
Fig. 1. Comparative morphology of some important characters among analysed populations. (**A** and **B**) *Leptodiaptomus garciai* from Lake Alchichica. (**C** and **D**) *Leptodiaptomus novamexicanus* s. l. from Castle Lake. (**E** and **F**) *Leptodiaptomus novamexicanus* s. l. from Ixtlahuaca Pond. Left panels (A, C and E) detail of process at the right posterior margin of the female genital segment. Right panels (B, D and F) detail of the process on the antepenultimate segment 19 of the male right antennule.

other populations this was relatively longer, i.e. more than a half of the length of segment 20 (Fig. 1D and F).

Although we concluded that *L. garciai* is a valid taxon on the basis of barcoding, salinity tolerances and interbreeding trials, we provide here a morphological description of this species.

Redescription of *Leptodiaptomus garciai* (Osorio-Tafall, 1942) (Figs 1A–B and 2–7)

Family Diaptomidae.

Genus *Leptodiaptomus*.

Diaptomus garciai, Osorio-Tafall, 1942; *Diaptomus (Leptodiaptomus) novamexicanus*, Wilson and Yeatman, 1959: 789 (*partim*); *Leptodiaptomus novamexicanus*, Reid, 1990: 179 (*partim*); *Leptodiaptomus novamexicanus*, Suárez-Morales *et al.*, 1996: 100–102, 108 (*partim*).

Revised material

Neotype. An ethanol–glycerine (5%) preserved specimen labelled *L. garciai*, Alchichica Lake (Type locality), collection date 8 February 2007, female. Accession number ECO-CH-Z-03 592. Additional material preserved in glasses with ethanol–glycerine (5%) represented by 10 females and 10 males (collected: 8 February 2007; accession numbers: ECO-CH-Z-03 593 and ECO-CH-Z-03 594) and 5 males and 5 females (collected: 17 May 2007; accession numbers: ECO-CH-Z-03 601 and ECO-CH-Z-03 602) were deposited in the Zooplankton Collection from El Colegio de la Frontera Sur, Chetumal Unit, México. Additionally, 10 males and 10 females (collected: 17 May 2007) in glasses with ethanol–glycerine (accession numbers: CNCR 25 101 and CNCR 25 102) and two dissected males and two dissected females (collected: 8 February 2007; accession numbers: CNCR 25 103 and CNCR 25 104) on permanent glycerine glass slide sealed with Permount[TM] mounting medium were deposited in the Colección Nacional de Crustáceos (CNCR), Instituto de Biología, Universidad Nacional Autónoma de México. Sequences for the DNA barcodes in BOLD (www.boldsystems.org) and GenBank accession numbers are EU701068 to EU701099 for all materials studied here.

Female. Length (mean ± SE) 0.96 ± 0.04 mm, range from 0.87 to 1.02 mm ($n = 20$). Prosome narrow anteriorly, symmetric, with a slight constriction just below the cephalic region (Fig. 2B). Rostral points acute, semi-triangular, with a spine-like process between them (Fig. 2I). Thoracic wings (i. e., lateral processes of fifth thoracic segment) vaguely asymmetrical, left slightly shorter than the right one; each with two processes directed posteriorly and bearing a distal sensilla (FigS 2C–E and 3B and C). 3-segmented urosome (Fig. 2A–D). Genital somite larger than the rest of the abdomen (Figs 2C–E and 3B and C), strongly asymmetrical with lateral processes at both sides, each with a terminal spine; the right one being the longest, whereas the left with a wider base. Right side of the genital segment with dorsal digitiform process at the posterior margin, about a third of length if compared with the second segment (Figs 1A, 2C and 3B). Second segment short, about one-eighth, whereas the anal somite is about one-third the length of genital segment. Caudal rami more than twice as long as wide with four terminals, one dorsal and one lateral plumose seta, relatively long, about 2.5 times as long as the caudal rami, with a row of hairs (Figs 2A–C and 3A).

Antennules (Fig. 2F–H) long, 25-segmented, reaching beyond posterior margin of caudal rami by at least two segments (Figs 2A and 3A). Appendages per segment as follow (Arabic numerals=segment, Arabic numerals in parentheses=number of setae, ae=aesthetasc, sp=spine): 1(1+ae), 2(3+ae), 3(1+ae), 4(1), 5(1+ae), 6(1), 7(1+ae), 8(1+sp), 9(2+ae), 10(1), 11(1), 12(1+ae+sp), 13(1), 14(1+ae), 15(1), 16(1+ae), 17(1)
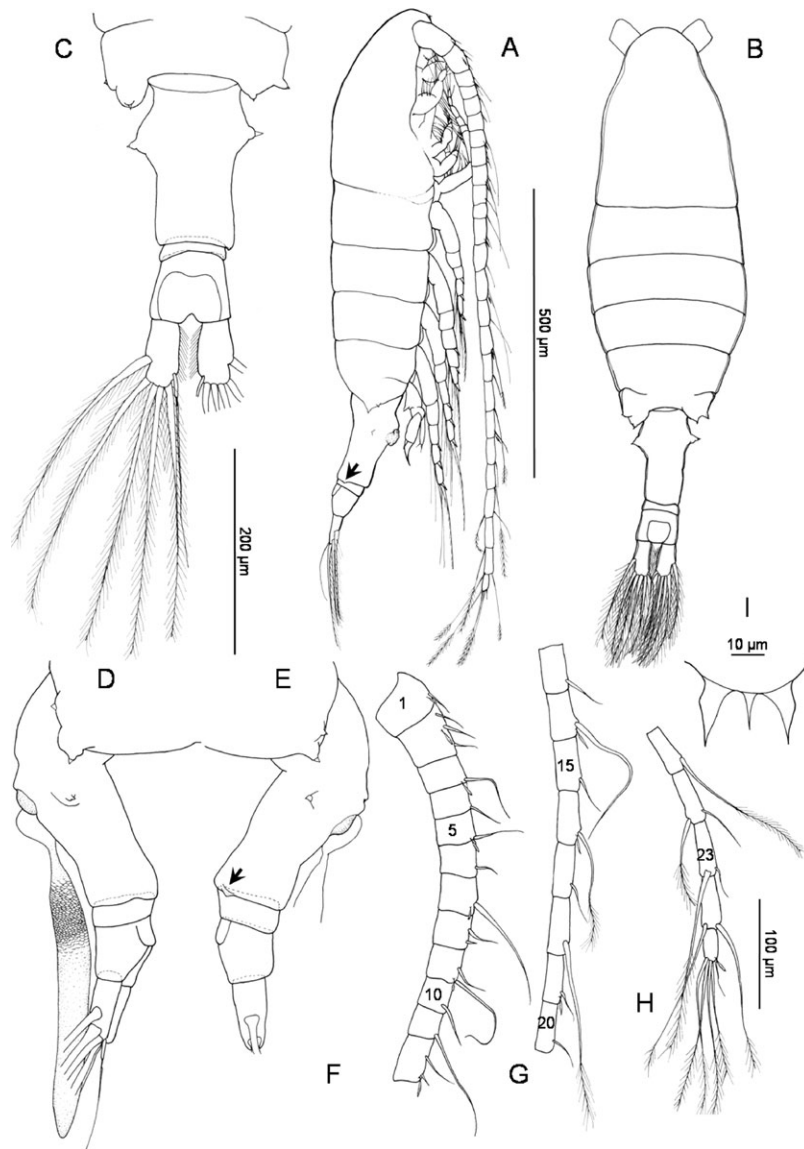
Fig. 2. *Leptodiaptomus garciai*, female. (**A**) Habitus, right lateral view (arrow points to the small process at the right posterior margin of genital segment). (**B**) Habitus, dorsal view. (**C**) Pediger 5 and urosome, dorsal. (**D**) Same with spermatophore, left lateral. (**E**) Same, right lateral. (**F**) Antennule, segments 1–12. (**G**) Same, segments 13–20. (**H**) Same, segments 21–25. (**I**) Rostral spines.

18(1), 19(1+ae), 20(1), 21(1), 22(2), 23(2), 24(2), 25(5+ae).

Antenna, maxilla, maxillule and maxilliped (Fig. 4A and C–E) similar in structure to those described for other *Leptodiaptomus* by Elías-Gutiérrez *et al*. (1999) and Suárez-Morales *et al*. (2000).

Mandible (Fig. 4B) with 6–7 teeth on gnathobase, most of them with a single point; ventral outermost tooth larger with a short spine-like projection on tip; while the second is wide and blunt. Distal end on internal margin with short setulated seta-like projection on the tip. Basis with four setae; endopod bisegmented, proximal segment with four setae; distal segment with

nine setae. Exopod 4-segmented, with a 1, 1, 1, 3 setation pattern.

First thoracic leg (armature formula in brackets) (Fig. 5A) with 3-segmented exopod (I-1; 0–1; I-3-2) and 2-segmented endopod (0–1; 0–1,2,3), coxa (0–1; equal for the rest of legs) with a plumose seta on internal margin, reaching middle of first endopodal segment. Second, third and fourth legs (Figs 5B–D) with exopods (I-1; I-1; I-3,3) and endopods 3-segmented (0–1; 0–2; 0–1,2,3).

Leg 5 (Figs 3E–H and 5E and F): coxa with lateral processes on each side, both with a terminal sensilla, with their tips reaching the distal end of basis. Basis
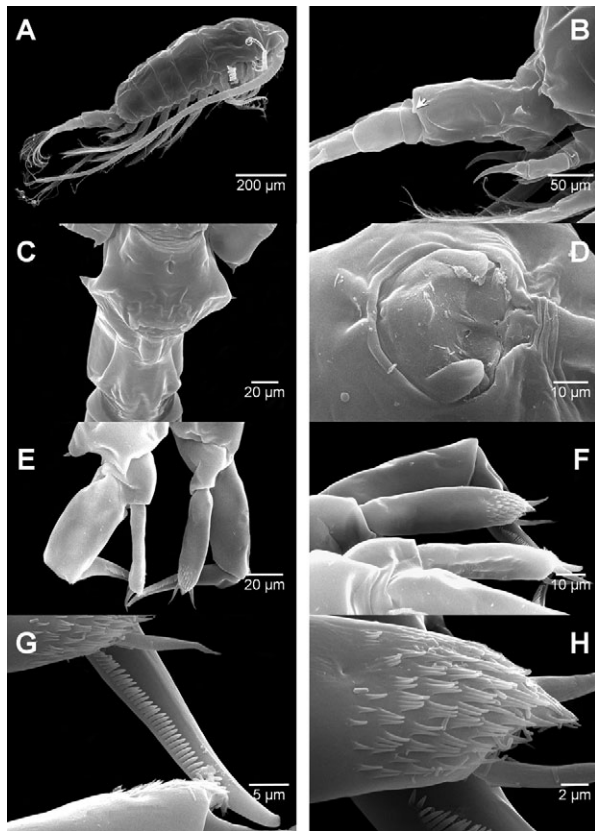
Fig. 3. SEM microphotographs of *L. garciai*, female. (**A**) Habitus, right lateral view. (**B**) Pediger 5 and urosome, right lateral. (**C**) Genital somite, ventral (arrow points to the small process at the right posterior margin of genital segment). (**D**) Detail of the genital aperture, ventral. (**E**) P5, anterior. (**F**) P5, left endopod (arrow). (**G**) P5, detail of terminal claw. (**H**) P5, detail of distal part of endopod.

with internal margin almost straight, with a long lateral seta reaching midlength of first exopodal segment. Endopod unisegmented, long and slender (4.5 times long as wide), its distal end reaching beyond distal margin of first exopodal segment; armed with two subequal in length subterminal setae. Distal part of the endopod tapering distally. Internal margin of distal quarter of endopod armed with short hair-like setae. First exopodal segment as long as second. Claw of second exopod with a blunt tip; internal margin armed with a row of teeth-like setae from about mid-margin but not reaching the tip. Third exopodal segment fused to the second, with two subequal in length spines.

Male. Length (mean $\pm$ SE) 0.91 ± 0.04 μm, range from 0.84 to 1.03 μm ($n = 20$). Prosome similar to female, more narrow (Figs 6A and 7A). Rostral points triangular in shape, close, with a spine-like projection in between (Fig. 6J). Pediger 5 tapering posteriorly (Fig. 6A–C), asymmetrical, with relatively smaller lateral wings when compared with the female. Right

lateral process oblong in shape, directed outwards, posterior end reaching about the half way along the first urosomite, with two slight processes each bearing a distal sensilla, similar to those described in the female. Left wing reduced, with a single rounded lobe pointed posteriorly bearing a distal sensilla. Urosome (Fig. 6B–C) slightly asymmetrical, 5-segmented. First urosomite with a small rounded process on left margin. Second and third segments with groups of 2–5 spinules arranged in a row on the distal dorsal margin. Caudal rami as in female.

Antennules somewhat longer than in female (Figs 6D–F and 7A). Appendages per segment as follow (Arabic numerals=segment, Arabic numerals in parentheses=number of setae, ae=aesthetasc, sp= spine): 1(1+ae), 2(3+ae), 3(1+ae), 4(1), 5(1+ae), 6(1), 7(1), 8(1+sp), 9(2+sp), 10(1), 11(1), 12(1+ae+sp), 13(1+ae), 14(2+ae), 15(1+ae+sp), 16(1+ae+sp), 17(sp) 18(1), 19(2+sp), 20(2), 21(5+ae). Segments 7–21 of right antennule modified, main articulation between segments 17 and 19. Setae on segments 7, 9 and 14 the largest. Segments 10, 11 and 13 each with one short stout spine-like process, the former being the largest reaching midlength of the next segment. Spines on segments 15, 16 and 17 biacuminate. Segments 17 and 18 elongated with a spine-like process pointing distally, and running along the internal margin of segments. Segment 19 with elongated knob-like process on distal half of the internal margin reaching proximal third of the next segment. Setation of left antennule, mouth-appendages and swimming legs as for female.

Left leg 5 (Fig. 6G and H): relatively short, roughly reaching distal margin of right endopod. Coxa with a small distal process on external margin. Basis with a long lateral seta reaching beyond distal margin of first exopodal segment. First exopodal segment similar in size as the second, with a group of relatively long hairs on distal half of internal margin (Figs 6H and 7B). Second exopodal segment ending in a distal process with a relatively large, slender and smooth terminal digitform process (Figs 6G and H and 7B and C), and a similar but shorter subterminal process which bears some papilla-like growths in the anterior margin (Fig. 7D). Internal margin of second exopod provided with long hairs on the proximal third and papilla-like processes on distal rounded third (Figs 6H and 7B and D). Endopod 1-segmented, long and slender, reaching beyond two-thirds of second exopodal segment, narrowing distally at distal third, which is covered in its internal margin with small hair-like setae (Fig. 7C).

Right leg 5 (Figs 6G–I and 7E and F): coxa with a process on distal external margin bearing a strong spine. Basis almost twice as long as first exopodal
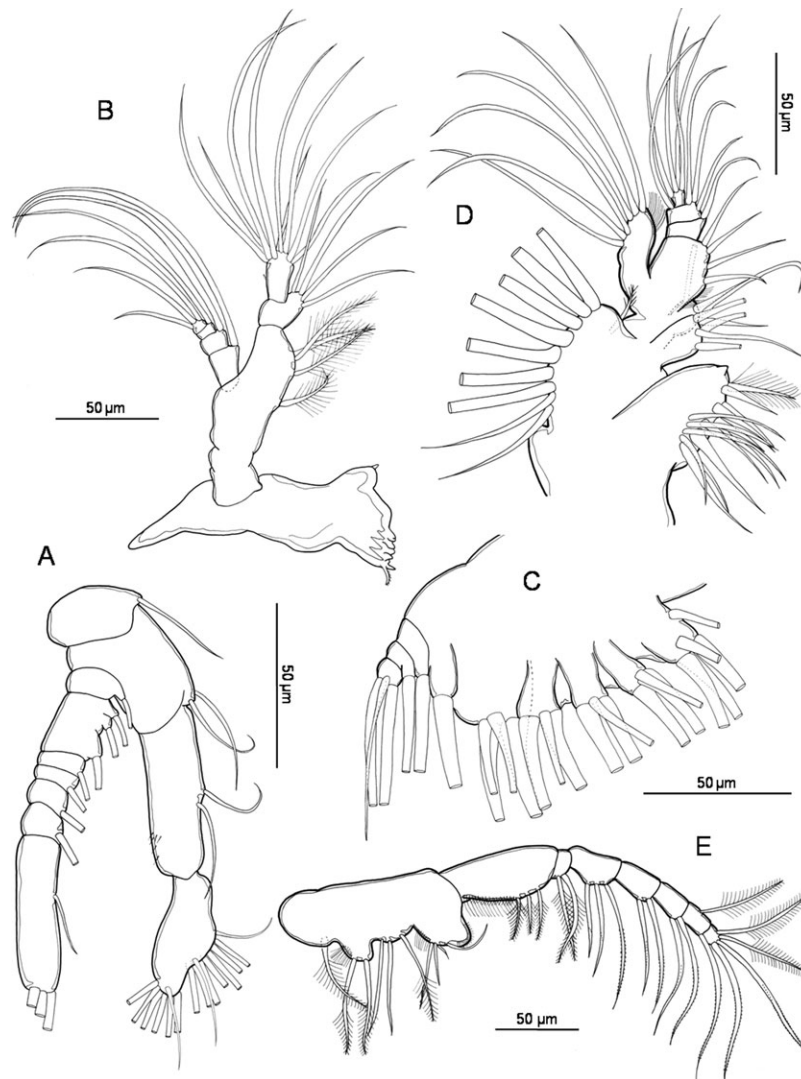
Fig. 4. *Leptodiaptomus garciai*, female. (**A**) Antenna. (**B**) Mandible. (**C**) Maxilla. (**D**) Maxillule. (**E**) Maxilliped.

segment with a seta inserted on middle of the external margin, and a rounded hyaline process on internal anterior margin directed upwards (Fig. 6H). First segment of exopod with two rounded, short processes at distal external and internal margins, the former relatively larger. Second exopodal segment long and slender (about three times as long as wide), and about three times as long as the first. Lateral spine slender and short (as long as the segment width), slightly curved, naked, tapering distally with a blunt tip, inserted in the middle of segment. Terminal claw slender and curved (Fig. 7F), tapering gradually from the enlarged base, about the same length as the exopod (segments 1 and 2 together), with a row of teeth on internal margin, increasing in size distally, which starts from about the midlength of claw and ends before reaching the claw

tip. Endopod (Figs 6G and H and 7E) 1-segmented, relatively large, reaching beyond the distal margin of first exopodal segment, compressed dorsal-ventrally, and tapering distally from the proximal third, which gives to it a characteristic semi-triangular shape, naked in the posterior surface, but with a group of setules on the tip of anterior surface (Fig. 7E).

DNA barcodes

In total, 586–657 nucleotide sequences of the section of COI gene were obtained for seven specimens from Lake Alchichica and 25 from Ixtlahuaca, Borregos and Invernadero. Results are available in BOLD (www.boldsystems.org) (Ratnasingham and Hebert, 2007). The simplified tree is given in the Fig. 8,

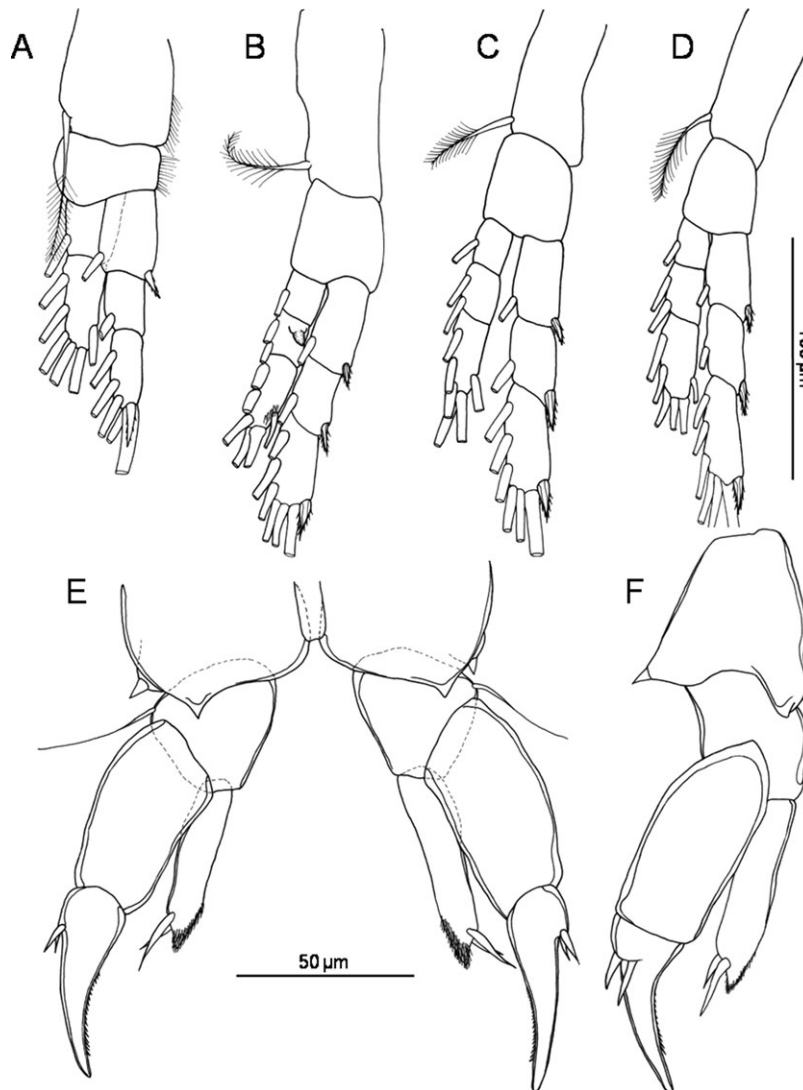**Fig. 5.** *Leptodiaptomus garciai*, female. (**A**) P1. (**B**) P2. (**C**) P3. (**D**) P4. (**E**) P5, all in posterior view. (**F**) P5, right lateral.

where three clusters clearly appear, one composed exclusively by Alchichica individuals and the other two integrated by Borregos, Invernadero, and Ixtlahuaca organisms. Intraspecific variation in each cluster was quite low averaging <0.18%. The divergence between the species within the genus was 6.04% on average (Table II). The Alchichica population formed a cluster apart (*L. garciai*) with at least 4.4% divergence from the closest neighbour. Moreover, *L. novamexicanus* s. l. formed two clusters, one of them closer to *L. garciai*. The GC content was between 38.02 and 39.14% in all specimens from the three clusters barcoded. Inside the cluster represented by Alchichica population, the GC content was from 38.40 to 38.78%.

The GenBank accession number of the 654 bp sequence ($5' - 3'$) of a topotype *L. garciai* (ZPLMX942-06) is EU701078.

Salinity tolerance

At reduced salinities, survivorship and moulting of Alchichica copepods were drastically reduced. After a week, ~70% of the animals remained alive at $9\,\mathrm{g\,L^{-1}}$ (lake salinity), but as salinity decreased a clear pattern of reduction in survivorship was observed (ANOVAR, $P \leq 0.001$; Fig. 9), registering a 0% survivorship on the fourth day in the freshwater treatment. Somatic growth was also affected (Fig. 10). At $4.5\,\mathrm{g\,L^{-1}}$, only $37.5 \pm 4.9\%$ of CIII copepodids could moult to CIV
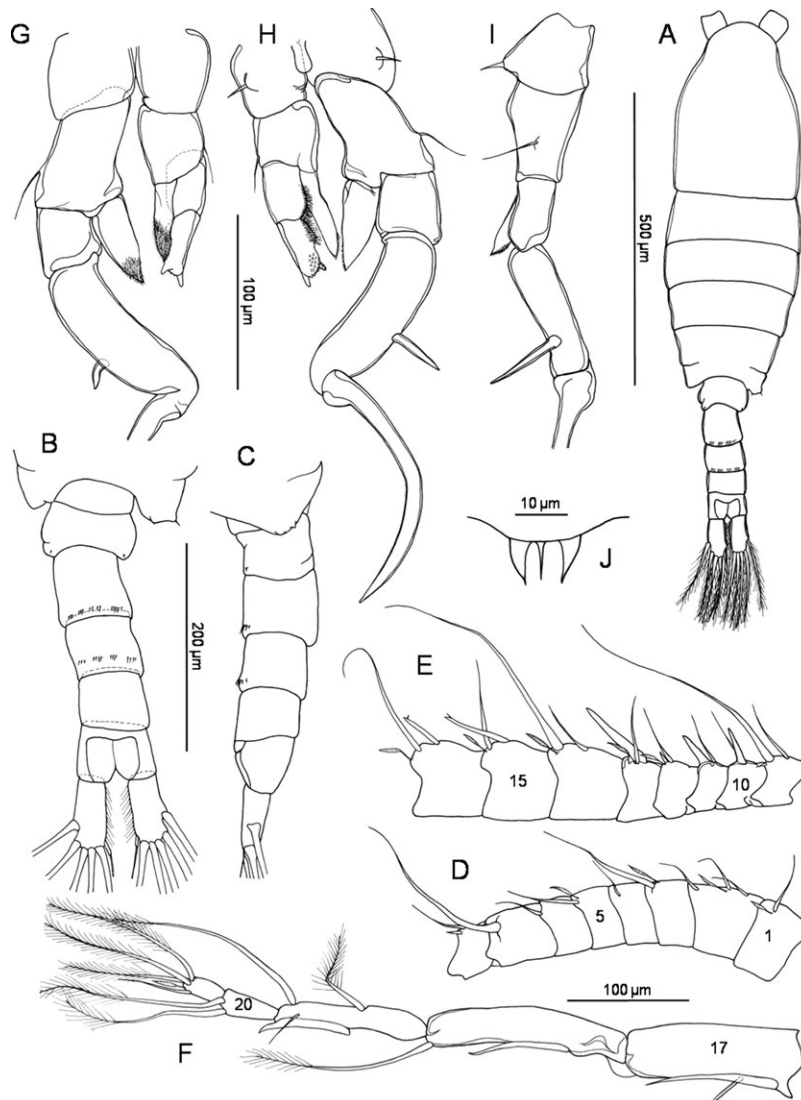
Fig. 6. *Leptodiaptomus garciai*, male. (**A**) Habitus, dorsal view. (**B**) Pediger 5 and urosome, dorsal. (**C**) Same, right lateral. (**D**) Right antennule, segments 1–8. (**E**) Same, segments 9–16. (**F**) Same, segments 17–21. (**G**) Fifth leg, anterior. (**H**) Same, posterior. (**I**) Same, right lateral.

stage, compared with $77.5 \pm 3.1\%$ at $9 \, \mathrm{g \, L^{-1}}$ (ANOVA, $P \leq 0.05$). At $9 \, \mathrm{g \, L^{-1}}$, $55 \pm 2.6\%$ of copepodids became adults, whereas only $8.7 \pm 2.9\%$ did so at $4.5 \, \mathrm{g \, L^{-1}}$. At $0 \, \mathrm{g \, L^{-1}}$ only $2.5 \pm 1.6\%$ of organisms moulted, and none of copepodids reached the adult stage (Kruskal–Wallis test, $P \leq 0.05$).

Survivorship and growth were more dramatically affected for the Ixtlahuaca Pond population at higher salinities than in freshwater (i.e. $>0 \, \mathrm{g \, L^{-1}}$; Figs 9 and 10). In freshwater (field salinity) $59.7 \pm 5.8\%$ of copepods survived after 7 days, whereas at $2.5 \, \mathrm{g \, L^{-1}}$ all the copepodids were dead on the fourth experimental day (ANOVAR, $P \leq 0.001$). At the two highest salinities (4.5 and $9.0 \, \mathrm{g \, L^{-1}}$) none of the organisms could survive even 24 h. Under freshwater conditions,

$22.2 \pm 4.9\%$ of copepodids reached adulthood, but moulting was not successful at higher salinities than freshwater (Fig. 10); consequently, no copepodids reached the adult stage.

Intrapopulation and interpopulation mating

All control crosses (intrapopulation) were successful for both populations at its corresponding field salinity, as 100% of females were copulated and fertilized. However, both populations differed widely in their reproductive characteristics (eggs per female and percentage of hatching; Fig. 11). At optimum salinity (Alchichica at $9.0 \, \mathrm{g \, L^{-1}}$ and Ixtlahuaca at $0 \, \mathrm{g \, L^{-1}}$) Alchichica females produced 5.0 ± 0.4 eggs per clutch
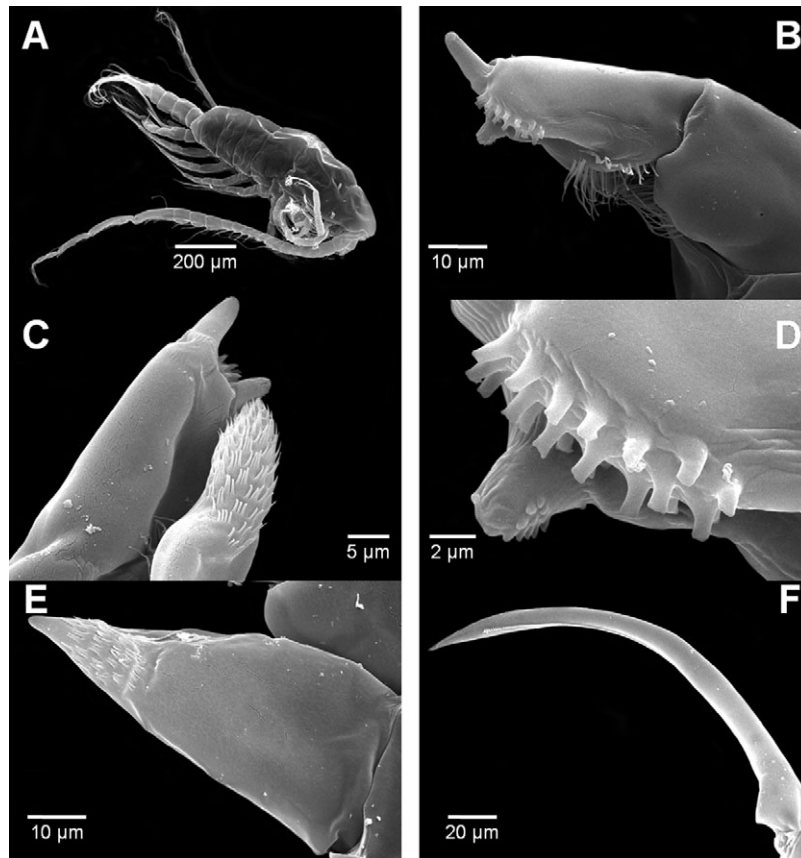
Fig. 7. SEM microphotographs of *L. garciai*, male. (**A**) Habitus, right lateral view. (**B**) Left P5, exopods 1−2, posterior. (**C**) Left P5, exopod 2 and distal part of endopod, anterior. (**D**) Left P5, exopod 2, detail of the internal distal margin with papilla-like processes and subterminal process. (**E**) Right P5, endopod, anterior. (**F**) Right P5, terminal claw.

whereas Ixtlahuaca females produced 10.1 ± 0.6 eggs per sac (ANOVA, $P \leq 0.05$). Likewise, the percentage of hatching was higher in Alchichica nauplii ($92.9 \pm 4.1\%$) than in the Ixtlahuaca ($70.0 \pm 12\%$) population.

Mating in copepods from Alchichica was not affected by reduced salinity: all of females were copulated in all salinity treatments. Moreover, at $4.5 \, \mathrm{g \, L^{-1}}$ neither clutch size nor hatching was affected significantly (Fig. 11). However, at the two lowest salinities, 2.5 and $0 \, \mathrm{g \, L^{-1}}$, average clutch size was reduced to 3.2 ± 0.5 and 1.1 ± 0.2 eggs, respectively (ANOVA, $P < 0.05$). Furthermore, the percentage of hatching decreased to 21 ± 7.9 at $2.5 \, \mathrm{g \, L^{-1}}$ and it was zero in the freshwater

treatment (ANOVA, $P \leq 0.05$). The Ixtlahuaca population was even more affected. At $2.5 \, \mathrm{g \, L^{-1}}$ and at higher salinities, females were not copulated and thus they produced no eggs (Fig. 11).

Most freshwater calanoids require mating for each clutch, and mating must occur when the female is gravid (Buskey, 1998; Berger and Maier, 2001). Our results show that all Alchichica females had mature oocytes at the intermediate salinity ($2.5 \, \mathrm{g \, L^{-1}}$) but only $14 \pm 14\%$ of Ixtlahuaca females were gravid under the
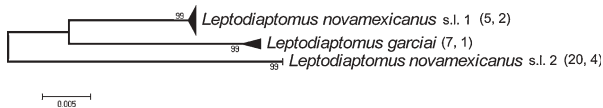

Fig. 8. Neighbour-joining tree of 32 COI sequences from the *Leptodiaptomus* specimens included in this study using K2P distances. The number of sequenced specimens and number of localities are in brackets.

Table II: Genetic divergences between distinct clades within L. novamexicanus s. l. and L. garciai

	L. novamexicanus s. l. 1	*L. novamexicanus* s. l. 2	*L. garciai*
L. novamexicanus s. l. 1	<0.34%		
L. novamexicanus s. l. 2	6.45–7.45%	<0.35%	
L. garciai	7.31–8.4%	4.4–5.27%	<0.82%

Distances are Kimura-2-parameter distance (%), with diagonal values indicating intra-clade genetic variation. Clades as in Fig. 8.
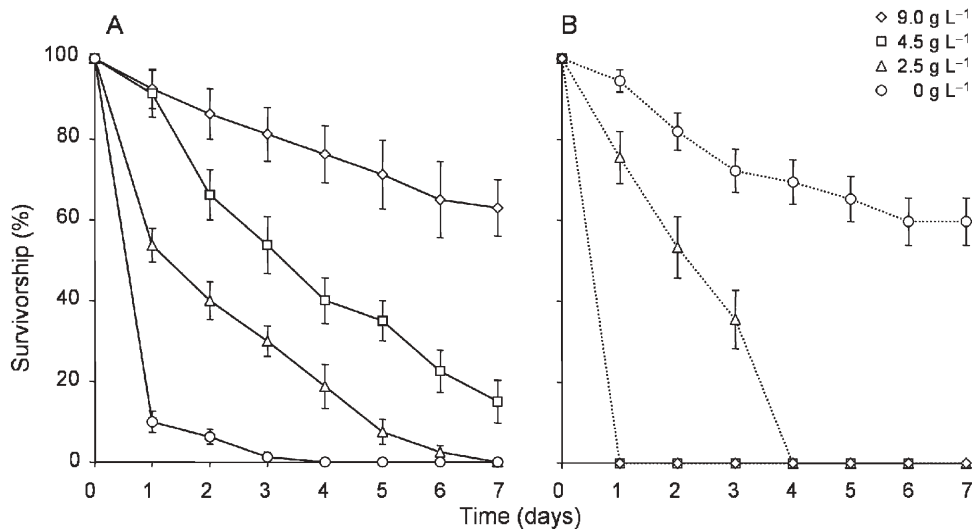
Fig. 9. Survivorship (%) of CIII copepodids of *L. novamexicanus* s. l. from two ecologically contrasting populations: (**A**) Lake Alchichica and (**B**) Ixtlahuaca Pond at several salinity treatments. Values are means ± SE.

same conditions. However, although some Ixtlahuaca females reached maturity, all interpopulation crosses were unsuccessful at the experimental salinity: copulated or fertilized females were not observed.

DISCUSSION

In this work we employed different complementary approaches to elucidate the biological and taxonomic status of a calanoid copepod population that, according to limited morphological criteria, belongs to the freshwater *L. novamexicanus* s. l., but that inhabits a brackish environment, Lake Alchichica. Our results reveal that the Alchichica population is very similar in general structure and morphology to the individuals from freshwater populations, although some slight but constant characters can be observed in both females and males that allow us to distinguish the Alchichica population from the rest of *L. novamexicanus* s. l. populations. The main differences were characters associated with reproductive structures (Defaye *et al.*, 2000) and may be
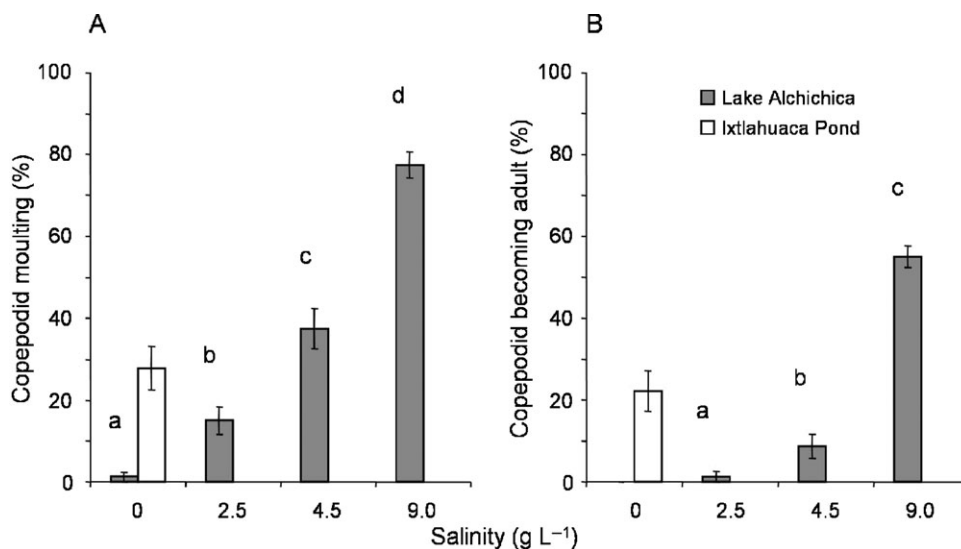
Fig. 10. (**A**) Effect of salinity on CIII copepodids determined as percentage of individual moulting to CIV stage, and (**B**) effect of salinity as percentage of individuals reaching the adult stage. Values are means ± SE. Identical letters indicate homogeneous groups that are not different at the 95% level (Student Newman Keuls *post hoc* test). In the case of Ixtlahuaca Pond, copepods moulted only at 0 g L^{-1}, thus statistical analyses were not performed.
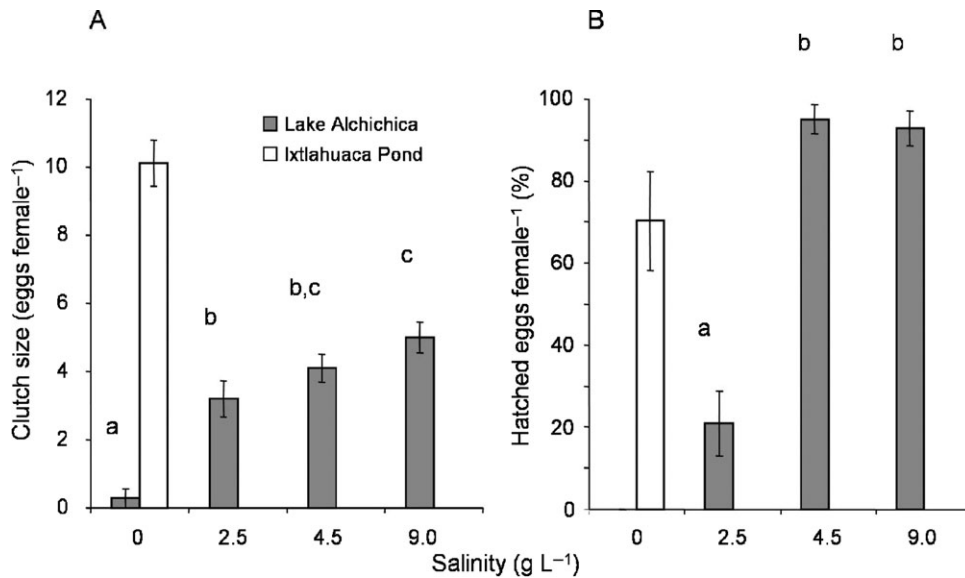
Fig. 11. Effect of salinity on the clutch size (number of eggs per female) and the hatching success (% of eggs hatched per female) obtained from intrapopulation mating trials of the two *L. novamexicanus* s. l. analysed. Values are means ± SE. Identical letters indicate homogeneous groups that are not different at the 95% level (Student Newman Keuls *post hoc* test). Ixtlahuaca Pond copepods produced eggs only at 0 g L^{-1}, thus statistical analyses were not performed.

related to sexual recognition patterns (Lonsdale *et al.*, 1998; Ohtsuka and Huys, 2001). However, the use of subtle morphological differences as a criterion in the separation of copepod species is not a straightforward decision, given the probable intraspecific variability. In the case of Alchichica *Leptodiaptomus*, Osorio-Tafall (1942) judged there were several morphological differences to consider it as a new species, although he used characters that in fact are variable and similar among populations. Later on Wilson and Yeatman (1959), quite probably without examining biological material (they did not give any details), synonymized it with *L. novamexicanus*. This situation makes a good instance in which additional, complementary tools are needed to guarantee an unambiguous discrimination among closely related species.

The next step in our study was the use of DNA barcodes based on nucleotide variation in a fragment of the mitochondrial gene cytochrome oxidase I (COI), which has been demonstrated to be a powerful species identification tool and is becoming a standard technique (i.e. Hebert *et al.*, 2003; Costa *et al.*, 2007). In the resulting tree (Fig. 8) all specimens from Alchichica grouped in a separate cluster from other *L. novamexicanus* s. l. which had small divergences, comprising a close group. Congeneric average divergences ranked among the lower values found by Costa *et al.* (2007) for crustaceans (the lowest they found was 4.9%) but enough to consider these populations as separate species. It should be noted that *L. novamexicanus* s. l. from Ixtlahuaca,

Borregos and Invernadero formed two separate clusters, suggesting another cryptic complex specialized to freshwater environments, leading the way to further research to clarify the status of this species.

As morphological and genetic analyses showed subtle but consistent differences among copepods from Alchichica and other populations, we performed some experiments to evaluate whether such a variation is related to differences in biological fitness along a salinity gradient, as salinity is the main dividing factor between Lake Alchichica and the rest of systems where *L. novamexicanus* s. l. has been reported up today.

Our results clearly show that neither the copepods from Alchichica nor from Ixtlahuaca have phenotypic plasticity to tolerate wide ranges of salinity. Salinity shock severely affected Ixtlahuaca copepods in terms of survivorship, moulting and intrapopulation reproduction. They were not able to survive more than four days and they did not moult at higher salinities than freshwater. Moreover, Ixtlahuaca females did not produce eggs at higher salinities than freshwater in intrapopulation crosses. Although Lake Alchichica copepods were more tolerant to salinity shock than those from Ixtlahuaca (females produced eggs at salinities lower than 9 g L^{-1}), fitness was also drastically affected.

Salinity has been shown to be one of the most important factors affecting the distribution and performance of aquatic organisms (Williams, 1998; Rokneddine, 2004), and it quite probably determines the distributional patterns and ability to colonize new

environments in *L. novamexicanus* s. l. Most important, according to our results we suggest that Ixtlahuaca and Alchichica copepods are unlikely to interact in nature as they do not have a common salinity range, and consequently, gene flow between both populations is improbable.

Ixtlahuaca and Alchichica interbreeding provided additional evidence as no fertilized females resulted. At $2.5\,\mathrm{g\,L}^{-1}$ (experimental salinity for interpopulation crosses) Ixtlahuaca copepods were the most negatively affected, but considering that the active role in mating behaviour is played by the males, and that in intrapopulation crosses Alchichica males successfully copulated females of their own population even at $2.5\,\mathrm{g\,L}^{-1}$, if both populations were the same biological species it would be likely that spermatophore transference would be observed between Alchichica males and females from Ixtlahuaca even if females were inactive (Kelly and Snell, 1998). However this did not occur at all, presumably because of a lack of mate recognition. Therefore, the lack of gene flow between both populations must be due to non-overlapping salinity tolerances and probably also reproductive incompatibility as well.

Thus, although differences in morphology are subtle, taking into account the significant genetic divergence, the reduced salinity tolerance, and the most likely absence of gene flow and reproductive incompatibility between the populations tested, we conclude that the Alchichica copepod is a different species from *L. novamexicanus* s. l., and therefore that *L. garciai* (Osorio-Tafall, 1942) is a biospecies and a valid, separate taxon.

In general terms, results from this study show that the analysed populations are different biospecies, and this reinforces the value of integrated multi-scope studies to elucidate the taxonomic status of cryptic species. Such differences reveal that the copepod *L. novamexicanus* is in fact a complex of sibling species, at least in Central Mexico, from which *L. garciai* (Osorio-Tafall, 1942) living in Lake Alchichica represents a valid taxon and most probably an endemic species.

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