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Morphological and genetic variation in seven species of the endangered *Chirostoma* "*humboldtianum* species group" (Atheriniformes: Atherinopsidae)

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Abstract: The *Chirostoma* "*humboldtianum*" group includes seven silverside species considered as a monophyletic assemblage because of their high genetic and morphological similarities. The group includes five moderately large species, "peces blancos" (117 - 300 mm standard length - SL) and two smaller species, "charales" (70 - 142 mm SL). These species are of great economical, cultural and ichthyological interest for local populations, and their management practices are controversial. We investigated the morphometric, meristic and allozyme variations of the seven species (13 populations) and related the variations with life history, habitat and management procedures. Nineteen morphometric variables, eight meristic variables (by multivariate analysis) and 23 allozyme loci of the seven species and populations of *Chirostoma* were compared. Principal component analysis (PC) of morphometric and meristic data indicate that both sets of data provided information to differentiate among the seven species. The variables that accounted for most of this differentiation were head length (HL), predorsal 1 length (P1L) and length of pelvic fin base (PfbL). PC and Discriminant Analysis (DA) with morphometric data also suggested the differentiation of populations within *C. grandocule* (83% correctly classified organisms), whereas PC and DA with meristic data differentiated populations of *C. humboldtianum* (80% correctly classified organisms). The most important morphometric variables for the differentiation were anal fin height (AfH), length of anal fin base (AfbL) and predorsal 2 length (P2L) and the meristic variables D2fR, PdS and AfR. The genetic variability data indicate changes in values of some of the species in relation to previously reported data. The present populations of *C. grandocule* show a reduction in H_e (0.002 vs. 0.009). Other species showed an increase; for instance, *C. consocium consocium*, *C. humboldtianum*, *C. lucius*, *C. promelas* and *C. sphyraena* averaged H_e = 0.069 vs. 0.027. θ indicated significant genetic differentiation among the analysed species (0.247, S.D. 0.159) and θ_S supported the morphological data that suggest intra-specific differentiation (0.360, S.D. 0.154).

Key words: Morphometric, meristic, genetic variability, inter-specific, *Chirostoma*, México.

The species of the genus *Chirostoma* Swanson are largely endemic to the Mesa Central de México. It has been suggested that they make up a "species flock" (Barbour 1973a, b, Barbour and Chernoff 1984, Echelle and Echelle 1984) because they have characteristics such as extremely high levels of local sympatry and morphological evolution and because of their unusually high speciosity in a relatively small geographic area (Echelle and Echelle 1984, Greenwood 1984).

According to Barbour (1973a, b), the Jordani group is comprised of 11 primarily lacustrine species and two subspecies with moderate to large sizes (70 - 142 mm to 117 - 300 mm). Osteological and genetic data supported the hypothesis that seven species from the Jordani group are a monophyletic assemblage, descendant from a *Chirostoma humboldtianum*-like ancestor (Barbour 1973b, Barbour and Chernoff 1984, Echelle and Echelle 1984, White 1985). Thus, the seven

species included in the present study are considered as the "*humboldtianum* group". The species of the present study are *C. humboldtianum* Valenciennes (H), *C. grandocule* Steindachner (G), *C. estor estor* Jordan (E), *C. lucius* Boulenger (L), *C. sphyraena* Boulenger (S) (Barbour 1973a: 108, 112, 114, and 118, respectively), *C. consocium consocium* (Jordan and Hubbs, 1919) (C), and *C. promelas* (Jordan and Snyder, 1899) (P).

Genetic changes in freshwater fish populations or species can arise just in a few years (Carvalho 1993, Ward *et al.* 1994) when populations or species are exposed to contrasting environmental conditions (Barriga-Sosa *et al*. in prep.), even when they exchange only a few migrants. Most members of the "*humboldtianum* group" are species that have been dispersed out of their natural range, because of their great economical and ichthyological relevance. Also some of the species (those species from Lake Pátzcuaro) are exposed to contrasting environmental conditions and to high fishing pressure. Thus, in this paper we investigated the levels of morphological and allozyme variation of the members of the "*humboldtianum* group" several years after they were first genetically monitored and under the consideration that most of the species have been distributed out of their natural endemic habitats. It is noteworthy that *C. promelas* and *C. compressum* (the latter considered a distinct form of *C. grandocule*) are nowadays considered endangered species (NOM-059-ECOL-1984), because of their actual low population densities in the first case and because of the "extinction" during a temporary dryness of lake Cuitzeo in the later. The larger forms *C. e. estor*, *C. lucius* and *C. sphyraena* are also found in very low densities at the present times (Alaye 1993a, Jiménez y Gracia 1995, Villicaña- Vázquez pers. comm.)

MATERIALS AND METHODS

Sampling: Samples were taken from commercial artisanal catches from 11 sites out of four lakes (Fig. 1): 1- five localities situated along the southern region at Lake Chapala (Cha) (a, b, c, d and e), the largest and most polluted of the lakes. 2- Zacapú Lagoon (Zac), Michoacán, a remnant of a larger lake that has been recently proposed as an ecological reserve (Moncayo 1996). 3- Lake Pátzcuaro (Ptz), Michoacán, characterised as highly eroded and perturbed. The collecting sites at this lake were Ojo de Agua (a), San Jerónimo (b) and Ichupio (c) (I), which belong to the northern basin of the lake, the deepest section and is free of aquatic vegetation (De Buen 1941, Chacón *et al*. 1991), and Janitzio (d) (J), which belongs to the northern portion of the southeastern basin, characterised by shallow waters and the abundance of aquatic vegetation. 4- Lake Zirahuén (Zir), characterised as the youngest and deepest of the four lakes (Chacón-Torres 1993). Sample size for the genetic analysis was 176 fishes, and 367 for the morphometric and meristic analysis.

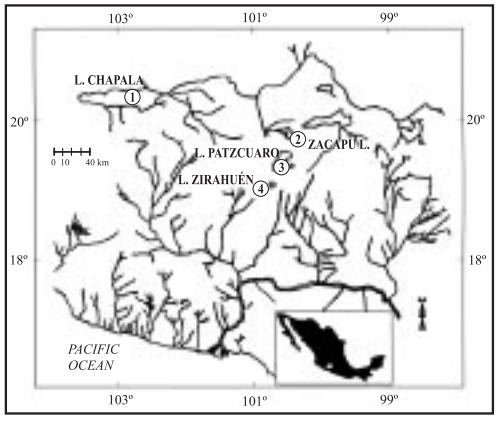

Fig. 1. Map that shows the Iakes from which Chirostoma samples were obtained.

Morphometric (M) and meristic (m) analyses: We used 27 variables for species identification, 23 were reported by Barbour (1973a) and Barbour and Chernoff (1984), the remaining four variables were reported as informative for the separation of 16 species of *Chirostoma* (Barriga-Sosa in prep.). Nineteen variables were morphometric (M): total length (TL), standard length (SL), head length (HL),

orbit diameter (OrB), snout length (SnL), mandible length (ML), postorbital head length (PoHL), caudal peduncle length (CpL), anal fin length of base (AfbL), predorsal 1 length (P1L), predorsal 2 length (P2L), prepelvic length (PpL), pelvic fin length of base (PfbL), body depth (BD), caudal peduncle length least depth (LdCp), anal fin height (AfH), pectoral fin height (PfH), first dorsal height (D1H), and second dorsal height (D2H). All characters were measured to the nearest 0.1 mm with dial callipers. Eight meristic variables (m) were also recorded: lateral line scales (LlS), predorsal scales (PdS), interdorsal scales (IdS), pectoral fin rays (PfR), first dorsal fin spines (D1fS), second dorsal fin rays (D2fR), anal fin rays (AfR), and gill rakers (GR).

Each category of data (M) and (m) was analysed separately. To eliminate differences related to size, morphometric data were transformed (Corti *et al*. 1988, Cuadras 1991) according to two different criteria: i) logarithmic transformation of each character, and ii) proportion, representing each character relative to length. We also analysed the original data (o) of each of the categories (Mo and mo). The data were submitted to multivariate analyses with two descriptive and exploratory methods that do not require of previous hypotheses: Principal Component (PC) and Reciprocal Averaging (RA). Discriminant Analysis (DA) was also utilised with both data sets to investigate discrimination among samples. Multivariate Analysis was carried out with the computer program STATGRAPHIC Plus version 2.1.

Allozyme data analyses: Homogenates of liver and muscle tissues were subjected to horizontal, cellulose acetate gel electrophoresis of allozymes for a standardised time of 20-35 min at 220-350 volts and at room temperature (RT°). All samples were screened for 12 enzymes: alcohol dehydrogenase (Adh, E.C. 1.1.1.1), glucose dehydrogenase (Gdh, E.C. 1.1.1.47), glycerol-3-phosphate dehydrogenase (G3pdh, E.C. 1.2.1.12), glucose-6-phosphate isomerase (Gpi, E.C. 5.3.1.9), hidroxyburate dehydrogenase (Hbd), isocitrate dehydrogenase (Idh, E.C. 1.1.1.42), L-lactate dehydrogenase (Ldh, E.C. 1.1.1.27), malate dehydrogenase (Mdh, E.C. 1.1.1.37), malic enzyme (Me, E.C. 1.1.1.40), phosphogluconate dehydrogenase (6Pgdh, E.C. 1.1.1.44), phosphoglucomutase (Pgm, E.C. 5.4.2.2), and xantin dehydrogenase (Xdh, E.C. 1.1.1.204). Five electrophoretic buffers were used (Tris-EDTA borate pH 8.5, Tris-citrate pH 6.5, Tris-citrate pH 8, Tris citrate-borate pH 8.2, and Tris-glycine pH 8.5). The staining procedures were those described by Hebert and Beaton (1989). The allele designation used in this study correspond with that of Barriga-Sosa (in prep.)

The allele frequencies, the proportion of polymorphic loci ($P_{.99}$ criteria), the mean number of alleles per locus, and the expected (H_e) heterozygosity for each locus analysed were calculated for each species and sample. Deviations from the expected Hardy-Weinberg proportions were tested using Fisher's exact test (GENEPOP 3.1, Raymond and Rousset 1995). Fisher's combined probability test (Sokal and Rohlf 1995) was used to test the significance of allele frequency differences between species and samples (TFPGA 1.3, Miller 1998). The probabilities of *F* indices (Weir and Cockerham 1984) associated with each locus and sample were significantly different from zero and were computed with GENEPOP. The estimate of Weir and Cockerham (1984) of *F*-statistics was used to measure preliminary levels of genetic population structuring (TFPGA 1.3, Miller 1998). The following parameters were estimated: *f*, the correlation of genes within individuals within populations; and θ and θ_S, correlation of genes of different individuals in the same population and subpopulation. Estimates of the variance of these parameters were obtained by jack-knifing across loci. *F*-statistics were tested for differences from zero permuting (2000 replicates) alleles within samples (*f*) and alleles between samples (*F*, θ and θ_S) over all loci. It is known that the species show high genetic identities, thus we estimated the genetic relationships between pairs of species and samples (populations) using Reynolds *et al.* (1984) coancestry estimate of genetic identity and distance (I_c and

D_c). Cluster analysis was performed with the UPGMA method of the TFPGA 1.3 program (Miller 1998) using bootstrap sampling of loci and a consistency index (Felsenstein 1985). The restricted maximum likelihood (ML) method (Felsenstein 1973) was performed with the PHYLIP package (Felsenstein 1995). Evaluation of individual branches within trees was performed by bootstrapping of loci (bootstrapping proportions > 70% were considered as highly significant and > 50% as considerably accurate, Hillis and Bull 1993).

RESULTS

The species examined, total sample size per species (N) utilised in each analysis (Ng = genetic, NM = morphometric, Nm = meristic), and collecting sites are summarised in Table 1. Differences in sample size between morphometric/meristic and allozyme analyses were due to the quality of the organisms collected, since some of them were not available *in situ* because of their very low actual densities and some samples were obtained from previously

TABLE 1
Species of Chirostoma *analysed and collecting sites*

Species Locality and collecting site number	N (G)	N (M) and (m)	Collecting date
C. consocium consocium			
1b, 1c and 1d (CCha)	20	92	May, Aug.1996 and 1997
C. estor estor			
1a, 1c, 1d and 1e (ECha)	8	12	May and Aug. 1996
3a and 3b (EPtz)	24	28	May 1996 and Aug. 1997
C. grandocule			
3c (GI-97)	21	50	May 1997
3d (GJ-96)	20	50	June 1996
C. humboldtianum			
1e and 1d (HCha)	8	7	Aug. 1997
3a (HPtz)	30	39	Sept. 1999
2 (HZac)	6	6	May 1996
4 (HZir)	6	40	Sept. 1996
C. lucius			
1d and 1e (LCha)	9	11	May 1996
3b (LPtz)	7	8	Aug. 1996
C. promelas			
1a (PCha)	12	12	Apr. and Aug. 1996 and 1997
C. sphyraena			
1a (SCha)	5	12	Aug. 1996 and 1997
N_{TOTAL}	176	367	

N = sample size; (G) = genetic analysis; (M) (m) = morphometric and meristic analysis; 1 = Lake Chapala, Jalisco-Michoacán, 1a = Centro Acuícola "Tizapán el Alto", 1b = Agua Caliente, Poncitlán, 1c = Mismaloya, Tizapán, 1d = Tepeguaje, Tuxcueca, 1e = Isla de Petatán, Cojumatlán; 2 = Zacapú Lagoon, Michoacán; 3 = Lake Pátzcuaro Michoacán, 3a = Ojo de Agua, 3b = San Jerónimo, 3c = Ichupio, 3d = Janitzio; 4 = Lake Zirahuén, Michoacán.

TABLE 2
Cumulated percentage of the variance in components I, II and III for multivariate analyses RA (Reciprocal Averaging) and PC (Principal Component)

Measure	Data	Multivariate analyses	Component I	Component II	Component III
Morphometric (M)	Original (o)	RA	70.1	95.6	96.5
		PC	56.4	91.9	94.2
	Log (i)	RA	74.1	92.8	96.8
		PC	89.6	97.2	98.6
	Proportion (ii)	RA	58.1	68.1	81.1
		PC	66.2	79.5	84.8
Meristic (m)	Original (o)	RA	48.1	67.3	84.5
		PC	71.5	86.9	94.7

frozen (-10° to -20°C) catches. Therefore, sample size in each location for most of the species except *C. consocium consocium* (CCha), *C. humboldtianum* (HPtz and HZir) and *C. grandocule* (GI-97 and GJ-96) were smaller for the genetic analysis compared to the morphometric and meristic analyses.

Morphometric (M) and meristic (m) data analyses: Table 2 summarises the cumulated percent of the variance resulted from PC and RA analyses, it includes the cumulated percent for the first three components (I, II and III) with the transformed (i and ii) and original (o) morphometric (M) and meristic (m) data.

Inter- and intra-specific variation: For the morphometric data, the first two components of the PC and RA analyses with data (ii) suggested an interesting arrangement within species. The correlation matrix of both analyses showed that these were different in sign and magnitude, a pattern that expresses variation in "shape" but not in "size" (data upon request), we utilised PC analysis because it explained the highest percentage of the variance

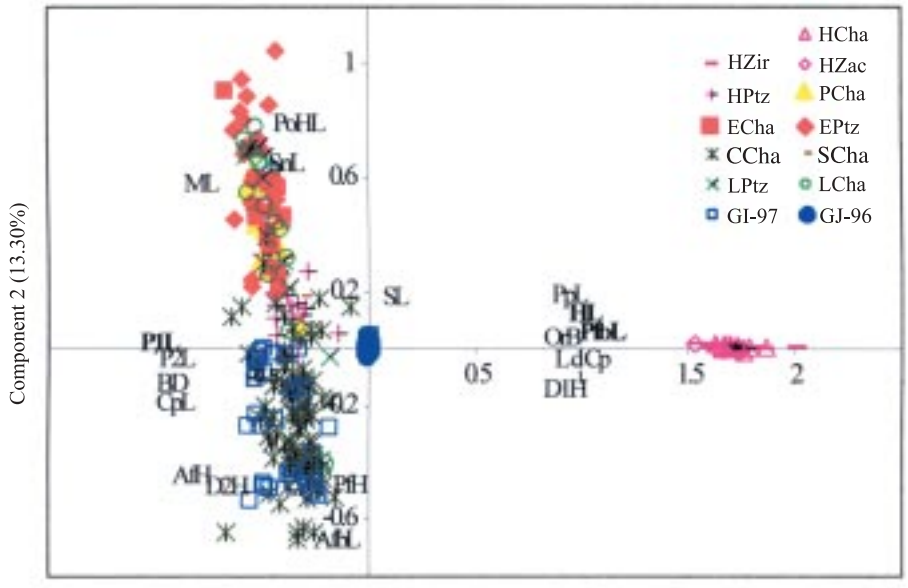

Fig. 2. Plot of first and second axis of a principal component analysis using 19 (Mii) variables for 13 samples/populations of seven species of *Chirostoma*.

(see Table 2). Fig. 2 shows an arrangement where most of the *C. humboldtianum* organisms are grouped in the positive area, except for those from Lake Pátzcuaro (HPtz), which are shown in the negative section of the plot. The same tendency was observed for *C. grandocule*, where a group of organisms was in the origin of the coordinates (GJ-96) and another group was in the negative area of the plot (GI-97). The remaining species did not show any special arrangement, although *C. c. consocium* did not show much dispersion. Variables that accounted the highest percentage of the variance and contributed to the separation of groups were PfbL (0.99), HL (0.98) and P1L (-0.97).

Due to the results observed in *C. humboldtianum* and *C. grandocule* we proceeded to analyse each of these species and samples following the (ii) criteria with PC and DA. PC

TABLE 3
Classification results from DA with M (ii) data for C. humboldtianum *and* C. grandocule

Species/sample	HZir	HPtz	GI-97	GJ-96	N
HZir	26 (65%)	11	0	3	40
HPtz	3	35 (90%)	0	1	39
GI-97	0	0	43 (86%)	7	50
GJ-96	3	0	3	44 (88%)	50

Percent of cases correctly classified: 83%

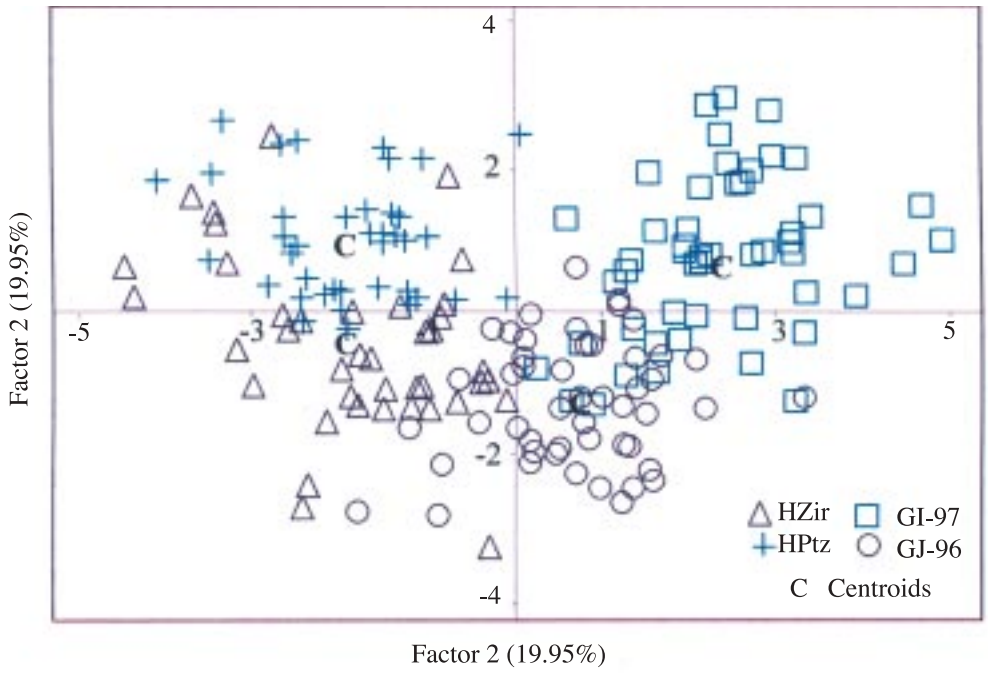

Fig. 3. Plot of first and second axis of a discriminant analysis using 19 (Mii) variables for four samples/populations of two *Chirostoma* species, two for *C. humboldtianum* (HPtz and HZir) and two for *C. grandocule* (GJ-96 and GI-97).

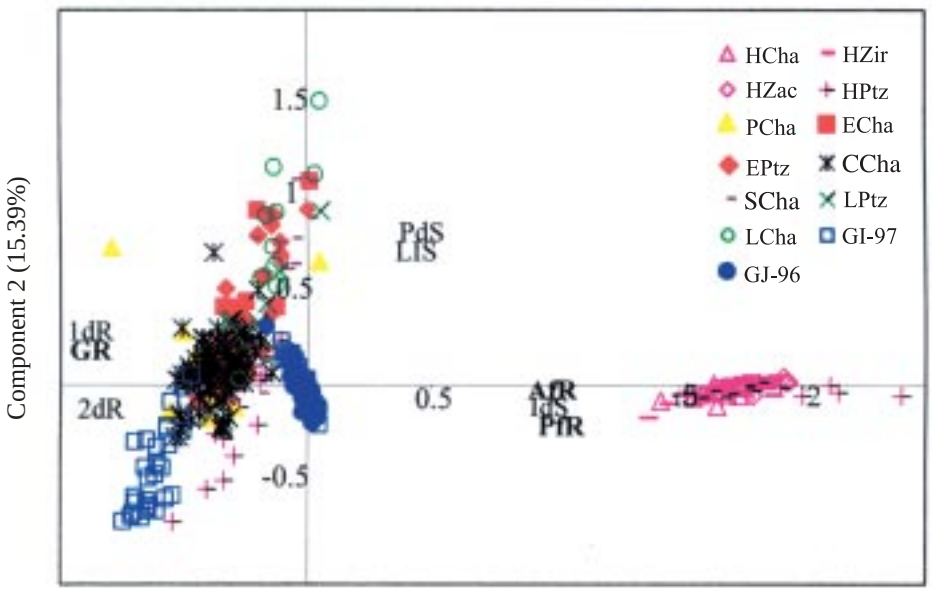

Fig. 4. Plot of first and second axis of a principal component analysis using eight meristic (m o) variables for 13 samples/populations of seven species of *Chirostoma*.

analysis indicated the separation of groups thus, we further analysed these species with DA. Discriminate functions I and II contributed with 74 and 20% of the total variance (Fig. 3), the variables that contributed with the highest variation to the separation of these groups were AfH (0.47) and AfbL (0.45) for the first discriminant function and P2L (0.63) for the second. Table 3 shows that the correct classification percentages for these organisms were 65% and 90% for HZir and HPtz, respectively, and that *C. grandocule* organisms were better classified with 86 and 88% for GI-97 and GJ-96, respectively.

For the meristic data we utilised PC because it explained the highest variance (see Table 2). As observed with PC analysis of M (ii) data, most of *C. humboldtianum* organisms are shown in the positive area of the plot (Fig. 4), except those from lake Pátzcuaro (HPtz), which are shown in the negative section of the plot. Two groups are also observed with *C. grandocule*, one in the origin of the coordinates (GJ-96) and a second group in the negative section of the plot (GI-97). In this analysis as well as in the PC with M (ii) data the latest species showed an overlap area with 17 organisms from GI-97 mixed with those from GJ-96. The remaining species do not show a particular arrangement except for *C. c. consocium*, which does not showed much dispersion. The variables that accounted with the highest variance were AfR (0.97), GR (-0.96) and PfR (0.95).

Similarly to the M (ii) PC analysis, the m (o) data for *C. humboldtianum* and *C. grandocule* prompted for further analysis with PC and DA. Similar results to those detected with PC of M (ii) data were observed, thus, we proceeded with DA analysis. Discriminant functions I and II accounted with 84 and 14% of the variance, respectively (Fig. 5). The most important variables in this analysis were D2fR (0.87) and PdS (-0.45) for the first discriminant function and LlS (-0.68) and AfR (0.66) for the second. Table 4 shows that the correct classification percentage was 80 and 72% for *C. grandocule* samples (GI-97 and GJ-96, respectively) and that *C. humboldtianum* was better classified with 77 and 92% for HZir and HPtz, respectively.

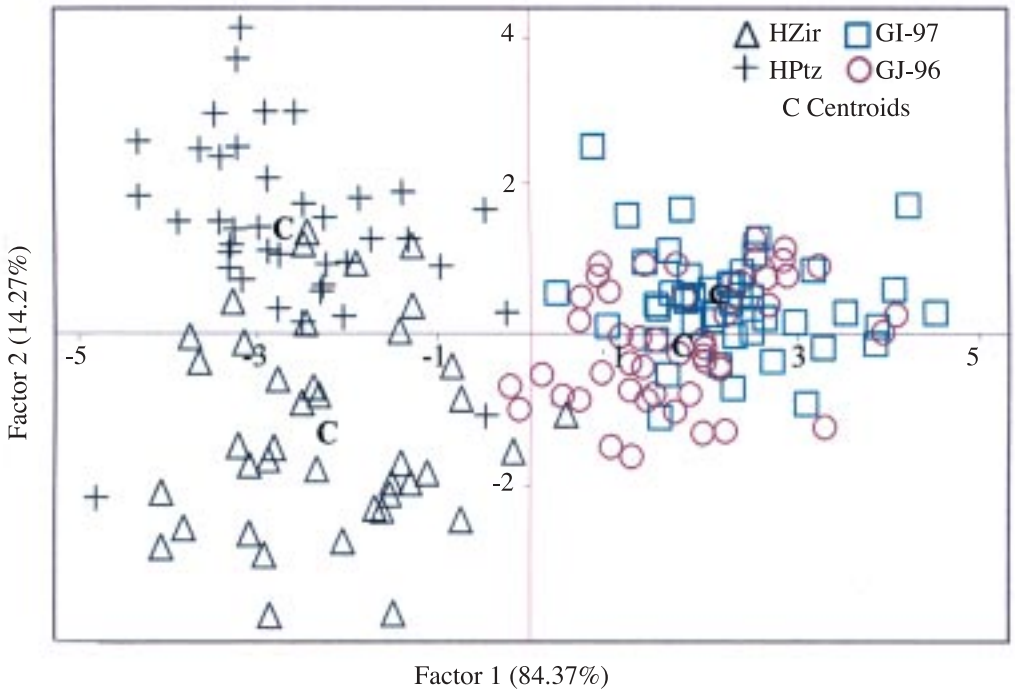

Fig. 5. Plot of first and second axis of a discriminant analysis using 19 (Mii) variables for four samples/populations of two *Chirostoma* species, two for *C. humboldtianum* (HPtz and HZir) and two for *C. grandocule* (GJ-96 and GI-97).

TABLE 4
Classification results from DA with m (o) data for C. humboldtianum *and* C. grandocule

Species/sample	HZir	HPtz	GI-97	GJ-96	N
HZir	31 (77%)	8	0	1	40
HPtz	2	36 (92%)	0	1	39
GI-97	0	0	40 (80%)	10	50
GJ-96	0	0	14	36 (72%)	50

Percent of cases correctly classified: 80%

Allozyme inter-specific variation: From the enzyme systems assayed, 23 presumptive loci were detected. Only one locus was detected for Gdh, G3pdh, Hbd, and Pgm. Two loci were observed for Adh, Idh, Ldh, 6Pgdh and Xdh and three loci for Gpi, Mdh and Me. The allelic frequencies of the polymorphic loci are shown in Table 5. Locus 1 for Gdh, G3pdh, Hbd and loci 1 and 2 for Adh, Idh and Xdh were monomorphic in all samples. The average $P_{.99}$ for the group was 14. At the species level the highest average $P_{.99}$ value was found in *C. e. estor* ($P_{.99}$ = 22), followed by *C. humboldtianum* (18), *C. promelas* (17), *C. c. consocium* and *C. sphyraena* (13), *C. lucius* (11) and *C. grandocule* (2). All polymorphic loci had significant differences in allelic frequencies among the species ($P < 0.05$, data upon request),

TABLE 5

Allelic frequencies of polymorphic loci for the seven species (13 samples) of the genus Chirostoma

	CCha	ECha	EPtz	GI-97	GJ-96	HCha	HPtz	HZac	HZir	LCha	LPtz	PCha	SCha	P_s	P_p
6Gpdh1	(20)	(8)	(24)	(21)	(20)	(8)	(30)	(6)	(6)	(9)	(7)	(12)	(5)	1.0000	1.0000(1)
a	0.500	0.500	0.500	0.000	0.000	0.500	0.500	0.500	0.500	0.500	0.500	0.458	0.500		1.0000(2)
b	0.500	0.500	0.500	0.000	0.000	0.500	0.500	0.500	0.500	0.500	0.500	0.542	0.500		1.0000(3)
6Gpdh2	(20)	(8)	(24)	(21)	(20)	(8)	(30)	(6)	(6)	(9)	(7)	(12)	(5)	0.0007	
a	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000		
b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.917	1.000		
Gpi-1	(20)	(8)	(24)	(21)	(20)	(8)	(30)	(6)	(6)	(9)	(7)	(12)	(5)	0.0000	0.0002(1)
a	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.0056(2)
b	0.275	0.375	0.854	1.000	1.000	0.812	0.350	0.583	0.750	0.167	1.000	0.333	0.400		0.0000(3)
c	0.700	0.625	0.146	0.000	0.000	0.188	0.417	0.250	0.250	0.833	0.000	0.667	0.600		
d	0.000	0.000	0.000	0.000	0.000	0.000	0.233	0.167	0.000	0.000	0.000	0.000	0.000		
Gpi-2	(20)	(8)	(24)	(21)	(20)	(8)	(30)	(6)	(6)	(9)	(7)	(12)	(5)	0.0000	0.0000(1)
a	0.300	0.000	0.583	0.000	0.000	0.250	0.700	0.250	0.417	0.000	0.000	0.333	0.400		0.0352(2)
b	0.700	0.375	0.375	1.000	1.000	0.625	0.300	0.417	0.583	0.167	1.000	0.667	0.600		0.0000(3)
c	0.000	0.625	0.042	0.000	0.000	0.125	0.000	0.333	0.000	0.833	0.000	0.000	0.000		
Gpi-3	(20)	(8)	(24)	(21)	(20)	(8)	(30)	(6)	(6)	(9)	(7)	(12)	(5)	0.0000	0.5695(1)
a	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000		0.0105(2)
b	1.000	1.000	0.937	1.000	1.000	0.750	0.750	0.917	0.917	1.000	1.000	0.958	1.000		
c	0.000	0.000	0.000	0.000	0.000	0.250	0.083	0.083	0.083	0.000	0.000	0.000	0.000		
d	0.000	0.000	0.063	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.000		
Ldh-2	(20)	(8)	(24)	(21)	(20)	(8)	(30)	(6)	(6)	(9)	(7)	(12)	(5)	0.0413	0.5679(1)
a	1.000	1.000	0.937	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.857	1.000	1.000		0.1828(3)
b	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.143	0.000	0.000		
Pgm	(20)	(8)	(24)	(21)	(20)	(8)	(30)	(6)	(6)	(9)	(7)	(12)	(5)	0.0000	0.0673(1)
a	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.1940(2)
b	0.000	0.250	0.167	1.000	0.975	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
c	1.000	0.687	0.833	0.000	0.000	0.938	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
d	0.000	0.063	0.000	0.000	0.000	0.062	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
													Aver.		
$P_{.99}$ 13	17	26	0.0	4	22	17	17	17	13	9	17	13	14		
H_e 0.060	0.088	0.079	0.000	0.002	0.085	0.087	0.089	0.072	0.049	0.035	0.076	0.071	(.030)		

A locus is polymorphic if the frequency of the most common allele does not exceed 99%. J-96 = Janitzio 1996; I-97 = Ichupio 1997; Cha = Lake Chapala; Zir = Lake Zirahuén; Zac = Centro Acuícola de Zacapu; Ptz = Lake Pátzcuaro; sample size in parenthesis; P_S = Fihser's *P* among samples; P_P = Fihser's *P* among populations: (1) *C. estor*, (2) *C. humboldtianum*, (3) *C. lucius*.

except 6Gpdh-1. The mean heterozygosity observed within the group was 0.060.

Intra-specific variation: At the intra-specific level, genetic variation differences ($P_{.99}$ and H_e) were observed among samples; for instance, in *C. e. estor* samples EPtz ($P_{.99}$ 26 and H_e 0.079) and ECha (17 and 0.088), in *C. humboldtianum* samples HCha (22 and 0.084) in relation to samples HPtz, HZir and HZac (17 and 0.087, 0.072 and 0.089, respectively), in *C. lucius* samples LCha (13 and 0.049) in relation to LPtz (9 and 0.035), and in *C. grandocule* samples GJ-96 (4 and 0.002), whereas GI-97 showed no polymorphic loci. The genetic variability values for PCha were $P_{.99}$ 17 and H_e 0.076; CCha and SCha had intermediate levels of polymorphism (13) and heterozygosity values of 0.060 and 0.071, respectively. Differences in genetic variability values within species can be due to the presence of more alleles in EPtz, HCha and LCha and less in ECha, HPtz, HZac, HZir and LPtz. In ECha, HPtz and HZac samples, the alleles had more intermediate allelic frequencies, while in EPtz some rare alleles were in very few frequencies. PCha is the only species with intermediate allele frequencies in locus 6Gpdh-1 and with relatively low frequencies at locus 6Gpdh-2, the rest of the species/samples showed intermediate fixed frequencies at these loci. EPtz and LPtz samples showed intermediate allele frequencies for locus Ldh-2 (Table 5). All polymorphic loci had significant differences in allelic frequencies among samples (P_s, Table 5). Significant differences in allelic frequencies within populations of *C. e. estor*, *C. humboldtianum* and *C. lucius* were also observed in one or another polymorphic loci and population (see P_P, Table 5).

We found a significant departure from the H-W equilibrium in most samples (Table 6), although it is noteworthy that the sample size in our study for this estimate is a limiting factor. Especially in the larger species, we consider it because we believe it could be related to the management practices carried out for the species during the last century (to be discussed latter on). The significant results in samples EPtz, HZac, LCha and PCha are due to a deficit of heterozygous at one, two or three loci in one or another sample, suggesting that inbreeding may occur in these samples at lakes Pátzcuaro, Chapala and Zacapú Lagoon. For samples HCha and SCha the significant results were due to an excess of heterozygotes, due perhaps to outbreeding (hybridisation) occurring within these "species" in lake Chapala. The averages indicate that inbreeding was stronger in LCha and HZac and outbreeding in SCha.

Genetic differentiation, distances and cluster analyses: Preliminary Weir and Cockerham estimates of *F*-statistics support our previous results, which suggest that inbreeding as well as outbreeding may occur within this group of species. It also supports the genetic differentiation within the group. Average jack-knifed θ and θ_s indicate significant, genetic differentiation among species and samples (0.247, S.D. 0.159 and 0.360, S.D. 154, respectively) (Table 7a). Differentiation within samples (populations) of *C. e. estor, C. lucius* and *C. humboldtianum* are also observed (Table 7b). The average jack-knifed θ_S values within these populations ranged from 0.604, S.D. 0.471 for *C. lucius*; 0.277, S.D. 0.139 for *C. e. estor*, to 0.072, S.D. 0.032 for *C. humboldtianum.* In the case of *C. lucius* and *C. e. estor* samples (LCha, LPtz, ECha, and EPtz), processes such as genetic isolation and local inbreeding (F = 0.490 – 0.148, Table 7b) might be important on the differentiation of such populations, although the θ_S value of the second species is of lower magnitude. The *C. humboldtianum* populations in the other hand (HCha, HPtz, HZir and HZac) showed lower but significant θ_S values (0.072, S.D. 0.032).

To visualise differences in allelic frequencies between species we obtained the coancestry estimate of genetic identity and distance (I_c and D_c) for the seven species and 13 samples. Genetic identity values between samples/populations ranged from 0.0006 for PCha/ECha to 2.7990 for LCha/GI-97 and distance values ranged from 0.0006 for PCha/ECha to 0.9303 for LPtz/GI-97. Negative values were also observed between closely related samples, for

TABLE 6
Fixation indices (probability values associated with the F inbreeding coefficients, for each sample and polymorphic loci

Species/sample	6Gpdh-2	Gpi-1	Gpi-2	Gpi-3	Ldh-2	Pgm	Average *F*
CCha	—	+0.103*	+0.073*	—	—	—	+0.088
ECha	—	+0.000*	+0.000*	—	—	-0.296*	-0.099
EPtz	—	-0.150*	+1.000**	-0.045*		-0.179*	+0.156
HCha	—	-0.167*	+0.125*	-0.273*	—	—	-0.105
HPtz	—	-0.267*	+0.223*	+0.106*	—	—	+0.021
HZac	—	+0.211*	+0.318*	—	—	—	+0.264
HZir	—	-0.250*	+0.063*	—	—	—	-0.093
LCha	—	+0.636*	+0.636*	—	—	—	+0.636
LPtz	—	—	—	—	-0.091*	—	-0.091
PCha	+0.500*	-0.095*	-0.095*	—	—	—	+0.103
SCha	—	-0.600*	-0.600*	—	—	—	-0.600

* P < 0.05; ** P < 0.001

TABLE 7
Estimates of genetic heterogeneity (θ, θ_S, F *and* f*) a) for the 13 samples of* Chirostoma *analysed, b) for eight populations of three species*

a)

θ	θ_S	*F*	*f*
0.247	0.360	0.138	-0.412
±0.159	±0.154	±0.394	±0.418

b)

Species populations	θ_S	*F*	*f*
C. e. estor (2)	0.277	0.148	-0.228
	±0.139	±0.406	±0.385
C. humboldtianum (4)	0.072	-0.217	-0.320
	±0.032	±0.304	±0.293
C. lucius (2)	0.604	0.490	-0.829
	±0.471	±1.051	±0.794

±S.D., 95% confidence interval with 10 000 bootstrapped replicates. P < 0.001

instance PCha/LCha (-0.0030) or PCha/SCha (-0.0630). The genetic identity and distance values for *C. grandocule* samples are low and prompted for further investigations (Barriga-Sosa *et al*., in prep.). For *C. humboldtianum* samples, the values, although low, are significantly different from zero, for instance, HPtz/HCha (0.1212) and HZir/HCha/Ptz (0.0633) (Table 8).

The UPGMA dendogram generated by using the coancestry genetic distances for the thirteen samples clearly illustrates such relationships. Thus, one main cluster contains two groups from which *C. grandocule* is equidistant with the highest distance because of the low genetic variability observed in the species. The first group includes the large species/samples from lake Chapala (ECha/SCha/PCha/LCha), whereas the second cluster includes the *C. humboldtianum* samples (HZir/HZac/HCha) from which HPtz separates as a distinct sample. Samples CCha, EPtz and LPtz are also found in this cluster. Bootstrapping proportions for the main branches of the UPGMA tree were

TABLE 8
Matrix of coancestry genetic identities (above) and distances (below) for the 13 samples/populations of Chirostoma

Species	CCha	ECha	EPtz	GI-97	GJ-96	HCha	HPtz	HZac	HZir	LCha	LPtz	PCha	SCha
CCha	—	0.1674	0.2032	0.7838	0.7597	0.1566	0.1211	0.1071	0.0977	0.2756	0.3103	0.1655	0.1191
ECha	0.1833	—	0.2165	0.7558	0.7197	0.1604	0.2129	0.0563	0.1755	0.0480	0.3504	0.0006	-0.0382
EPtz	0.2272	0.2440	—	0.6007	0.5769	0.0469	0.1042	0.0488	-0.0047	0.3684	0.1807	0.2606	0.2050
GI-97	1.5315	1.1099	0.9180	—	0.0256	0.7419	0.6718	0.8068	0.8348	0.9303	0.9391	0.8126	0.8952
GJ-96	1.4260	1.2719	0.8601	0.0260	—	0.6997	0.6565	0.7656	0.7868	0.9044	0.8742	0.7873	0.8557
HCha	0.1703	0.1748	0.0480	1.3545	1.2028	—	0.1212	0.0625	0.0633	0.3414	0.0878	0.1911	0.1235
HPtz	0.1290	0.2394	0.1101	1.1142	1.0686	0.1292	—	0.0625	0.0633	0.2836	0.3105	0.2131	0.1733
HZac	0.1133	0.0579	0.0500	1.6440	1.4509	0.0645	0.0645	—	-0.0238	0.1965	0.1843	0.0640	-0.0023
HZir	0.1028	0.1929	-0.0047	1.8008	1.5454	0.0653	0.0653	-0.0235	—	0.3689	0.1257	0.2015	0.1383
LCha	0.3224	0.0492	0.4595	2.6629	2.3477	0.4177	0.3335	0.2187	0.4603	—	0.5786	-0.0030	0.0064
LPtz	0.3715	0.4314	0.1993	2.7990	2.0732	0.0919	0.3719	0.2038	0.1343	0.8642	—	0.3810	0.3611
PCha	0.1809	0.0006	0.6020	1.6748	1.5478	0.2120	0.2397	0.0662	0.2250	-0.0030	0.4797	—	-0.0630
SCha	0.1268	-0.0375	0.2294	2.2552	1.9357	0.1318	0.1903	-0.0023	0.1488	0.0065	0.4480	-0.0611	—

above 50% (Fig. 6), but for the internal branches were lower than 50%; thus, are not reliable. A maximum likelihood tree grouped all samples into two distinct assemblages from which HZac is equidistant, with bootstrap probabilities of > 50% (Fig. 7).

Fig. 6. UPGMA tree derived from co-ancestry genetic distances of seven species of *Chirostoma* and based on 23 allozyme loci. Bootstrap values estimated from 1000 replications are reported when equal or higher than 50%.

Fig. 7. Consensus tree of a bootstrapped maximum likelihood phenogram (100 runs) constructed from allelic frequencies of 23 allozyme loci. Bootstrap results are shown for nodes with a probability of 50%.

DISCUSSION

The members of the *Chirostoma* "*humboldtianum* species group" have been for centuries of great economical and ethnic importance, but despite these facts little is known about their basic biology. Further knowledge is critical for the conservation and for developing ecologically sound and stable management practices for these species. These species were for a long time restricted to Chapala, Pátzcuaro, Zirahuén and Zacapu lakes, except for *C. humboldtianum* with a wider range of distribution. However, inadequate fishing practices and the increased human settlements in the surrounding areas have led to the dissemination of these species into other water basins and as a result we observed an increase in the genetic variation and differentiation in some of these species and a decrease in others. From Echelle and Echelle (1984) allele frequency data (Appendix 2, pages 108-109) we obtained the levels of genetic variation ($P_{.95}$ and H_e) for the seven species and compared those to the results obtained in the present study. The overall $P_{.95}$ and H_e estimates in our study are among those reported by Nevo (1978 in Hartl 1987) in a review of 51 species of fishes (15.2 and 0.051, respectively). However they are slightly higher to those from Echelle and Echelle's (1984) data (12 and 0.022) (Table 9). Although these differences can be

TABLE 9
Genetic variability of 23 loci of six species of the genus Chirostoma

Species	Sample size		Alleles/locus		$P_{.95}$		H_e	
	I	II	I	II	I	II	I	II
C. c. consocium (Cha)	30	20	1.2 (0.1)	1.2 (0.10)	17	13	0.049 (0.026)	0.060 (0.033)
C. e. estor (Cha)	—	8	—	1.2 (0.11)	—	17	—	0.088 (0.041)
C. e. estor (Ptz)	—	24	—	1.3 (0.12)	—	26.	—	0.079 (0.034)
C. grandocule (I-97)	—	21	—	1.0 (0.00)	—	0.0	—	0.000 (0.000)
C. grandocule (J-96)	10*	20	1.1 (0.1)	1.0 (0.04)	9	4	0.009 (0.006)	0.002 (0.002)
C. humboldtianum (Cha)	—	8	—	1.3 (0.11)	—	22	—	0.085 (0.038)
C. humboldtianum (Ptz)	—	30	—	1.3 (0.13)	—	17	—	0.087 (0.041)
C. humboldtianum (Zac)	10	6	1.2 (0.1)	1.3 (0.13)	22	17	0.026 (0.011)	0.089 (0.045)
C. humboldtianum (Zir)	—	6	—	1.2 (0.08)	—	17	—	0.072 (0.036)
C. lucius (Cha)	31	9	1.1 (0.1)	1.1 (0.07)	4	13	0.013 (0.012)	0.049 (0.028)
C. lucius (Ptz)	—	7	—	1.1 (0.06)	—	9	—	0.035 (0.026)
C. promelas (Cha)	8	12	1.1 (0.1)	1.2 (0.08)	9	17	0.028 (0.023)	0.076 (0.037)
C. sphyraena (Cha)	30	5	1.1 (0.1)	1.1 (0.07)	9	13	0.022 (0.14)	0.071 (0.039)
Average	19.8	13.5	1.1	1.2	12	14	0.022	0.061

A locus is considered polymorphic (P) if the frequency of the most common allele does not exceed 95%. I = estimated from data of Echelle and Echelle (1984); II = from the present study. S.D. is in parentheses. * Corresponds to organisms collected at Ihuatzio, Lake Pátzcuaro, Michoacán.

related to sample size in some of the species, other factors can also be considered. For instance, hybridisation between species has been suggested since the early 80's (Barbour and Chernoff 1984, Toledo 1987) and experimentally demonstrated (Pérez 1987, Andrade 1990, Estrada 1991, Alaye 1993a, b); the introduction of unidentified eggs into other water basins (Toledo 1987, Alaye 1993a, b) and to fishing pressure (Jiménez 1992, Rojas *et al*. 1993a, b, Chacón and Rosas 1995, Jiménez and Gracia 1995).

Inter-specific morphological and genetic variation: The PC results with M (ii) and m (o) data show that five morphometric and two meristic variables contributed the most in discriminating among the seven species. Two (M) variables are from the head (head length and postorbital head length), the remaining related to the swimming and stability of the animals in the water column (pelvic fin length of base, predorsal 1 length, anal fin length of base, anal fin rays, and pectoral fin rays). The variability detected in these measurements is associated to the diverse feeding habits of these organisms and to their different swimming requirements in the water column, since several of these species co-exist in the same habitat. Indeed, differentiation in the mandible apparatus has been earlier reported for species from Lake Chapala (Rodríguez and Granado 1987, 1988). The limitation of a small sample size of *C. promelas*, *C. sphyraena*, *C. e. estor* and *C. lucius* in this study is directly related to the decrease in their abundance in recent years (Alaye 1993b, Jiménez and Gracia 1995, Villicaña-Vázquez pers. comm.). It also restricted our conclusions on the morphological analysis of these species. However, Barbour and Chernoff (1984) suggested that eight morphometric variables related to the head and the swimming ability of these fishes were powerful discriminators of *C. promelas* (mean n = 24), *C. sphyraena* (mean n = 40) and *C. lucius* (mean n = 66) from lake Chapala. Nevertheless, in spite of the limitation in sample size, our allozyme results suggest significant differences in the genetic variability of these four species (H_e = 0.035±0.028 – 0.076±0.037, Table 9) as well as an inter-specific differentiation (θ = 0.137, S.D. 0.052). It should be considered that inter-specific differentiation was found in samples of *C. e. estor* and *C. lucius* from different lakes. Differentiation at the molecular level has been suggested for the latest species, for instance, the differentiation on the haemoglobin molecule of *C. lucius* in relation to that from other *Chirostoma* species from lake Pátzcuaro (Alaye 1996a, b). The possible causes of the H-W deviation (in addition to small sample size) resulting from a high significant deficit of heterozygous in LCha sample (+0.636), suggests that both inbreeding as well as a possible bottleneck might be the causes of such deviation. Indeed the actual LCha population is reduced to few organisms that do not reach large sizes (119-179 mm SL). This suggests that effective population size might be highly reduced. *Chirostoma promelas* and *C. sphyraena* are also rare in Lake Chapala (Villicaña-Vázquez pers. comm.) and are not found elsewhere in the country. The presence of these species in the lake is limited to organisms recruited for a rescue program (Centro Acuícola "Tizapán el Alto", Jalisco, Secretaría de Medio Ambiente, Recursos Naturales y Pesca-SEMARNAP).

Chirostoma c. consocium, the most abundant species from Lake Chapala (Rodríguez and Granado 1987, Morelos and Guzmán 1995) does not show significant morphological or meristic variation, and the levels of genetic variation are considered as intermediate compared to those observed within the group of species studied. Rodríguez and Granado (1987) analysed morphological characters of the mandibular apparatus with PC and DA analyses of species of *Chirostoma* from Lake Chapala and reported similar results to those we obtained here for *C. c. consocium*. Our allozyme analysis indicates that the species showed slightly lower levels of polymorphism but higher heterozygosity values compared to those reported by Echelle and Echelle (1984) ($P_{.99}$ 13 vs. 17, H_e 0.060 vs. 0.049, respectively, Table 9). Differences among

these values are due to the intermediate frequencies of the heterozygous alleles. The genetic identity values observed between *C. c. consocium* and the large species from lake Chapala ranged from 0.0879 to 0.2035 (*C. humboldtianum* and *C. e. estor*, respectively) and supports the separation of this species in the cladogram. The relatively low levels of morphological and allozyme variation observed in this species can relate to the homogenisation on its distribution within the lake, which might indeed favour random mating (F = 0.088, Table 6).

Intra-specific morphological and genetic variation: The DA analysis with both (M) and (m) data indicate the separation of *C. humboldtianum* samples into two distinct groups (HZir and HPtz, with higher sample sizes) suggesting differentiation within the species. Better results are shown with the meristic data. The discrimination results are supported by the allozyme data (genetic differentiation, identity and cluster data). Although the genetic differentiation value is lower (θ_S = 0.072, S.D. 0.032) to those observed among other samples, it is significantly different from zero. This value is similar to the low end found in G_{st}/F_{st} estimates of other freshwater fish species. For instance, for catostomids (0.035-0.507), cyprinodontids (0.015-0.189), atherinids (0.014-0.461) (Ward *et al*. 1994 and references therein) and for *C. grandocule* (0.025) (Barriga-Sosa *et al*. in prep.). The morphological, meristic and allozyme variation observed within these populations must relate to their geographic isolation. For instance, Lake Zirahuén, characterised as relatively stable, young, homogenous water mass, supports only two species of the genus *Chirostoma*, and the fishing practices are oriented to sport fishing of larger species. Concordant to this characteristics, sample HZir showed the lowest heterozygosity value (0.072, S.D. 0.036) among those observed for the species and an F value (probability value associated with the F inbreeding coefficients, 0.264) which suggests inbreeding in this population. In contrast, samples HPtz and HCha had the highest heterozygosity values (0.085-0.087, S.D. 0.038-0.041, respectively), and the identity values for HPtz and the other samples (0.062, 0.063 to 0.121 for HZac/HZir/HCha, respectively) are concordant with an earlier hypothesis that suggested that the population found in Pátzcuaro is indeed distinct (Alaye 1993b). Lakes Pátzcuaro and Chapala in contrast with Lake Zirahuen and Zacapu Lagoon, are the largest lakes and are highly polluted, eroded and support a highly heterogeneous assemblage of *Chirostoma* and other fish species (Berlanga *et al*. 1997). An increase of heterozygous organisms in these populations can be partially explained by the fact that they are exposed to a higher selective pressure (competition for food and space and high fishing pressure). Moreover, it is noteworthy that *C. humboldtianum* has not been reported for lake Chapala, and that the negative values related to inbreeding (F = -0.105) indicate to us that hybridisation between this and other "charal" or "peces blancos" from this lake can not be ruled out.

The morphometric and meristic data also indicate that *C. grandocule* samples GI-97 and GJ-96 may be distinct subpopulations, as observed with the DA M (ii) data analysis (86 and 88% correctly classified organisms). The morphological variation observed within these "subpopulations" from Lake Pátzcuaro can be explained by a high phenotypic plasticity as a response to the contrasting environmental conditions that prevail within the lake. Barriga-Sosa *et al*. (in prep.) showed with a substantially larger sample size, a clear morphological and allozyme differentiation of these "subpopulations" due apparently in part to the contrasting environmental conditions of the Northern and Southern regions of the lake.

In general it appears that most species of the "*humboldtianum* group" are encountering drastic pressures, especially the larger forms *C. promelas* and *C. sphyraena* which are restricted to Lake Chapala. It is critical to establish for this group of species a long term population ecology and genetics study, employing more polymorphic genetic markers, such as RAPD's, AFLP's and microsatellites in addition to allozymes.

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RESUMEN

El grupo *Chirostoma* "*humboldtianum*" incluye siete especies de peces considerados como un conjunto monofilético por sus altas similaridades genéticas y morfológicas. El grupo incluye cinco especies moderadamente grandes, "peces blancos" (117 – 300 mm longitud estándar – SL) y dos especies más pequeñas, "charales" (70 – 142 mm SL). Estas especies son de gran interés económico, cultural e ictiológico para las poblaciones locales y sus prácticas de manejo son controversiales. Nosotros investigamos las variaciones morfométricas, merísticas y de isoenzimas para las siete especies (13 poblaciones) y relacionamos las variaciones con la listoria de vida, el hábitat y los procedimientos de manejo. De las siete especies y poblacines de *Chirostoma*, se compararon 19 variables morfométricas, ocho variables merísticas (por análisis multivariado) y 23 loci de isoenzimas. El análisis de componentes principales (PC) de los datos morfométricos y merísticos indica que ambos juegos de datos da información para diferenciar entre las siete especies. Las variables que cuentan por casi toda esta diferenciación fueron longitud de la cabeza (HL), longitud predorsal 1 (P1L) y longitud de la base de la aleta pélvica (PfbL). Los análisis discriminantes (DA) y de PC con los datos morfométricos también sugieren la diferenciación de poblaciones dentro de *C. grandocule* (83% organismos correctamente clasificados), mientras que los análisis DA y PC con los datos merísticos diferenciaron poblaciones de *C. humboldtianum* (80% organismos correctamente clasificados). Las variables morfométricas más importantes para la diferenciación fueron el alto de la aleta anal (AfH), la longitud de la base de la aleta anal (AfbL) y la longitud predorsal 2 (P2L), y las variables merísticas D2fR, PdS y AfR. Los datos de variabilidad genética indican cambios en los valores de algunas de las especies en relación a datos informados previamente. Las poblaciones presentes de *C. grandocule* muestran una reducción en *H*e (0.002 vs. 0.009). Otras especies mostraron un incremento; por ejemplo, *C. consocium consocium*, *C. humboldtianum*, *C. lucius*, *C. promelas* y *C. sphyraena* promediaron *H*e = 0.069 vs. 0.027. q indicó una diferenciación genética significante entre las especies analizadas (0.247, S.D. 0.159) y q_s apoyó los datos morfológicos que sugieren diferenciación intraespecífica (0.360, S.D. 0.154).

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