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Introgressive hybridisation between two Iberian *Chondrostoma* species (Teleostei, Cyprinidae) revisited: new evidence from morphology, mitochondrial DNA, allozymes and NOR-phenotypes

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A b s t r a c t . Analysis of the hybridisation events between two Iberian *Chondrostoma* species in the Távora River (Douro Basin) suggests different levels of trait introgression. Nuclear traits studied showed different introgression levels, whereas mitochondrial DNA introgression was not found. Lack of mtDNA introgression suggests that male and female hybrids may not equally fit or that possibly backcross matings may not be random. This could be contributing to the maintenance of a relative morphologic cohesion of hybridizing species, in spite of differences relative to allopatric populations. The hybrid zone was possibly originated by secondary contacts between populations of the species involved, motivated by connectivity between adjacent basins. Reanalysis of the hybridizing taxa revealed that *Chondrostoma macrolepidotum* is the species involved in the interspecific crosses with *C. duriense*, instead of *C. arcasii* as previously proposed.

Key words: *Chondrostoma arcasii*, *Chondrostoma duriense*, *Chondrostoma macrolepidotum*, hybrid zone, river capture, multivariate

Introduction

The evolutionary consequences of introgressive hybridisation, defined as the incorporation of alien genes in parental genotypes through backcrossing, have received increased attention both by botanists and, more recently, by zoologists. Early studies in freshwater fishes suggested that hybridisation is quite common, noting however that most of the hybrids produced are sterile (H u b b s 1955). According to A r n o l d & H o d g e s (1995), limited production of mixed-ancestry individuals does not necessarily yield inconsequential evolutionary results, since they may act as bridges for new hybrid generations with more fit genotypes, i.e. the Evolutionary Novelty Model.

Hybridisation in Cyprinidae, the most speciose family of freshwater fishes, is a common phenomenon (reviewed by H u b b s 1955, S c h w a r t z 1972, 1981, A r g u e & D u n h a m 1999, Y a k o v l e v et al. 2000). Hybridisation has long been hypothesised to occur among Iberian cyprinids (e.g. S t e i n d a c h n e r 1866, A l m a ç a 1965). Within the genus *Chondrostoma*, which has several endemic representatives in the Iberian Peninsula, some natural hybrids have been described (S t e i n d a c h n e r 1866, A l m a ç a 1965, C o l l a r e s - P e r e i r a & C o e l h o 1983, E l v i r a 1986, E l v i r a et al. 1990).

The hybridisation between the straight-mouth nase, *C. duriense* Coelho, 1985 (once recognized as a subspecies of *C. polylepis* Steindachner, 1865) and the curved-mouth nase,

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C. arcasii (Steindachner, 1866) (previously included in the genus *Rutilus*), was inferred based on morphological analysis of both putative parental taxa and their hybrids from the Távora River, Douro River Basin, (Collares-Pereira & Coelho 1983). It was suggested that hybrids were morphologically intermediate to parental species, showing character displacement towards *C. arcasii*, thus indicating recurrent backcrossing. They were distinguished from the putative parental *C. arcasii* by the presence of a horny blade, which is characteristic of *C. duriense*, and by intermediate numbers of scales and gill-rakers (Collares-Pereira & Coelho 1983, Coelho 1987, Coelho & Collares-Pereira 1990).

The application of morphological and molecular characters in hybridisation studies has proved very useful since they provide independent data sets that can be compared (e.g. Dowling & Moore 1984, Dowling et al. 1989, Rand & Harrison 1989, DeMarais et al. 1992, Gerber et al. 2001). Therefore, in the present study a combined analysis of morphology, mitochondrial DNA (cytochrome *b*), allozymes and nucleolus organizer regions phenotypes (NORs) was used to explore hybridisation and introgression patterns in the Távora River.

Materials and Methods

A total of 33 specimens belonging to both putative parental species and their hybrids were collected by electrofishing in the Távora River (Douro River Basin) in May 2001 (Fig. 1). Owing to identification difficulties involving *C. arcasii* and its sister species, *C. macrolepidotum*, museum representative specimens of this last species were also used in the morphological analyses (Table 1). Both species show varying degrees of orange coloration at the base of pelvics and anal fins, and overlap of several meristic traits (Collares-Pereira 1983). Additional museum specimens of *C. arcasii* and *C. duriense* were used as references in the morphological analysis (see below). A previous definition of species involved in the hybrid zone was made by the analysis of mitochondrial DNA, and these results are firstly presented.

Total DNA was extracted from fin and muscle tissues (Sambrook et al. 1989). Amplification and sequencing of the entire cytochrome *b* (cyt *b*) gene was undertaken

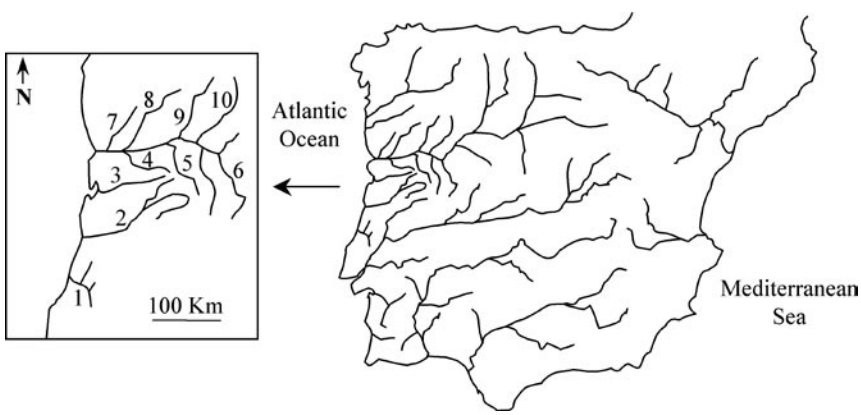

Fig. 1. Map of Iberian Rivers, depicting sampling sites and relevant adjacent rivers. 1 Alcoa River Basin; 2 Mondego River Basin; 3 Vouga River Basin and from Douro River Basin – 4 Paiva River, 5 Távora River, 6 Águeda River, 7 Sousa River, 8 Tâmega River, 9 Tua River, 10 Sabor River.

for a subsample of ten freshly collected specimens from the Távora River, encompassing the morphological variation observed, using primers LCB1 (Brito et al. 1997) and HA (Schmidt & Gold 1993), following the methods of Mesquita et al. (2001). Additional specimens of *C. macrolepidotum* from Alcoa and Mondego River Basins were also sequenced and included in the analysis. Sequences obtained here were deposited at the EMBL/GenBank/DDBJ databases under the accession numbers AJ854047-AJ854054. They were aligned by hand in BioEdit v.5.0.6 (Hall 1999) using published cyt *b* sequences of *C. arcasii* and *C. duriense* from Douro River Basin, *C. polylepis* from Tejo River Basin, and *C. macrolepidotum* from Mondego River Basin, retrieved from EMBL databank (Alves et al. 1997, Briolay et al. 1998, Zardoya & Doadrio 1998). Maximum-Parsimony (MP), Maximum-Likelihood (ML) and Distance (D) trees were generated in PAUP* (Swofford 2002).

Sequences of *Squalius carolitertii* and *S. pyrenaicus* retrieved from EMBL databank (Zardoya & Doadrio 1998) were used as outgroups to root the trees. For MP and ML, a heuristic search was conducted with 100 random step-wise additions of taxa, TBR branch swapping. For MP analysis, constant sites were excluded from analysis and only variable sites were used. Modeltest 3.06 (Posada & Crandal 1998) was implemented to find the best model of sequence evolution that fit our data. Therefore, ML analysis was conducted using the GTR+I model with empirical base frequencies (0.2817, 0.2863, 0.1511), empirical proportion of invariable sites (0.6960) and estimated rate matrix (1.0000, 38.8448, 1.0000, 9.6275). For D analysis, a Neighbour-Joining tree (NJ) (Saitou & Nei 1987) based on GTR+I distance matrices was inferred. Robustness of the inferred MP, NJ and ML trees was tested by bootstrap analysis (Felsenstein 1985) with 1000 pseudoreplications each, using stepwise-additions of taxa.

In the morphological analysis, four meristic traits that discriminate parental species – lateral line scales, transverse rows above and below lateral line and gill-rakers – were analysed (Collares-Pereira & Coelho 1983). Presence of horny blade and orange fins insertions was recorded for each of the freshly collected individuals. Specimens of both curved-mouth nase species, *C. arcasii* and *C. macrolepidotum*, and straight-mouth nase, *C. duriense*, deposited in the Museu Bocage collections were used as references, mostly from allopatric locations (Table 1). Institutional code follows Leviton et al. (1985). Small sample size of

Table 1. Origin and sample sizes of reference museum specimens used in the morphological analysis.

Species	Basin	River	Sample size	Collection no.
C. arcasii	Douro	Sabor	10	MB05-1440 MB05-1441 MB05-1442
C. duriense	Douro	Águeda	3	MB05-394
		Sousa	33	MB05-442
		Tâmega	22	MB05-401 MB05-464
		Távora	26	MB05-435 MB05-487 MB05-568
		Tua	14	MB05-440 MB05-441 MB05-443
C. macrolepidotum	Alcoa	Areia	35	MB05-1258
		Nasce Água	53	MB05-1455
	Mondego	Arunca	30	MB05-1436

reference *C. arcasii* reflects its reduced availability from the area where it has been shown to occur, based on molecular data (Alves et al. 1997, Coelho et al. 1997).

Principal Component Analysis (PCA) of the correlation matrix of standardized meristic data was performed to obtain an objective ordination of specimens from the Távora River, since *a priori* identification of hybrids based on intermediacy can be misleading, particularly for backcross specimens. Pairwise t-tests of first Principal Component scores were performed to test for morphological differences among *C. macrolepidotum* populations, among *C. macrolepidotum* and *C. arcasii* populations, and among *C. duriense* populations. *C. duriense* specimens from Távora collected in this study were pooled with the respective reference sample from Távora to increase sample size. All calculations were performed in SYSTAT v.10.

For a allozyme analysis, we followed Coelho et al. (1997) who described fixed different mobilities of *PGM-1** (phosphoglucomutase; EC 5.4.2.2) and *sSOD-1** alleles (superoxide dismutase; EC 1.15.1.1) between *C. duriense* and the two sister curved-mouth nase species, *C. arcasii* and *C. macrolepidotum*. Livers of 22 specimens were homogenized and stored at -80˚C for no longer than one month before screening of both loci. Liver tissue of the remaining 11 specimens was suspended in whole for cell culture. Allozyme starch electrophoresis of liver homogenates followed methods of Coelho (1992) and Alves & Coelho (1994). Deviations from Hardy-Weinberg equilibrium (HWE) were tested in polymorphic loci using the one-tailed exact probability test (Weir 1990).

For chromosome banding, metaphase chromosomes were prepared from cephalic kidney and liver following the short-term culture method of Fenocchio et al. (1991), which was successful in 24 specimens from the Távora River. NOR-phenotypes were assigned using Chromomycin A_3 (CMA_3) fluorescent banding, following the procedure described by Sola et al. (1992), which allows for identification of rDNA clusters in fish chromosomes (e.g. Rodrigues & Collares-Pereira 1996). Slides were left at least 3 days at 37 ºC before inspection. Double NORs have been found to be a fixed condition in karyotypes of *C. macrolepidotum* from Alcoa River Basin, instead of a single terminal NOR found in other cyprinids (Fig. 2; Gante & Collares-Pereira, unpublished data). Deviations from HWE in NOR-phenotype frequencies were tested using the one-tailed exact probability test (Weir 1990).

Results

Phylogenetic analysis used only 16 different cytochrome *b* sequences (1140 bp), since some of the specimens had the same sequence for that gene. 889 bp were constant sites and 251 bp were variable, 192 bp of which were phylogenetically informative under the parsimony criterion. None of the sequences obtained were assigned to *C. arcasii*. Instead, seven sequences were assigned to *C. macrolepidotum* and three sequences to *C. duriense* (Fig. 3). Robustness of mtDNA assignment was supported by high bootstrap values in every tree generated, which yielded similar topologies. Six out of the seven *C. macrolepidotum* sequences from the Távora River obtained were identical (haplotypes M1 and M2). Every *C. macrolepidotum* from Mondego and Alcoa River Basins yielded a different haplotype (M3, M4, M5, and M6, respectively), as did *C. duriense* (D1, D2, D3 and D4). Phylogenetic reconstructions placed *C. macrolepidotum* sequence from Alcoa River Basin in a basal position relative to the other *C. macrolepidotum* sequences. Every sampled specimen with *C. duriense* phenotype had *C. duriense* mtDNA, whereas every curved-mouth and morphological intermediate specimens sampled showed *C. macrolepidotum* mtDNA.

The first and second Principal Components explained 98.0% of the observed meristic variation, 95.3% and 2.7%, respectively, (Fig. 4). *Chondrostoma arcasii* reference specimens

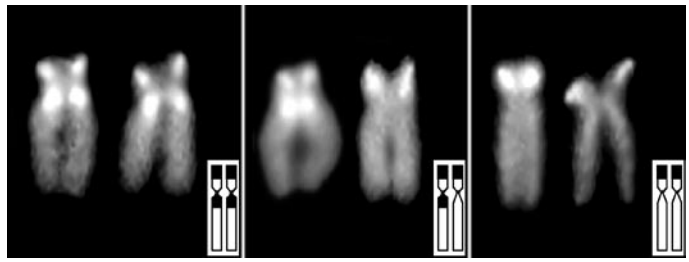

Fig. 2. NOR-bearing submetacentric chromosomes, stained with CMA_3 (light areas) Left – Double NOR homozygote. Both chromosomes show double NORs, below centromere and in the small arms. Center – Heterozygote. One chromosome shows double NORs, below the centromere and in the small arms, whereas the other has single NORs. Right – Single NOR homozygote. Both chromosomes show single NORs in the small arms. Insets show chromosome diagrams with NORs locations in black.

were intermediate to *C. duriense* and *C. macrolepidotum*, being the most similar to *C. macrolepidotum* from Távora, although significantly different (*T = 3.214, df = 34, P = 0.003*). *Chondrostoma macrolepidotum* from Távora was also significantly different from Alcoa and Mondego populations (*T = 9.642, df = 112, P <0.001, and T = 6.951, df = 54, P <0.001, respectively*). *Chondrostoma duriense* from Távora was also significantly

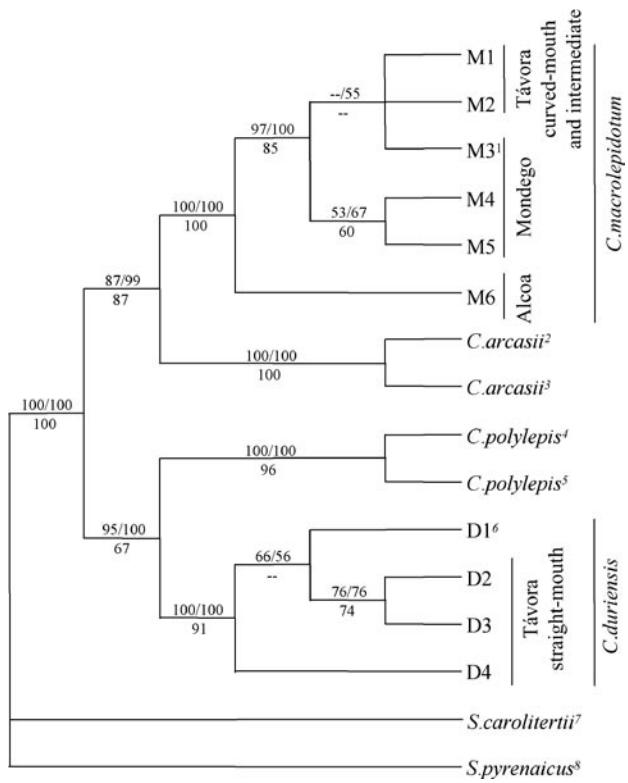

Fig. 3. Phylogenetic tree of taxa analysed based on cytochrome *b* sequences. Numbers above branches represent bootstrap values obtained for 1000 pseudo-replications for Maximum-Parsimony and Neighbour-Joining. Values below branches represent those for Maximum-Likelihood. Nodes with bootstrap values below 50% were forced to collapse and yield polytomies. Hyphens indicate a particular branch not recovered by a given method. [1]AF045986; [2]AF045979; [3]X99424; [4]AF045982; [5]Z75108; [6]AF045983; [7]AF045993; [8]AF045994.

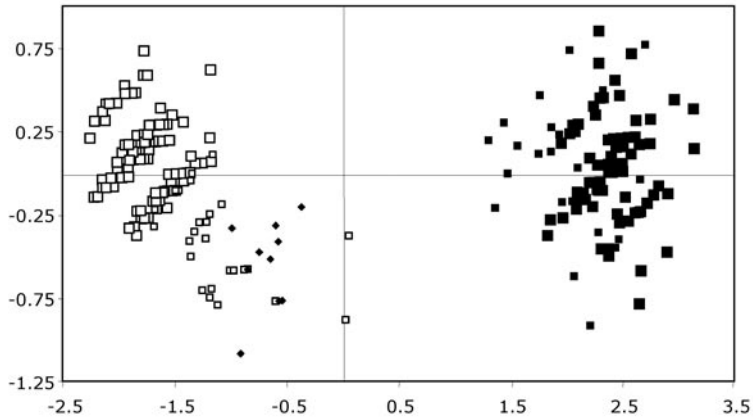

Fig. 4. Scatterplot of first and second Principal Components derived from meristic characters. □ *C. macrolepidotum* (Alcoa and Mondego basins – museum material); □ *C. macrolepidotum* (Távora River – freshly collected); ■ *C. duriense* (excluding Távora River – museum material); ■ *C. duriense* (Távora River – fresh and museum material); ◆ *C. arcasii* (Sabor River – museum material).

different from all allopatric populations of the same species studied (P = 0.024 to P <0.001). The two intermediate individuals had orange fins insertions and horny blade, simultaneously.

In the allozyme analysis, specimens with *C. duriense* phenotype yielded *C. duriense* typical alleles for both *PGM-1** and *sSOD-1**. Specimens with *C. macrolepidotum* and intermediate phenotype showed *C. macrolepidotum* typical alleles, except for one specimen with *C. macrolepidotum* phenotype which exhibited the *C. duriense sSOD-1** and the *C. macrolepidotum PGM-1** alleles, both in homozygosity. This polymorphic locus showed significant deviation from HWE expectations in *C. macrolepidotum* and intermediate phenotype specimens, with heterozygotes deficiency (exact P=0.026).

Diploid chromosome number was invariably 2n=50 for all specimens. The cytogenetic analysis showed the presence of chromosomes with double NORs in the karyotypes of 20 out of 24 specimens analysed (Fig. 2). Five *C. macrolepidotum* specimens showed homozygozity for this chromosome marker, whereas 12 others, including both intermediate specimens, where heterozygous. Three out of seven *C. duriense* specimens were heterozygotes, whereas the others were homozygous for the single NORs (Table 2). Marginally significant deviation from HWE was found for *C. macrolepidotum* specimens, with an excess of heterozygotes (exact *P = 0.046*), while NORs in *C. duriense* specimens conformed to HWE expectations (exact *P = 0.769*).

The acquisition of positive results in every specimen for all the markers used had some constraints, although there was a minimum of at least two-three markers per individual (Fig. 5).

Table 2. Observed and expected number of NOR-genotypes in *C. macrolepidotum* and *C. duriense* specimens from Távora. D – double NORs; S – single NORs.

	Genotype	Observed	Expected
C. macrolepidotum	DD	5	7.12
	DS	12	7.76
	SS	0	2.12
C. duriense	DD	0	0.32
	DS	3	2.36
	SS	4	4.32

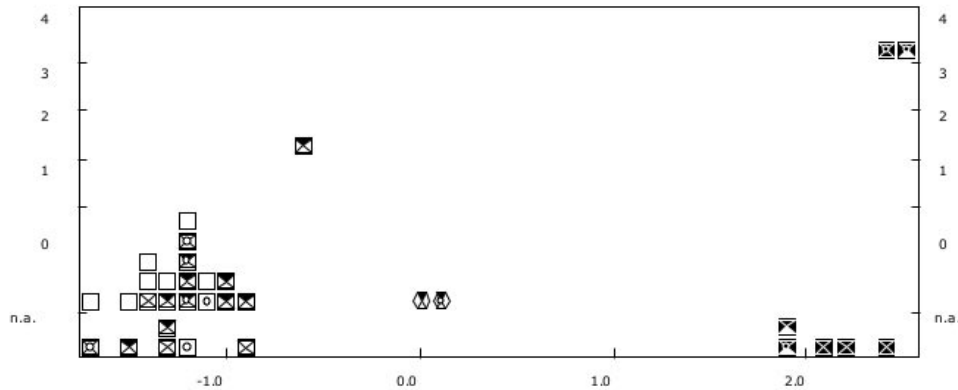

Fig. 5. Multicharacter representation summary of the 33 individuals sampled. First Principal Component extracted from meristic characters was used as x-axis. Allozyme genotype was used to define the y-axis, by tabulating the number of *C. duriense* alleles. n.a. – non-available data. Open symbols represent traits characteristic of *C. macrolepidotum*, whereas solid symbols represent traits characteristic of *C. duriense*. Open squares represent individuals with orange fins insertions, solid squares represent individuals with horny blade, whereas hexagons represent individuals with both traits. NOR genotypes are superimposed – allele "double" is represented by open triangle, and allele "single" is represented by solid triangle. Superimposed circles represent mtDNA lineages.

Discussion

The original suggestion, that *C. arcasii* is one of the parental species based on morphology (Collares-Pereira & Coelho 1983), was not supported by the morphological or molecular data. MtDNA sequences obtained showed that the species involved in the hybridisation event with *C. duriense* is in fact *C. macrolepidotum* (Fig. 3). Morphological analysis based on meristic traits revealed a striking resemblance, even to the naked eye, between *C. arcasii* and the *C. macrolepidotum* hybridizing population from Távora, which likely have induced this probable misidentification. The present findings raise doubts over the presence of *C. arcasii* in the Távora River, whose hypothetical presence was based on morphological features. Misidentification probably occurred because specimens of mixed ancestry closely mimic *C. arcasii* phenotypical traits, such as the numbers of scales and gill-rakers, and mouth position.

Diploid chromosome number was 2n=50 for all specimens, which is the most common condition in cyprinids belonging to the subfamily Leuciscinae (Ráb & Collares-Pereira 1995), indicating that no change in chromosome number occurred in hybridisation events. Double NORs were found to be a fixed trait in karyotypes of *C. macrolepidotum* from Alcoa River Basin (Fig. 2; Gante & Collares-Pereira unpublished data), which may be a basal population of this species as suggested by the mtDNA phylogeny. The presence in Távora River of heterozygotes for this chromosome marker in populations of both species suggests the existence of introgression in both directions – double NOR chromosomes introgressing *C. duriense* and single NOR chromosomes introgressing *C. macrolepidotum*. These might also be accompanied by introgression of morphological traits, as revealed by differences relative to reference samples used (Fig. 4; t-Test values). These differences in morphology were most probably generated by introgression, as opposed to species polymorphisms in this area, since the traits used are clearly different between them and it would call for local convergence.

The allozyme data indicated that none of the analysed specimens were F_1 hybrids, and suggested little protein introgression. In fact, only one specimen, exhibiting *C.*

macrolepidotum mtDNA, was homozygous for the *C. duriense* allele at sSOD-1*, making this specimen a F_2 or a backcross hybrid. Miller (2000) noted that errors in assigning individuals to genealogical classes may arise because of the overlap of genotypic constitution of backcross specimens with parental taxa.

Deficiency of heterozygotes in allozyme locus *sSOD-1** in *C. macrolepidotum* specimens may be indicative of some degree of reproductive isolation between the two species, either in the form of premating isolation (positive assortative mating) or postmating isolation. Also, a 50% decrease in heterozygous loci per generation is expected should backcrosses occur consistently to the same species (Avise 2001), as suggested both by low allozyme introgression and by the lack of mtDNA introgression. However, a significant excess of NOR heterozygotes was found in *C. macrolepidotum* specimens. This discrepancy could be due to gene interactions within mixed genomes. Experiments with *Gambusia* revealed that selection, acting on hybrid genotypes, can be intense and consistent (Avise 2001); the result is various hybrid combinations in specific proportions, some of which can be fitter than their parents (Arnold & Hodges 1995, Arnold & Emms 1998). Alternatively, the observed heterozygotes excess could be the result of continuous input from *C. duriense* males since mtDNA introgression was not observed, though this hypothesis seems less probable since no heterozygous enzyme locus was found.

The present data suggest that the nuclear markers studied have differential introgression, whereas mtDNA introgression was not found. Lack of mtDNA introgression suggests that male and female hybrids are not equally fit or that possibly backcross matings are not random, involving females of each species in each direction and/or hybrid females with each parent, with which they share mtDNA. This may also be contributing to the maintenance of a relative cohesion within each morphological group.

Origin of hybridisation

Hybrid zones are defined as the areas in which genetically distinct populations overlap, mate and produce offspring (Barton & Hewitt 1985) that are viable and at least partially fertile (Arnold 1997). Many of the present day hybrid zones are located in zones of secondary contact (see Hewitt 2001), i.e. where contact was established after allopatric differentiation. This may be the case of the hybridising *Chondrostoma* populations now revisited, which might have come into contact after convergence of tributaries of the Douro and Mondego River basins (Fig. 1). Putative hybrids between *C. macrolepidotum* and *C. duriense* or *C. polylepis* have been reported in Vouga River Basin (Almaça 1965), Paiva and Távora Rivers (Douro River Basin; Collares-Pereira & Coelho 1983) and Mondego River Basin (Collares-Pereira 1983) – all have their origin at Serra da Lapa where headwater convergence might have occurred. No evidence of contacts involving *C. polylepis* from Mondego or Vouga River Basins, where the species replace *C. duriense*, was found in our data.

Owing to sample size and marginal levels of significance for some tests, these results should be seen as preliminary and indicative of the processes stated above. Larger samples should be used to verify the proposed scenario, namely for the introgression of allozymes and mitochondrial loci. Most importantly, these results call for further work on this hybrid zone, including the Mondego and Vouga River systems, with these and other markers to understand fully the causes, dynamics and consequences of these hybridisation events. The present study suggests that introgressive hybridisation may have an impact on the evolutionary trajectories of mixed-ancestry populations, making these cyprinids a very good model for speciation studies.

A c k n o w l e d g m e n t s

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