Phylogenetic relationships of the newly described species Chondrostoma olisiponensis (Teleostei: Cyprinidae)

H. F. Gante*†‡, C. D. Santos§ and M. J. Alves*

*Centro de Biologia Ambiental and Museu Bocage – Museu Nacional de História Natural, Lisboa, Portugal, †School of Life Sciences, Arizona State University, Tempe, Arizona, U.S.A. and §Centro de Biologia Ambiental, Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

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Phylogenies were generated using mitochondrial cytochrome b and nuclear ß-actin gene DNA sequences to infer the phylogenetic relationships of the newly described Chondrostoma olisiponensis. Results indicate that the species is monophyletic with species of the lemmingii-group in mtDNA phylogenies, while it is monophyletic with species of the arcasii-group in the nuclear ß-actin trees. This is in agreement with the morphological resemblance of C. olisiponensis to both species groups. Results from nuclear but not mitochondrial DNA indicate that one population could be currently hybridizing with sympatric Chondrostoma lusitanicum. Based on a relaxed clock calibration of cytochrome b, it is estimated that C. olisiponensis split 12.5–7.9 million years ago (middle–upper Miocene) from its most recent ancestor, which coincides with a period of endorheism in the Iberian Peninsula.

Key words: Achondrostoma; cytochrome b; Iberochondrostoma; molecular phylogeny; nuclear ß-actin gene.

INTRODUCTION

The genus Chondrostoma Agassiz is composed of small to medium-sized cyprinids distributed in the northern Mediterranean drainages across Europe, western Asia and Middle East (Elvira, 1987, 1991, 1997; Durand et al., 2003). Phylogenetic analyses suggest that Chondrostoma is a monophyletic taxon composed of seven monophyletic lineages or groups of species with and without a horny blade on the lower lip (Coelho et al., 1997; Zardoya & Doadrio, 1998; Doadrio & Carmona, 2003, 2004), hereafter referred to as Chondrostoma s.l. The genus is particularly diverse in the Iberian Peninsula, where over one-third of the known species are found. Recently, Robalo et al. (2007) suggested the species groups are in agreement with morphological criteria and proposed their elevation to different genera. According to this new classification, Chondrostoma s.st. consists only of the nasus and soetta-species groups, while the arcasii, genei, lemmingii, polylepis and toxostoma-species groups...
were each elevated to the generic level (respectively, Achondrostoma, Protochondrostoma, Iberochondrostoma, Pseudochondrostoma and Parachondrostoma).

Subsequent to this splitting, a new bladeless species was described from the lower Tejo basin in Portugal. The Lisbon arched-mouth nase Chondrostoma olisiponensis Gante, Santos & Alves is a small species that lacks a horny blade on the lower lip, a characteristic that makes it more similar to Achondrostoma and Iberochondrostoma (the arcasii and lemmingii-species groups, respectively). Nevertheless, the new species has a combination of characters that does not fit in the proposed genera and breaks the combinations of characters deemed diagnostic of these two new genera (Gante et al., 2007). These facts led the authors to include the new species in Chondrostoma s.l. before proceeding to further changes that could lead to instability in the taxonomy of the group.

The present study aims to assess the phylogenetic relationships of C. olisiponensis using mitochondrial and nuclear sequences, and to provide an estimate for time of divergence using a molecular clock calibration.

**MATERIALS AND METHODS**

Muscle and fin tissues from 17 specimens of C. olisiponensis deposited in the tissue collection Museu Bocage (MB) of the Museu Nacional de História Natural, Portugal (accession numbers MB55-0610, MB55-4678, MB55-4679, MB55-4756, MB55-4757, MB55-4833 through to 4840, MB55-5825, MB55-5826, MB55-5840 and MB55-5967) were used to extract total genomic DNA, using standard phenol–chloroform protocols (Sambrook et al., 1989). Specimens originated from Trancão, Maior and Muge River basins and include the type series (Tejo River basin, Portugal; Fig. 1).

Phylogenetic relationships of C. olisiponensis were investigated using sequences of two genes, the nuclear ß-actin gene and the mitochondrial cytochrome b (cyt b) gene. Amplification of the cyt b gene was accomplished using primers LCB1 (Brito et al., 1997) and HA (Schmidt & Gold, 1993) and the conditions of Mesquita et al. (2001). For amplification of the nuclear ß-actin gene fragment, conditions and primers in Robalo et al. (2006) were followed. Sequences were obtained by direct sequencing on an Applied Biosystems 3700 DNA sequencer (www.appliedbiosystems.com) following manufacturer’s instructions. Chromatograms were visually inspected for sequencing errors, and sequences were manually aligned and trimmed with BioEdit v.5.0.6 (Hall, 1999). Sequences representing the most common allele of each locus within C. olisiponensis were used in the phylogenetic analyses. In addition to C. olisiponensis, several Chondrostoma and out-group species for which sequence data of ß-actin and cyt b are available were used (Table I).

Bayesian phylogenies were constructed in BEAST v.1.4.8 (Drummond & Rambaut, 2007), and maximum likelihood (ML) and maximum parsimony (MP) phylogenies were constructed in PAUP® 4.0b10 (Swofford, 2002). Substitution models employed in Bayesian and ML analyses were selected using the corrected Akaike information criterion (AIC) implemented in Modeltest 3.07 (Posada & Crandall, 1998), following Posada & Buckley (2004). Bayesian analyses were run for 10 000 000 generations, sampled every 1000 generations, with a subsequent burn-in of 2501 trees (c. 25%) using TreeAnnotator v.1.4.8 (Rambaut & Drummond, 2002). Conversion and stability of the estimated parameters were checked using Tracer v.1.4 (Rambaut & Drummond, 2003). Phylogenetic trees were visualized using FigTree v.1.1.2 (Rambaut, 2006). ML and MP analyses were conducted using heuristic tree searches (10 replicates, random sequence addition and tree bisection-reconnection (TBR) branch swapping, and gaps were treated as a fifth state). Bootstrap support was estimated using 1000 (MP) and 100 (ML) pseudoreplicates (10 and five sequence addition replicates, respectively, for MP and ML).

The time of divergence of Chondrostoma species and their credibility intervals (highest posterior density, HPD) were calculated using a relaxed clock model implemented in BEAST.
v.1.4.8 (Drummond & Rambaut, 2007). Branch rates were drawn from an uncorrelated log-normal distribution (Drummond et al., 2006), and a Yule speciation prior was applied. The pair-wise rate of evolution of 1.05% per million years (Dowling et al., 2002), often applied to Chondrostoma cyt b data (Doadrio & Carmona, 2003, 2004; Robalo et al., 2006), was used.

**RESULTS**

The 17 specimens of *C. olisiponensis* originating from three localities in the lower Tejo River yield three β-actin alleles. Allele A is found in all sampled populations, allele B occurs in both Trancão and Muge Rivers and allele C is exclusively found in Maior River (Table II). Alleles A and B differ from each other by only one mutation, and C differs from B and A by 11 and 12 mutations, respectively. The entire nuclear data set consists of 912 sites (453 exonic and 459 intronic) of which 88 are variable and 30 are parsimony informative. The most common allele of *C. olisiponensis* (allele A) is closest to *Chondrostoma oligolepis* (Robalo, Doadrio, Almada & Kotttelat) with a genetic distance of 0.34%, and to *Chondrostoma arcasii* (Steindachner).
Table I. Taxa included in the phylogenetic analyses and sequence database accession numbers

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Cyt&lt;sup&gt;b&lt;/sup&gt;</th>
<th>β-actin</th>
<th>Taxon</th>
<th>Cyt&lt;sup&gt;b&lt;/sup&gt;</th>
<th>β-actin</th>
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<td>DQ447714&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Chondrostoma phoxinus</td>
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<td>DQ447715&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>DQ447718&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>DQ455049&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>DQ447725&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>AM886165&lt;sup&gt;k&lt;/sup&gt;</td>
<td>Telestes</td>
<td>AY838934&lt;sup&gt;d&lt;/sup&gt;</td>
<td>DQ061950&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Zardoya & Doadrio (1998); <sup>b</sup>Robalo et al. (2007); <sup>c</sup>Doadrio & Carmona (2004); <sup>d</sup>Robalo et al. (2006); <sup>e</sup>Zardoya & Doadrio (1999); <sup>f</sup>Hrbek et al. (2004); <sup>g</sup>Durand et al. (2003); <sup>h</sup>Durand et al. (2002); <sup>i</sup>Alves et al. (2001); <sup>j</sup>Briolay et al. (1998); <sup>k</sup>Present study and <sup>l</sup>Freyhof et al. (2006).

and *Chondrostoma occidentale* Robalo, Almada, Sousa-Santos, Moreira & Doadrio with a genetic distance of 0-45%, all of which belong to the *arcasii*-species group (*i.e.* *Achondrostoma*). Allele C is identical to the typical *Chondrostoma lusitanicum* Collares-Pereira allele used in this study and highly divergent (0-91%) relative to the other alleles found in *C. olisiponensis*.

Four cyt<sub>b</sub> haplotypes, differing by not more than three nucleotide substitutions, are found in the 17 specimens analysed. The most common haplotype (A) is found in the three populations sampled, haplotype B is absent from Maior River, while haplotypes C and D are found exclusively in Trancão and Muge Rivers, respectively (Table II). The entire mitochondrial data set (1140 bp) consists of 386 variable sites of which 272 are parsimony informative. The most common haplotype of *C. olisiponensis* (allele A) is closest to *C. lusitanicum* with a genetic distance of 7-11%, followed by *Chondrostoma lemmingii* (Steindachner) with a distance of 7-81%, both species belonging to the *lemmingii*-species group (*i.e.* *Iberochondrostoma*).
Table II. Geographic distribution (see Fig. 1) of different haplotypes found in Chondrostoma olisiponensis specimens

<table>
<thead>
<tr>
<th>Locality</th>
<th>Cyt b</th>
<th>ß-actin</th>
</tr>
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<tbody>
<tr>
<td>Maior (n = 3)</td>
<td>A = 3</td>
<td>A = 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C = 3</td>
</tr>
<tr>
<td>Trancão (n = 10)</td>
<td>A = 5</td>
<td>A = 14</td>
</tr>
<tr>
<td></td>
<td>B = 3</td>
<td>B = 6</td>
</tr>
<tr>
<td></td>
<td>C = 2</td>
<td></td>
</tr>
<tr>
<td>Muge (n = 4)</td>
<td>A = 2</td>
<td>A = 5</td>
</tr>
<tr>
<td></td>
<td>B = 1</td>
<td>B = 3</td>
</tr>
<tr>
<td></td>
<td>D = 1</td>
<td></td>
</tr>
</tbody>
</table>

The most common allele at each locus (allele A; Table II) was used to reconstruct phylogenetic relationships. The different tree-building methods give largely congruent results for each gene fragment; when a particular node is retrieved by one method, it is also retrieved with equivalent support by the other methods as well (Figs 2 and 3). Chondrostoma olisiponensis is monophyletic with the lemminii-species group in cyt b trees, while it is monophyletic with the arcasii and polylepis-species groups in ß-actin phylogenies. In both phylogenies, C. olisiponensis shares a common ancestor with the entirety of these groups.

According to the relaxed clock calibration, divergence within Chondrostoma initiated c. 15·1 million years before present (b.p.) (95% HPD: 17·8–12·4 M b.p.) during the lower–middle Miocene (late Burdigalian–Serravalian). In particular, the time of divergence leading to C. olisiponensis is estimated to have occurred c. 10·1 M b.p. (95% HPD: 12·5–7·9 M b.p.), during the middle–upper Miocene (Serravalian–Late Tortonian; Fig. 2).

DISCUSSION

Chondrostoma olisiponensis was recently described for the lower Tejo River basin in Portugal (Gante et al., 2007). Since the species shares many morphological characters deemed diagnostic of Achondrostoma and Iberochondrostoma, while others fit none of the two newly designated genera, molecular data could shed light on this taxonomic hurdle. This conflict, however, is also evident in the molecular data, since C. olisiponensis is placed in either of these species groups, depending on the marker. Chondrostoma olisiponensis is recovered as monophyletic with the lemminii-species group with high support by different methods in the mitochondrial trees (Fig. 2). Conversely, C. olisiponensis is recovered monophyletic with the arcasii and polylepis-species groups in the nuclear trees with high posterior probability (Fig. 3). This type of incongruence is usually due to incomplete lineage sorting of the nuclear gene fragment, biases associated with any of the markers or hybridization between two ancestral forms (Funk & Omland, 2003). Given the extensive contemporary hybridization between species belonging to different lineages of Chondrostoma s.l. (Collares-Pereira & Coelho, 1983; Elvira et al., 1990; Gante et al., 2004), it is very likely that hybridization and introgression have occurred in the past,
Fig. 2. Bayesian phylogenetic tree showing relationships among Chondrostoma s.l. species based on cyt b sequences. Values above branches refer to posterior probabilities. Values below branches refer to maximum likelihood (ML) and maximum parsimony (MP) bootstrap values, respectively. Grey bars represent the 95% highest posterior density (HPD) for each estimated age based on a relaxed molecular clock. The most common haplotype found in C. olisiponensis was used (haplotype A; see Table II).
Fig. 3. Bayesian phylogenetic tree showing relationships among Chondrostoma s.l. species based on β-actin sequences. Values above branches refer to posterior probabilities. Values below branches refer to maximum likelihood (ML) and maximum parsimony (MP) bootstrap values, respectively. The most common allele found in C. olisiponensis was used (allele A). Allele C found only in Maior River is identical to the typical C. lusitanicum allele (Table II).

potentially with persisting effects in the phylogeny (Smith, 1992), and causing the observed conflicting phylogenetic signals. Testing of these hypotheses will require additional sequences of β-actin from each species and further analysis of unlinked nuclear markers.

Evidence for contemporary hybridization and introgression is also present in this study. β-actin allele C, found in heterozygosity in all C. olisiponensis specimens from Maior River (Table II), is typical of the sympatric C. lusitanicum (Fig. 3). This pattern of allele sharing suggests the occurrence of gene flow between these species. Breakage of reproductive barriers could be associated with small population sizes of C. olisiponensis and environmental disturbance of its habitats (Gante et al., 2007).

The mean estimated ages of splitting of Chondrostoma species derived in the present study, using a relaxed molecular clock with a mean pair-wise rate of 1.05% per million years, are generally slightly older than that reported by Doadrio & Carmona (2003, 2004) using a strict clock calibration (Fig. 2). Nevertheless, many of these later estimates fall within the present 95% HPD. According to the present results, initial cladogenesis within Chondrostoma started between 17.8 and 12.4 M B.P. and that of C. olisiponensis between 12.5 and 7.9 M B.P., which was the first species to split from the ancestor of the lemmingii-species group. This timing of speciation for C. olisiponensis coincides with a period of endorrheism in the
Iberian Peninsula (Ribeiro et al., 1979), suggesting that speciation of *C. olisiponensis* occurred in Lower Tejo Tertiary basin. The influence of Tertiary endorheic basins in the evolution of Iberian *Chondrostoma* and other freshwater fishes has been recognized by several authors (Carmona et al., 2000; Doadrio & Carmona, 2003, 2004; Sanjur et al., 2003; Doadrio & Perdices, 2005; Robalo et al., 2006, 2008), since many speciation events coincide with this period. As noted by Doadrio & Carmona (2003, 2004) for other bladeless species of the *arcasii* and *lemmingii*-species groups, the distribution area of *C. olisiponensis* is restricted to a portion of the present-day hydrographic basin, implying its reduced vagility.

The molecular data presented in this study do not allow unequivocal placement of *C. olisiponensis* in either of the new designated genera, which is in agreement with the previous morphological results (Gante et al., 2007). In particular, *C. olisiponensis* has morphological traits deemed diagnostic of *Achondrostoma* and *Iberochondrostoma*, as well as molecular markers distinctive of each of these genera. This pattern could have originated either by incomplete sorting in a form ancestral to both groups or by ancient hybridization and deserves further investigation. In addition, extent and directionality of contemporary introgression between *C. olisiponensis* and *C. lusitanicum* should be investigated, as both species are rare and threatened.

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References


