Research Article

Phylogenetic position of the most endangered Chilean bird: the Masafuera Rayadito (*Aphrastura masafuerae*; Furnariidae)

Javier Gonzalez[1]

[1]Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Str. 24–25, 14476 Potsdam, Germany. Corresponding author: Dr. Javier Gonzalez Email: thinocorus@googlemail.com

Abstract

Masafuera Rayadito (*Aphrastura masafuerae*; Furnariidae) is a Critically Endangered species endemic to Alejandro Selkirk Island (Juan Fernández Archipelago, Chile). Categorized as probably extinct in 1980, later estimates, ranging from 140 (in 2002) to 500 individuals (in 2006–2007), showed a fluctuating population size of the species. The grazing of goats and cattle has increased habitat loss for the species. Other threats are lack of nesting sites, introduced species such as feral cats and rats (*Rattus rattus* and *R. norvegicus*), and increased populations of natural predators like the Masafuera Hawk. In order to increase the availability of nesting sites, 81 nest boxes were installed in different habitats in 2006, with evidence of use during subsequent breeding seasons. Despite conservation concerns, however, no genetic studies are yet available for this furnariid. This study reports for the first time the levels of genetic divergence of the species, based on nucleotide sequences of the mitochondrial DNA (cytochrome oxidase subunit 1 gene; COI). *Aphrastura masafuerae* is closely related to a widespread species of furnariid distributed mainly in Chile on the mainland, the Thorn-tailed Rayadito (*A. spinicauda*). The Masafuera Rayadito diverged from its mainland sister species probably during the Pleistocene 0.57 ± 0.19 Myr ago. Consistent with mitochondrial and nuclear molecular clocks, the estimated time of the split between *A. masafuerae* and *A. spinicauda* is in perfect agreement with the geological origin of the Juan Fernández Archipelago, which is of volcanic origin. In order to assess genetic variability within the population of this fragile bird, further studies using a multi-locus genetic approach at the population level are necessary.

Keywords: Furnariidae, Juan Fernandez Archipelago, mitochondrial DNA, molecular clock, Threatened species

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Introduction

Archaeological data show high rates of extinction on the Pacific islands [1]. However, molecular phylogenetic studies reveal radiations of several songbirds that might have started on islands, increasing avian diversity on nearby continental regions [2]. The high endemism found on islands [3] correlates often with high rates of extinction because of inbreeding and human impact [4]. Thus, island ecosystems become biodiversity hotspots and at the same time have a high conservation priority.

Usually found in dense vegetation cover [5], the Masafuera Rayadito (*Aphrastura masafuerae*; Furnariidae) is a small, buff-brownish bird characterized by reddish spine-like tail feathers and a light superciliary stripe ([6] and references therein). This species is endemic to Alejandro Selkirk Island (Juan Fernández Archipelago, Chile) and is declared a Critically Endangered species by the International Union for Conservation of Nature (IUCN) [7]. In 1980, Vaurie [8] thought that the species was probably extinct, and population size estimates of the early 1990s have been as low as 140 individuals [9]. Subsequent conservative estimates, during the post-breeding season of 2006 and 2007 [10], suggested a larger population size of 500 individuals, indicating a fluctuating population size of the species during recent decades. The grazing of goats and cattle has increased habitat loss for the species. The lack of nesting sites, introduced species such as feral cats and rats (*Rattus rattus* and *R. norvegicus*) and increased populations of natural predators like the Masafuera Hawk (*Buteo polyosoma exsul*) are threatening this bird as well [6,10].

Conservation efforts have concentrated on nesting and feeding behavior, breeding population size, censuses, critical habitat, and other ecological features of this furnariid [5,6,10]. In 2006, for example, 81 nest boxes were installed in order to increase the availability of nesting sites. Evidence of use of these nesting boxes by the Masafuera Rayadito was found in only seven boxes located in the southern part of the island [10]. Despite conservation concerns, genetic studies are available only for the sister species (*Aphrastura spinicauda*) and close relatives [11-13]. Nowadays, wildlife conservation programs nearly always integrate molecular techniques to explore the genetic makeup of species of concern. Considering that the Masafuera Rayadito is threatened with extinction, genetic data are needed for future conservation programs. Using nucleotide sequences of the mitochondrial DNA (cytochrome oxidase subunit 1 gene; COI), this study reports for the first time the levels of genetic divergence of this endangered species from its mainland sister species, the Thorn-tailed Rayadito.

Methods

Sampling

Two individuals were captured with mist-nets on Alejandro Selkirk (33°45' S, 80°45' W, see Fig. 1), the westernmost island of the Juan Fernández Archipelago. This steep volcanic island is located 181 km west of Robinson Crusoe Island and 835 km off the Chilean coast. See Castilla [14] for detailed information about climate and other geographical characteristics of the island. The birds were captured on the summit region of the island (Los Inocentes peak; 1,200 m above sea level) where the species normally reproduces (Fig. 2). The vegetation of the area consists of dominant fern *Lophosoria quadripinnata* (95–100% cover), tree-ferns (*Dicksonia externa* and *Blechnum cycadifolium*) and the tree *Drimys confertifolia*. See Hahn et al. [6] for detailed information regarding the nesting habitat of the species. Blood samples were obtained by puncturing the brachial vein and stored on FTA cards (Whatman, Germany). Immediately after blood sampling, the birds were released in the same place where they ware captured. The capture of the two individuals in Alejandro Selkirk Island was authorized by CONAF (Corporación Nacional Forestal, Archipiélago de Juan Fernández National Park).

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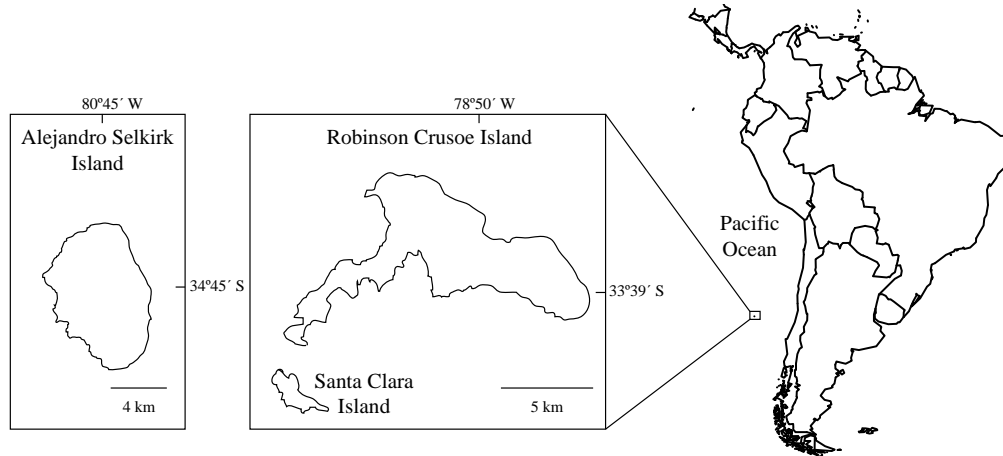
Fig. 1. Map of the study area. Alejandro Selkirk Island, Archipiélago de Juan Fernández National Park, Chile

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DNA isolation, PCR amplification and sequencing of COI

The DNA was extracted from FTA cards following the manufacturer's instructions. Briefly, the FTA card sample discs (1.2 mm) were placed in the PCR amplification tubes and washed two times with 200 μl of FTA purification reagent. After removal from the purification reagent, the discs were washed twice with 200 μl of TE buffer. FTA discs were dried at room temperature for 1 hr.

The primers BirdF1 and COIbirdR2 [15] were used to amplify about 700 base pairs (bp) of the mitochondrial cytochrome oxidase subunit 1 gene (COI). The PCR reaction mix (25 μL) contained the following components: 2 μL of DNA template (60 ng of DNA), 2.5 μL AmpliTaq® 360 Buffer (10✕), 2 μL of 25 mM $MgCl_2$, 2 μL of 10 mM solution of dNTP and 0.125 μL AmpliTaq® 360 DNA Polymerase (Applied Biosystems, Germany).

PCR cycles were performed as follows: 94°C for 5 min, 33 cycles of 94°C for 1 min, 50°C for 40 s, 72°C for 40 s, and a final extension at 72°C for 5 min. PCR products were visualized on 1.4% agarose gels. Sequencing of both strands was conducted using an ABI 3730XL Capillary Sequencer (Applied Biosystems, Germany) with the BigDye® Terminator Cycle Sequencing Kit version 3.1 by GATC Biotech AG (Konstanz, Germany).

The sequences generated in this study have been deposited in GenBank under accession numbers JQ739454 and JQ739455.

Data analysis

COI sequences were retrieved from the GenBank for two families of the Furnarioidea, i.e. Furnariidae and Dendrocolaptidae [13]. Sequences were aligned with BIOEDIT v. 7.0.9.0 [16], and phylogenetic trees were reconstructed using maximum likelihood (ML) in PAUP* v. 4.0b10a [17], and Bayesian inference (BI) in MRBAYES v. 3.1.2 [18]. We explored the model of sequence evolution that fits the data best with JMODELTEST v. 0.1.1 [19] and MRMODELTEST v. 2.3 [20]. ML heuristic searches were performed with closest stepwise sequence additions, tree-bisection-reconnection, branch-swapping (TBR), 'multrees' option and the best model found with JMODELTEST. In the ML analyses, the robustness of each node was

assessed by 1,000 bootstrap replicates. For BI analyses, two independent runs of 10,000,000 generations each were performed along with four Markov chains. Trees were sampled every 500 generations and the first 4,000 samples were discarded as 'burn-in'. Uncorrected genetic distances (*p*-distance) were calculated with MEGA v. 5 [21]. Phylogenetic trees were rooted with five representatives of the tapaculos (Rhinocryptidae) and the Elegant Crescentchest (*Melanopareia elegans*; see [13,22,23]).

For the molecular dating, an uncorrelated lognormal (UCLN) model of molecular evolutionary rate heterogeneity was also used in the computer program BEAST v. 1.6.2 [24,25]. The analysis was conducted using the model found by JMODELTEST v. 0.1.1 [19] that fits the data best. The universal avian clock of 2.1% sequence divergence per million years (0.0105 substitutions/site/lineage/million years) was employed in these analyses [26]. Two independent runs of 20,000,000 generations each were performed with sampling once every 1,000 trees. The number of generations required to reach convergence was assessed by TRACER v. 1.5 (http://beast.bio.ed.ac.uk/Tracer).

Fig. 2. View from "Los Inocentes" peak, Alejandro Selkirk Island, Archipiélago de Juan Fernández National Park, Chile. The vegetation cover consists mainly of fern *Lophosoria quadripinnata* and the tree *Drimys confertifolia* (left). The Masafuera Rayadito (*Aphrastura masafuerae*) caught by a mist-net for blood sampling (right)

Results

The alignment of the protein-encoding gene COI consisted of 694 bp. No internal stop codons or frame shifts were found in these sequences that translated entirely by using the chicken mitochondrial code. In both individuals of the Masafuera Rayadito, variation was found to be at only one site (0.14%) and corresponds to a synonymous transition (T/C) at the third position of the codon (GCT/GCC) that encodes the amino acid adenine.

Figure 3 shows the phylogeny based on COI nucleotide sequences. Maximum likelihood and Bayesian inference analyses recovered congruent topologies (see Appendices 1 and 2). Topological incongruence was due only to nodes supported by low bootstrap or posterior probability values. The phylogenetic reconstruction reveals a polytomy and a basal position of *Aphrastura* among the Furnariidae, and one major separate clade for the woodcreepers (Dendrocolaptidae). *Aphrastura masafuerae* is closely related

to *A. spinicauda* on the mainland. Both species clustered together with high bootstrap support (see Fig. 3).

The uncorrected genetic distance (*p*-distance) between *Aphrastura masafuerae* and *A. spinicauda* was 1.2 ± 0.4%. Assuming an avian clock of roughly 2.1%/Myr for the mitochondrial DNA [26] these taxa might have recently diverged 0.57 ± 0.19 Myr ago. The Bayesian inference molecular clock analysis shows a similar divergence time estimate of 0.71 Myr with a 95% confidence interval of 1.18–0.34 Myr (see Appendix 3).
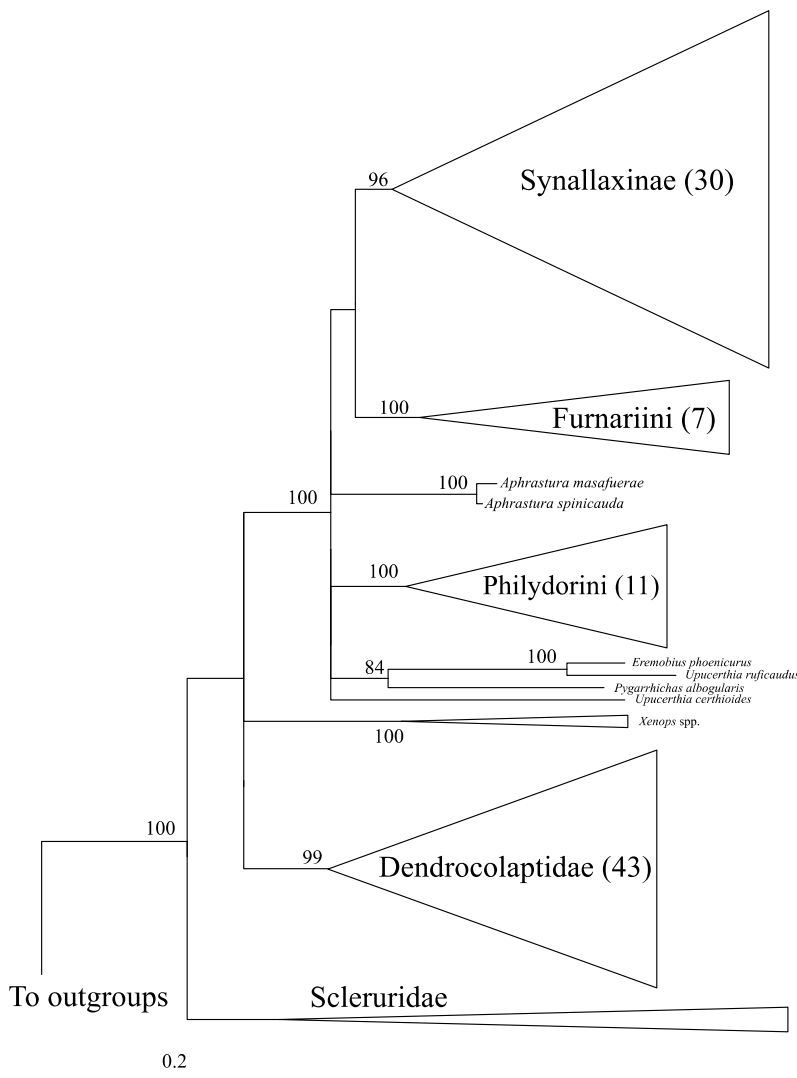
Fig. 3. Majority-rule consensus tree derived from Bayesian inference (BI) analysis and based on 694 bp of the cytochrome oxidase subunit 1 gene (COI). BI posterior probabilities (≥0.90) are indicated above the branches. The model selected for the BI analysis consisted of GTR + gamma distribution shape parameter (G) = 1.42 + proportion of invariable sites (I) = 0.60. In parentheses are the numbers of species represented by the terminal triangles. See Supplementary material for GenBank accession numbers and complete detailed phylogenies

Discussion

Phylogenetic relationships
The topology obtained in this study conforms to accepted knowledge about the infraorder Furnariides based on nuclear and mitochondrial DNA [13,27-29]. Regarding *Aphrastura*, the phylogenetic position of the genus has adopted different phylogenetic positions within the Furnariidae depending on the marker and taxa analyzed. Considering nuclear genes, nucleotide sequences from introns do not provide strong support for the relationship of this genus with any other furnariid species in particular, for instance, *Aphrastura* clusters with *Coryphistera* or *Leptasthenura* [30]. However, these analyses included a reduced number of species [30]. Although the phylogenetic relationships of *Aphrastura* and other furnariids are only partially clear, a larger data set (about 4,000 bp and additional taxa) based on recombination activating genes supports a basal position for the genus among the Synallaxinae with high/medium bootstrap support [13].

As in this study, the evidence recovered from another mitochondrial gene, cytochrome *b*, supports an unresolved polytomy at the base of the Furnariidae but a closer relationship of *Aphrastura* to Synallaxinae and the tribe Furnariini [30]. Combined data sets including sequences of nuclear introns and mitochondrial genes also support a basal position of this lineage among Synallaxinae [12,30].

Based on a molecular clock for the myoblogin intron 2 in passerines [29], the genus *Aphrastura* probably diverged from other furnariids at the Lower Miocene [12]. In this study, Bayesian molecular clock analyses show also similar ages (19–18 Myr) for the split between the Synallaxinae and Furnariinae (see Appendix 3).

Juan Fernández Archipelago colonization
The remote Pacific Island Alejandro Selkirk is located 181 km west of Robinson Crusoe and 835 km west of South America. Thus, for dispersing birds like the Thorn-tailed Rayadito [11], the Juan Fernández Archipelago represents a biogeographically remote group of islands isolated by a major but traversable oceanic barrier. The spatial separation of this archipelago has turned out to be a hotspot of avian endemism [31].

The level of genetic divergence between *Aphrastura masafuerae* and *A. spinicuada* supports a recent colonization of the Juan Fernández Archipelago by the Masafuera Rayadito in the South Pacific Ocean, approximately 0.65 Myr ago. These time estimates do not predate the origin of the Juan Fernández Archipelago and are in perfect agreement with the geological age based on potassium-argon dating [32]. The three main islands of the Juan Fernández Archipelago are not older than 5.8 ± 2.1 Myr, and Alejandro Selkirk Island is the youngest one with 2.44–1.01 Myr [32]. Previous genetic studies performed on other Juan Fernández passerines recovered similar time divergences. For instance, using mitochondrial genetic markers like the cytochrome *b* gene, colonization time estimates of 700,000 and 300,000 years have been suggested for the Juan Fernandez Firecrown (*Sephanoides fernandensis*; [33]) and the Juan Fernandez Tit-Tyrant (*Anairetes fernandezianus*; [34]), respectively.

The Masafuera Rayadito might have colonized Alejandro Selkirk Island after the emergence of the island during the Pleistocene. However, it remains unclear whether this avian species arrived at Alejandro Selkirk by a direct colonization from the mainland or from neighboring islands like Masatierra or Santa Clara, which are 3.79–4.23 and 5.8 Myr old, respectively. One plausible scenario for this cross-oceanic dispersal is an east-west colonization followed by later extinction events on older eastern islands. Modes of

speciation such as dispersals from Masatierra to Masafuera and back-dispersal events have been proposed for several species of plants [32,35]. Thus, a step-stone model of colonization for the Juan Fernández Islands by birds, as in other archipelagos (e.g. the Canary Islands; [36]), cannot be discarded.

Implications for conservation

Genetic data may provide critical information about population structure [37], genetic variability [38], taxonomic status [39] and conservation management units [40]. Estimation of the phylogenetic distance among species of a community is essential for determining phylogenetic diversity and the functioning of ecosystems [41]. The geographical isolation of the Juan Fernández Archipelago has resulted in the mere morphological description of several endemic species of birds, and only a few genetics studies conducted on Robinson Crusoe Island are available so far [33,34]. For the first time, the new genetic data obtained in the present study provide insights about the level of divergence between the endangered Masafuera Rayadito thriving on Alejandro Selkirk and the widespread Thorn-tailed Rayadito on the mainland.

With approximately 130 species, the Synallaxinae (spinetails and allies) contribute nearly half of the diversity found within the Furnariidae ([42]), and both species *Aphrastura masafuerae* and *A. spinicauda* are the sole representative of a probable basal lineage in the phylogeny of this speciose group of Neotropical birds ([12,13]).

Despite the existing hesitation to include phylogenetic diversity in conservation planning ([43]; but see [44]) and in order to keep evolutionary history to the highest level possible, many conservation studies incorporate phylogenetic diversity analyses in order to maximize the number of clades rather than the number of species conserved, giving a high relative weight to species which are taxonomically distinct [45]. Lineages containing few or no sister taxa, which is the case of *Aphrastura*, may contribute greatly to phylogenetic diversity ([46]). With relatively long-branch lengths leading to the two extant species of *Aphrastura* in all phylogenetic reconstructions (this study), this genus does contribute importantly to the phylogenetic diversity within the Furnariidae. Thus, coupled with its small population size and restricted distribution on a remote island, the phylogenetic position of the Masafuera Rayadito is an additional biodiversity component supporting the high conservation priority of the species.

Population size fluctuations (e.g. bottlenecks) may be caused by glaciations, climate change, or anthropogenic impact determining the demographic history of the species ([47]). Based on the level of genetic divergence estimated in this study, the Masafuera Rayadito has probably survived in isolation on Alejandro Selkirk Island during the last 600,000 years. We do not know how much the environment of this species has changed during the last hundred thousand years, but human impact has been documented on Alejandro Selkirk during recent decades ([6,10]). Census estimates indicate that the population of this endangered species has probably been fluctuating during this time (for instance, see [9] and [10]).

Genetic variation dynamics of small and isolated populations like that of Masafuera Rayadito are explained mainly by two phenomena: genetic drift, which may lead to loss of adaptive alleles or the fixation of deleterious alleles; and inbreeding, which may increase homozygosity within the population ([48]). As a consequence of these phenomena, the viability of populations may be affected by a fitness reduction or inbreeding depression. How did the founder population of the Masafuera Rayadito manage to overcome inbreeding depression or survive probably successive bottlenecks on a remote island like Alejandro Selkirk during the last hundred thousand years? The species has probably been able to maintain a certain level of genetic variability. For instance, the differences found on nucleotide sequences obtained from two individuals (this study) indicate some degree of intrapopulation genetic variability, enabling the

persistence and survival of the lineage. However, these data are not enough to determine the genetic makeup of the species, since they are based on only two individuals and one genetic marker. In order to understand the time and mode of evolution of the Masafuera Rayadito as part of an insular endemic avifauna inhabiting the remote Juan Fernández Archipelago, further studies using a multilocus genetic approach at the species and population level are necessary.

Acknowledgements

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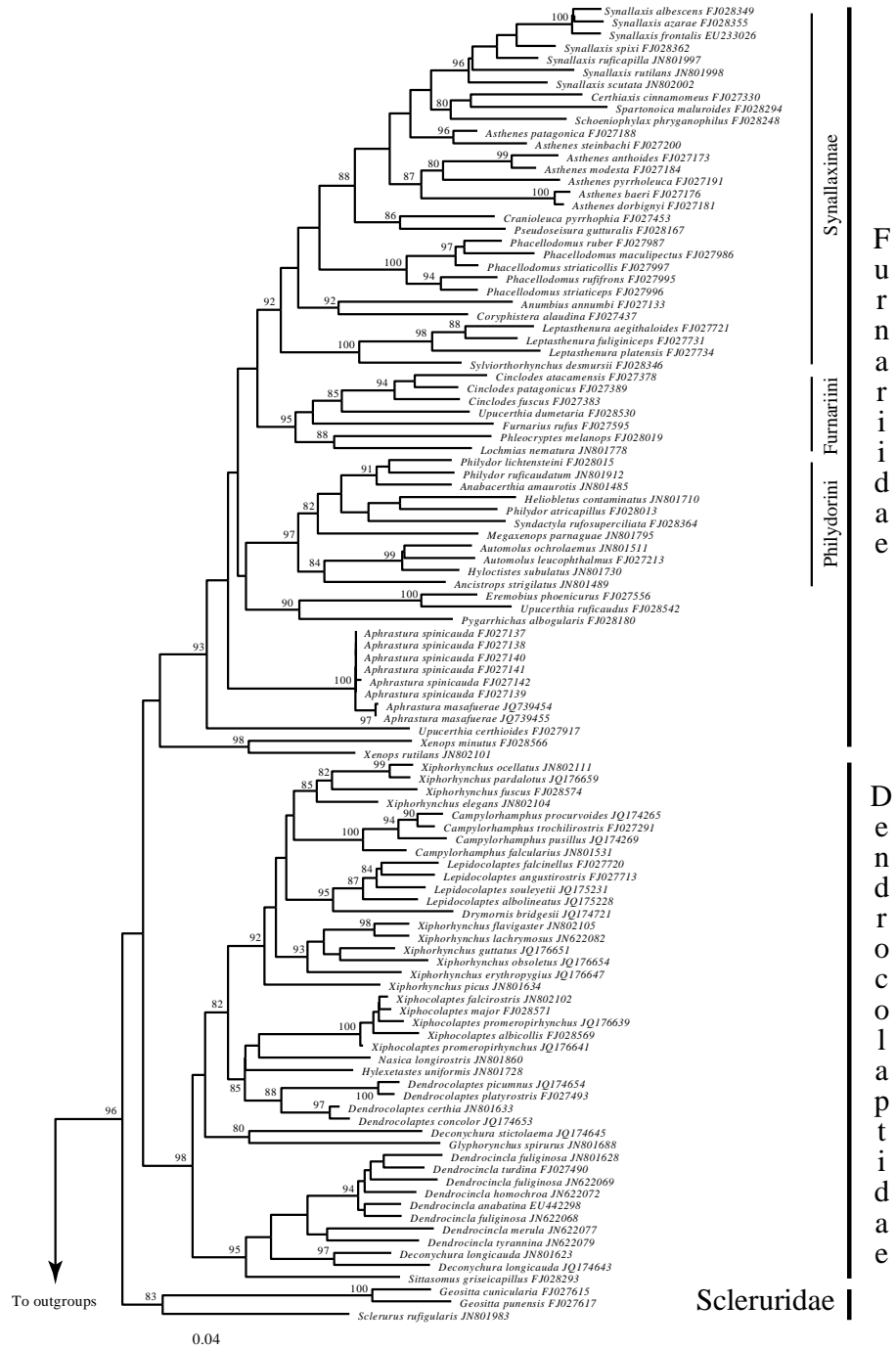
Appendix 1. Detailed maximum likelihood (ML) phylogram based on 694 base pairs of the cytochrome oxidase subunit 1 gene (COI). Bootstrap values (≥0.75%; 1,000 replicates) are indicated for each node. The model selected for the ML analysis consisted of GTR + gamma distribution shape parameter (G) = 0.87 + proportion of invariable sites (I) = 0.58. GenBank accession numbers indicated for each species.
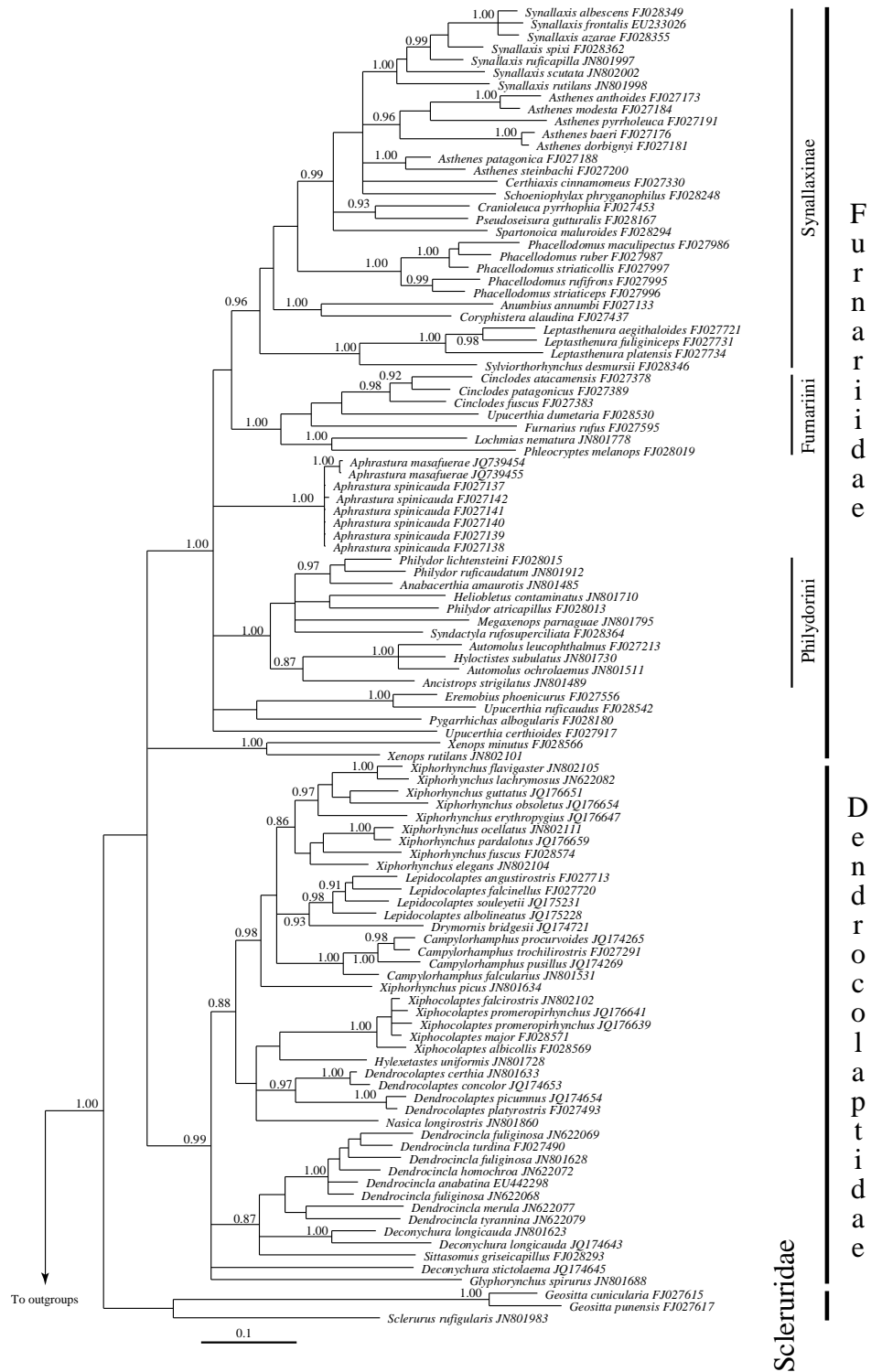
Appendix 2. Detailed Bayesian inference (BI) tree based on 694 base pairs of the cytochrome oxidase subunit 1 gene (COI). BI posterior probabilities (≥0.90) are indicated above the branches. The model selected for the BI analysis consisted of GTR + gamma distribution shape parameter (G) = 1.42 + proportion of invariable sites (I) = 0.60. GenBank accession numbers indicated for each species.
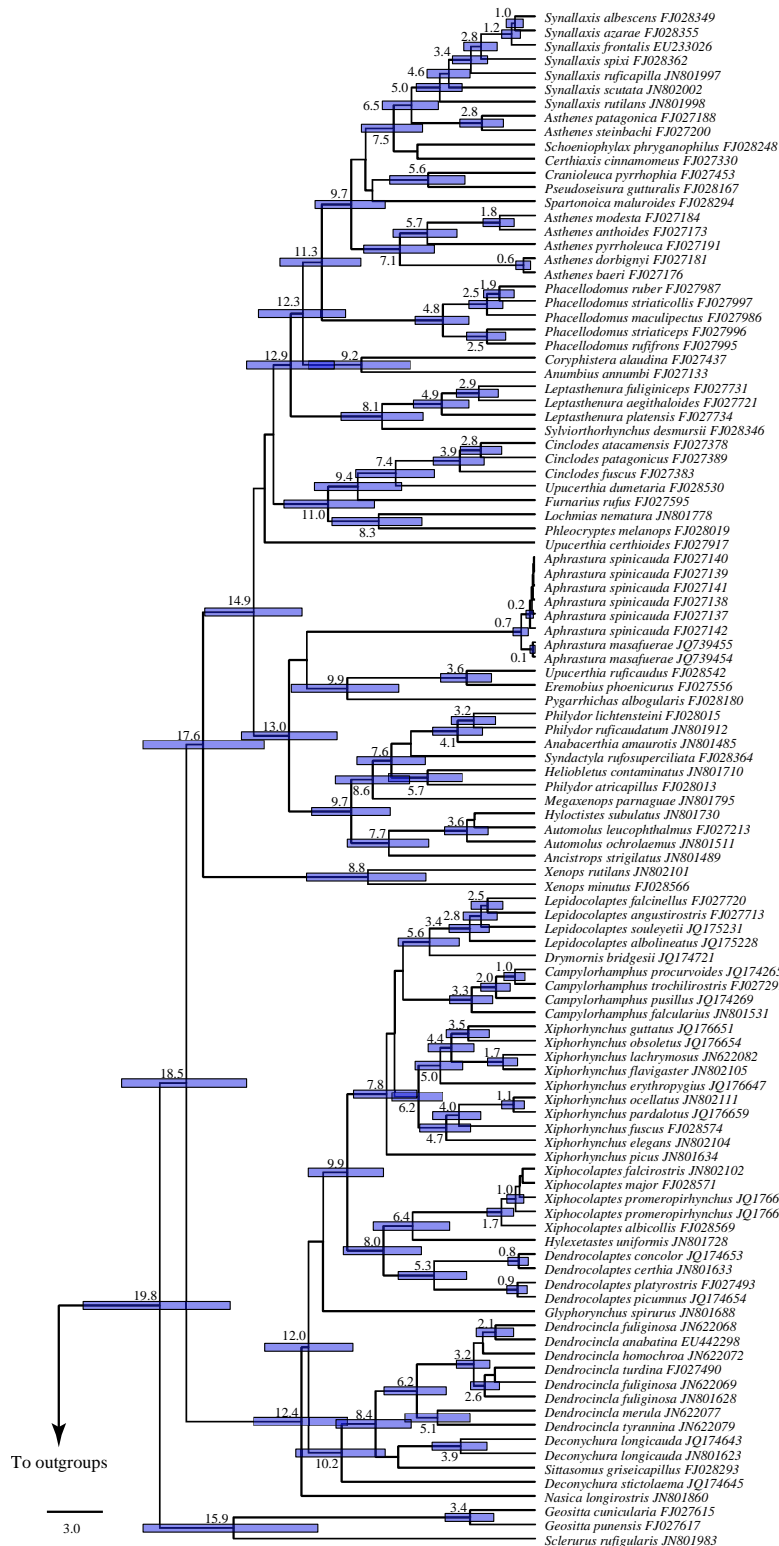
Appendix 3. Bayesian inference chronogram (GTR + G + I) inferred from COI in 114 species of the Furnarioidea. Bayesian age estimates are shown for each node. Bars indicate 95% highest posterior density (HPD) intervals. GenBank accession numbers indicated for each species.