



ACADEMIC
PRESS

Molecular Phylogenetics and Evolution 23 (2002) 408–421

MOLECULAR
PHYLOGENETICS
AND
EVOLUTION

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A phylogeny of the megapodes (Aves: Megapodiidae) based on nuclear and mitochondrial DNA sequences

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Received 02 July 2001; received in revised form 17 October 2001

Abstract

DNA sequences from the first intron of the nuclear gene rhodopsin (RDP1) and from the mitochondrial gene ND2 were used to construct a phylogeny of the avian family Megapodiidae. RDP1 sequences evolved about six times more slowly than ND2 and showed less homoplasy, substitution bias, and rate heterogeneity across sites. Analysis of RDP1 produced a phylogeny that was well resolved at the genus level, but RDP1 did not evolve rapidly enough for intrageneric comparisons. The ND2 phylogeny resolved intrageneric relationships and was congruent with the RDP1 phylogeny except for a single node: this node was the only aspect of tree topology sensitive to weighting in parsimony analyses. Despite differences in sequence evolution, RDP1 and ND2 contained congruent phylogenetic signal and were combined to produce a phylogeny that reflects the resolving power of both genes. This phylogeny shows an early split within the megapodes, leading to two major clades: (1) *Macrocephalon* and the mound-building genera *Talegalla*, *Leipoa*, *Aepyodius*, and *Alectura*, and (2) *Eulipoa* and *Megapodius*. It differs significantly from previous hypotheses based on morphology but is consistent with affiliations suggested by a recent study of parasitic chewing lice. © 2002 Elsevier Science (USA). All rights reserved.

1. Introduction

The megapodes are a fascinating group of ground-dwelling birds that use environmental heat sources rather than body heat to incubate their eggs (Frith, 1956; Jones and Birks, 1992; Jones et al., 1995). These heat sources include geothermal heat, solar radiation, and heat from microbial decomposition—the latter usually harnessed through mound building. Megapodes inhabit much of the South Pacific east of Wallace's Line, including Melanesia, Micronesia, Australia, and New Guinea (Jones et al., 1995). Their evolutionary path toward alternate incubation strategies has produced a wide array of ecological and behavioral adaptations unique to megapodes and which vary among species and genera in the family.

Because of these shared adaptations, the monophyly of the family Megapodiidae has never been seriously

questioned. The most recent taxonomic classification, which we use here, includes seven genera and 22 species, 13 of which belong to the genus *Megapodius* (Jones et al., 1995; Roselaar, 1994). One of the major challenges for taxonomists has been identifying species boundaries within this genus: from four to 19 species of *Megapodius* have been recognized by various authors in the past 50 years (Jones et al., 1995). *Megapodius* species nest in burrows or mounds; they are atypical in that they are capable of flying long distances (most other megapodes fly only short distances when escaping predators), they occur on many small islands, and they have an extended range from the Nicobar Islands in the northwest to Tonga in the southeast (Jones et al., 1995). Additionally, populations of different *Megapodius* species may hybridize where they overlap (Jones et al., 1995; Roselaar, 1994).

Other taxonomic challenges in megapodes include the phylogenetic affiliation of the Maleo, *Macrocephalon maleo*, from the island of Sulawesi. The Maleo nests in burrows in the sand, has black, white, and salmon-colored plumage and an unusual black head casque, and

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is the most morphologically distinct of the megapodes. Although it looks superficially more like *Alectura*, *Aepyodius*, *Leipoa*, and *Talegalla*—the genera we will refer to simplistically here as the “mound builders” because all species build mounds—than *Megapodius* or *Eulipoa*, it has been traditionally grouped with the latter two genera (Clark, 1964; Jones et al., 1995; but see Mey, 1999). Another area of contention centers around *Eulipoa*—a nocturnal, burrow-nesting megapode that resembles *Megapodius* species but has morphological differences such as striped plumage. Some have placed it within *Megapodius*, but others consider it different enough from other *Megapodius* species to give it full generic status (Jones et al., 1995; Roselaar, 1994).

Despite a long history of interest in megapodes, to date there is no well-resolved phylogeny with which to study the megapodes' unique adaptations within an historical context. Here, we present the first well-resolved phylogeny of megapodes that includes all seven genera and most (15 of 22) species.

1.1. Molecular markers

We used complete sequence from the first intron of the nuclear gene rhodopsin (hereafter RDP1) and from the mitochondrial gene NADH dehydrogenase subunit 2 (ND2). One goal of this study was to explore the potential of RDP1 as a marker in avian molecular phylogenetics by comparing patterns of molecular evolution of RDP1 and ND2. Rhodopsin is a visual pigment embedded in the outer portion of the rod cells in animal eyes (Goldsmith, 1990). The gene exists as a single copy whose structure usually includes five exons and four introns (Takao et al., 1988). Rhodopsin's conserved structure in most vertebrates (Okano et al., 1992) may make it a potentially useful gene for other studies.

As far as we are aware, ours is the first use of rhodopsin intron sequence in an avian phylogenetic study. We chose an intron from rhodopsin because of its likely utility for many avian taxa, its convenient size (~1 kb), and the ease with which it was amplified for a variety of galliform taxa. ND2 was chosen to complement RDP1. ND2 has been used in several recent phylogenetic studies of birds (Hackett, 1996; Johnson et al., 2000; Johnson and Sorenson, 1998; Omland et al., 1999) and apparently often evolves at a faster rate than cytochrome *b* (Meyer, 1994; Omland et al., 1999), which was desirable for comparisons among closely related *Megapodius* species (see below).

Although mtDNA generally has a smaller effective population size than nuclear DNA and is thus theoretically more likely to have a gene phylogeny concordant with organismal phylogeny (Moore, 1995), empirical studies suggest that single mtDNA gene sequences may provide idiosyncratic trees or may not always be supe-

rior to nuclear DNA in all cases (Allard and Carpenter, 1996; Baker et al., 2001; Springer et al., 2001). Furthermore, using a combination of nuclear and mitochondrial sequences has several advantages, providing genetically independent estimates of organismal phylogeny (Avise, 1989; Hudson, 1992; Moore, 1995; Pamilo and Nei, 1988; Slowinski, 1999; Wu, 1991), opportunity for tests of congruence (e.g., Pritchko and Moore, 1997; Johnson and Clayton, 2000), and complementary resolving powers due to differences in rate and substitution dynamics (Holmquist et al., 1983; Johnson and Clayton, 2000; Johnson and Sorenson, 1998). Here, we use maximum-likelihood analyses of RDP1 and ND2 to construct our primary hypotheses for megapode phylogeny, but we also take advantage of having two independent estimates of phylogeny to further explore differences in evolutionary dynamics between nuclear and mitochondrial sequences as well as the effects of weighting in parsimony analyses—a topic of ongoing debate (Allard and Carpenter, 1996; Allard et al., 1999; Barker and Lanyon, 2000; Björklund, 1999; Broughton et al., 2000; Voelker and Edwards, 1998; Wenzel and Siddall, 1999).

2. Methods

2.1. Taxon sampling

Because many megapodes occur in remote locations, complete taxonomic sampling is difficult. We obtained tissue or genomic DNA for 24 individuals representing 15 of the 22 species and all seven genera; other species were unavailable. However, *Aepyodius bruijnii*, presumed for decades to be possibly extinct, was very recently rediscovered (Heij and Post, 2001) and may be included in a future analysis. Whenever possible, two individuals were included for each species. For outgroup taxa we chose 4–5 galliform species, because previous phylogenetic studies have indicated that the megapodes' sister group is probably “all other galliforms” (Jones et al., 1995). The outgroup taxa included varied slightly between the ND2 and RDP1 analyses due to difficulties in amplifying DNAs from some taxa. Genetic samples and collection and sequencing information are listed in Table 1.

2.2. Amplification and sequencing

For most tissue samples, whole genomic DNA was extracted using standard phenol/chloroform techniques, followed by membrane dialysis. For a few samples, Qiagen DNeasy tissue kits or cesium chloride gradients (e.g., Edwards and Wilson, 1990) were used instead. After extraction, DNA was PCR amplified in 50- μ l reactions in a Perkin-Elmer Thermal Cycler 9600 with the

Table 1
Species, source, and sequence information for tissues or DNA

Genus species	Common name	Source	Voucher ID and/or museum ^a	RDP1 ^b	ND2
Megapodiidae					
<i>Aepyodius arfakianus</i>	Wattled brush-turkey	Papua New Guinea, Tetebedi	MV E147	X (814)	X
<i>Alectura lathamii</i>	Australian brush-turkey	Australia, Queensland, Brisbane	UWBM 55908	X (814)	X
<i>Alectura lathamii</i>		Australia, Queensland, Brisbane	UWBM 55907	X (814)	X
<i>Eulipoa wallacei</i> 1	Moluccan megapode	Indonesia, Moluccas, Haruku	RM 9997-00262		X
<i>Eulipoa wallacei</i> 2		Indonesia, Moluccas, Haruku	RM 9997-00261	X (816)	X
<i>Eulipoa wallacei</i> 3		Indonesia, Moluccas, Halmahera		X (816)	
<i>Leipoa ocellata</i> 1	Malleefowl	Australia, New South Wales	UWBM 62959	X (807)	X
<i>Leipoa ocellata</i> 2		Australia, New South Wales	UWBM 62984	X (807)	X
<i>Talegalla fuscirostris</i>	Black-billed talegalla	Papua New Guinea, Veimauri River	MV E651	X (814)	X
<i>Macrocephalon maleo</i>	Maleo	USA, Bronx Zoo captive	AMNH PRS 1204	X (807)	X
<i>Megapodius cumingii</i>	Philippine megapode	Sulawesi, Tangkoko-Dua Saudara Nature Reserve			X
<i>Megapodius decollatus</i>	New Guinea megapode	Papua New Guinea, Crater Mountain Biological Station			X
<i>Megapodius eremita</i> 1	Melanesian megapode	Solomon Islands, Isabel Island, Garanga River	AMNH MKL 67	X (818)	X
<i>Megapodius eremita</i> 2		Papua New Guinea, Ambitle Island	MV KNG16	X (818)	X
<i>Megapodius forstenii</i> 1	Forsten's megapode	Indonesia, Moluccas, South Ceram	RM 98-178		X
<i>Megapodius forstenii</i> 2		Indonesia, Moluccas, South Ceram	RM		X
<i>Megapodius freycinet quoyii</i>	Dusky megapode	Indonesia, Moluccas, Halmahera		X (818)	X
<i>Megapodius freycinet freycinet</i> 1		Indonesia, West Papuan Islands, Waigeu	RM 9997-00538		X
<i>Megapodius freycinet freycinet</i> 2		Indonesia, West Papuan Islands, Waigeu	RM 9997-00538		X
<i>Megapodius layardi</i> 1	Vanuatu megapode	Vanuatu, Ambrym Island	UWBM	X (818)	X
<i>Megapodius layardi</i> 2		Vanuatu, Ambrym Island	UWBM	X (818)	X
<i>Megapodius pritchardii</i>	Polynesian megapode	Tonga, Niuafo'ou, Motu MoleMole		X (818)	X
<i>Megapodius reinwardt</i>	Orange-footed megapode	Australia, Queensland, Mission Beach	MV C561	X (818)	X
<i>Megapodius tenimberensis</i>	Tanimbar megapode	Indonesia, Tanimbar Island	LM	X (818)	X
Outgroups					
<i>Alectoris chukar</i>	Chukar partridge	J. Kornegay ^c	GenBank L08378	X (906)	
<i>Bonasa bonasia</i>	Hazel grouse	Russia, Krasnoyarskiy Kray	UWBM 51758	X (863)	
<i>Dendragapus obscurus</i>	Blue grouse	USA, Washington	UWBM 53437	X (857)	X
<i>Pavo cristatus</i>	Common peafowl	J. Kornegay ^c	GenBank L08379	X (901)	X
<i>Numida meleagris</i>	Helmeted guineafowl	J. Kornegay ^c	GenBank L08383	X (892)	X
<i>Ortalis vetula</i>	Plain chachalaca	J. Kornegay ^c	GenBank L08384		X

^a AMNH, American Museum of Natural History; LM, Leiden Museum; MV, Museum of Victoria; RM, Rotterdam Museum; UWBM, University of Washington Burke Museum.

^b All taxa sequenced for each gene are marked with "X" (see Methods); for RDP1, intron length varied and follows in parentheses.

^c Several outgroup DNAs were from Kornegay et al. (1993); GenBank accession numbers refer to cytochrome *b* sequences for these taxa.

following reaction conditions: 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C (for RDP1) or 50 °C (for ND2), 30 s at 72 °C; followed by a final extension of 7 min at 72 °C. Because megapodes are relatively closely related to chickens (*Gallus*), primers for PCR amplification were designed based solely on published sequence from the chicken genome (Takao et al., 1988) using the computer program Primer (version 3.0,

Whitehead Institute: www.genome.wi.mit.edu/cgi-bin/primer/). For RDP1, primers were located in the exons flanking intron 1 (Fig. 1). Primers were placed so that some flanking exon sequence (about 40 bp) would be amplified with the intron, which helped to confirm the identity of the less conserved intron sequence (Prychitko and Moore, 1997). Two primers, RDP1.U and RDP1.L, amplified RDP1 for all megapodes and galliform out-

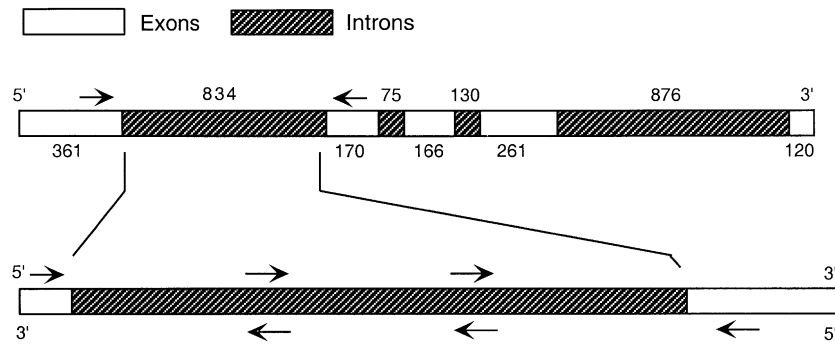


Fig. 1. Schematic diagram of the rhodopsin gene showing both coding and non coding regions in domestic chicken (*Gallus*; Takao et al., 1988), with intron 1 enlarged (below). Numbers below exons and above introns indicate length in bp for chicken. The two arrows shown above the entire gene correspond roughly to the location of the two amplification primers, RDPU1 and RDPL1, situated in the flanking exons 1 and 2, respectively. The 5' and 3' ends of the upper (sense) and lower (antisense) strands of the intron are labeled. The arrows in the lower part of the diagram correspond to positions of sequencing primers; the sequences derived from these were 350–450 bp in length, so that they overlapped significantly.

groups. These primers correspond to positions 1010–1029 in exon 1 and 1926–1945 in exon 2 in *Gallus* and have the following sequence:

RDPI.U1 (5'-GTAACAGGGTGCTACATCGA-3')

RDPI.L1 (5'-ACAGACCACCACATATCGTT-3')

Primers for amplifying ND2 were designed in the same way, based on chicken sequence (Desjardins and Morais, 1990). Two primers amplified ND2 for all taxa. These primers correspond to chicken genome positions 5119–5145 in the flanking tRNA^{Gln} and 6394–6416 in tRNA^{Ala}, as follows:

L5145: (5'-GAACCTACACAGAAGAGATCAAAA CTC-3')

H6394 (5'-ATTAAGCGTCTGATTTGCATTC-3')

PCR products were prepared for sequencing by centrifuging with Ultrafree-MC filters (Millipore) and sequenced in 10- μ l Dye Terminator Cycle Sequencing reactions (Applied Biosystems, Foster City, CA) or with ABI Prism BigDye Terminators, according to manufacturer's protocols. Both strands of DNA were sequenced for all taxa on either an ABI Model 373 automated sequencer (most taxa) or ABI model 377. Sequences were aligned by eye using Genetic Data Environment (developed and maintained by S. Smith, with compilation of programs by various authors; available free from ftp.bio.indiana.edu. in molbio/unix/GDE) or with Sequencher (Gene Codes Corp., Ann Arbor, MI). Various aspects of sequence evolution (e.g., number of steps at each nucleotide site) were explored using MacClade (Maddison and Maddison, 1992).

RDP1 amplified as a single fragment in all taxa, and an initial screening for the presence of heterozygous sites was done by cloning PCR amplifications using TA cloning kits (Invitrogen). The first 400 bp of RDP1 was sequenced for six clones each of four genera (*Alectura*, *Aepyodius*, *Talegalla*, and *Megapodius*). No evidence of heterozygosity was found, and all subsequent sequencing was direct. Because RDP1 amplification primers were not effective for sequencing most taxa, we designed

two sequencing primers, RDPI.U2 (5'-GGGTGCTAC ATCGAGGGCT-3') and RDPI.L2 (5'-CTGCAGTT GCTGGATTTGCAC-3') slightly (~10 bp) interior to each amplification primer and based on the initial cloned megapode sequence. Additional sequencing primers were designed as appropriate, some for specific taxa. Early on it was clear that RDP1 did not evolve quickly enough to provide useful phylogenetic signal within the genus *Megapodius* (six species; maximum *P* distance = 0.01), so all subsequent *Megapodius* samples were sequenced for ND2 only (Table 1).

2.3. Pairwise comparisons

We estimated relative substitution rates of the two genes by plotting uncorrected *P* distances of ND2 vs RDP1, and examined substitution dynamics graphically for ND2 and RDP1 by plotting transitions vs transversions, by codon position for ND2 (e.g., Edwards, 1997).

2.4. Maximum-likelihood analyses

All phylogenetic analyses were conducted using PAUP* (Swofford, 1998). Parsimony and likelihood trees were generated separately for RDP1 and ND2. Gaps (indels) were treated as missing data in analyses of RDP1. Because distantly related outgroups can cause problems with tree reconstructions (Halanych et al., 1999; Smith, 1994), we did a separate maximum-likelihood analysis with all outgroups removed to see if in-group topology remained stable; these trees were rooted at the midpoint for visual comparison. We conducted a partition homogeneity test on the ND2 and RDP1 sequences to test for congruence in phylogenetic signal (Cunningham, 1997; Farris et al., 1994). We chose maximum-likelihood as our preferred tree-building method because of its ability to incorporate explicit models of molecular evolution, including estimates of

important parameters taken directly from the data under consideration, and because of its robustness to differences in base composition and models of DNA substitution (Huelsenbeck, 1995). We used the likelihood ratio test (LRT) to determine the best model, with an initial tree based on neighbor-joining and Kimura two-parameter distances (Huelsenbeck and Crandall, 1997; Huelsenbeck and Rannala, 1997). Several likelihood models were evaluated using the program MODELTEST 3.0 (Posada and Crandall, 1998) including the F81 (Felsenstein, 1981), HKY85 (Hasegawa et al., 1985), and general time-reversible (Rodríguez et al., 1990); each model was tested under conditions allowing for invariant sites (I), Γ -distributed rate heterogeneity, or a combination of both (Sullivan et al., 1999). Comparison of likelihood scores indicated that the general time reversible model allowing for six substitution types, invariant sites, and rate heterogeneity (the most parameter-rich model currently available) offered a significant improvement in fit to the data over less complex models for both RDP1 and ND2. Thus, the GTR + I + Γ model was used for all likelihood analyses. Because rate heterogeneity parameters (the substitution rate-matrix, Γ shape parameter (α), and proportion of invariant sites (I) are sensitive to taxon sampling (Sullivan et al., 1999; Saunders and Edwards, 2000), they were estimated separately for each gene and set of taxa (including an analysis combining both ND2 and RDP1), with four rate categories for the Γ shape parameter α (Yang, 1994). To reduce computation time, single representatives of each species or population were used in the ND2 analysis and in the combined gene analysis, which included more *Megapodius* species. Within megapodes, we used the LRT to determine whether RDP1 and ND2 were evolving in a clock-like manner, holding all other GTR model parameters constant (Felsenstein, 1981; Huelsenbeck and Crandall, 1997; Huelsenbeck and Rannala, 1997). Evaluating clock-like sequence evolution is important for analyses comparing relative divergence dates among taxa (Arbogast and Slowinski, 1998).

2.5. Parsimony analyses

For comparison to our maximum-likelihood trees, we generated trees using heuristic parsimony searches. We chose to use a series of three weighting schemes based on estimates of transition/transversion bias present in the each gene sequence. We used maximum-likelihood trees to estimate the transition/transversion ratio (Ti/Tv) and kappa (κ). We weighted all positions in both genes equally (1:1), by the Ti/Tv estimated for that gene, and by κ . Flanking regions of the genes were not weighted. Support for inferred trees under these weighting schemes was evaluated using 1000 bootstrap replicates (Felsenstein, 1985) and by comparing the consistency index

(CI), retention index (RI), and the rescaled consistency index (RC) (Farris, 1989; Maddison and Maddison, 1992). We compared these values and the number of nodes with strong bootstrap support for trees derived from each gene and weighting scheme. We performed the same set of analyses for the combined dataset, with appropriate κ and Ti/Tv values used for each gene region.

3. Results

3.1. Characterization of RDP1 and ND2

Primers for RDP1 amplified a single fragment in all taxa. There was no amplification of related fragments such as processed pseudogenes (Slade et al., 1994). This fragment included 7 bp of flanking sequence from exon 1 and 42 bp from exon 2. For simplicity, the entire fragment is referred to as RDP1 here. RDP1 varied in length from 807 bp in *Macrocephalon* to 906 bp in *Alectoris* (Table 1) due to the presence of indels inferred from aligned sequences; the aligned fragment totaled 972 bp. Within megapodes, indels were small (1–7 bp) and few (six total) so that fragment length among the ingroup taxa varied by only 11 bp. In comparisons among outgroups and especially among megapodes and other galliforms, indels were larger and more common and were concentrated toward the middle of the intron (Fig. 2). For example, there were four large indels totaling 79 bp in the region from 500 to 700 bp, but only 1–2 indels of ≥ 10 bp before or after this region. Most indels (63%) were confined to single species, and, perhaps due partly to its genetic distance, a large proportion of indels (40%) were present in only a single outgroup species, (*Dendragapus*). Of the 18 indels shared by two or more species, all but one contained phylogenetic signal that was congruent with the trees inferred from sequence data (below). Thirteen of the 18 supported monophyly of the megapodes; two supported other ingroup clades, and two supported outgroup clades. Except for a few small conserved regions around the exon boundaries and in the first third of the intron, nucleotide substitutions were spread fairly evenly across the intron (Fig. 2).

ND2 also amplified as a single fragment for all taxa, but with no indels. The aligned fragment length was 1072 bp, including the entire ND2 gene (1041 bp) and 31 bp from flanking tRNA sequences (20 bp tRNA^{Met} and 11 bp tRNA^{Trp}); analyses other than tree building were based only on that portion of the fragment from the ND2 gene.

Mean relative base-pair frequencies for RDP1 were not strongly skewed (18.7% (A), 25.5% (T), 25.9% (C), and 30.0% (G)), nor A-T rich, as reported for the β -Fibrinogen 7 intron (Prychitko and Moore, 1997). A

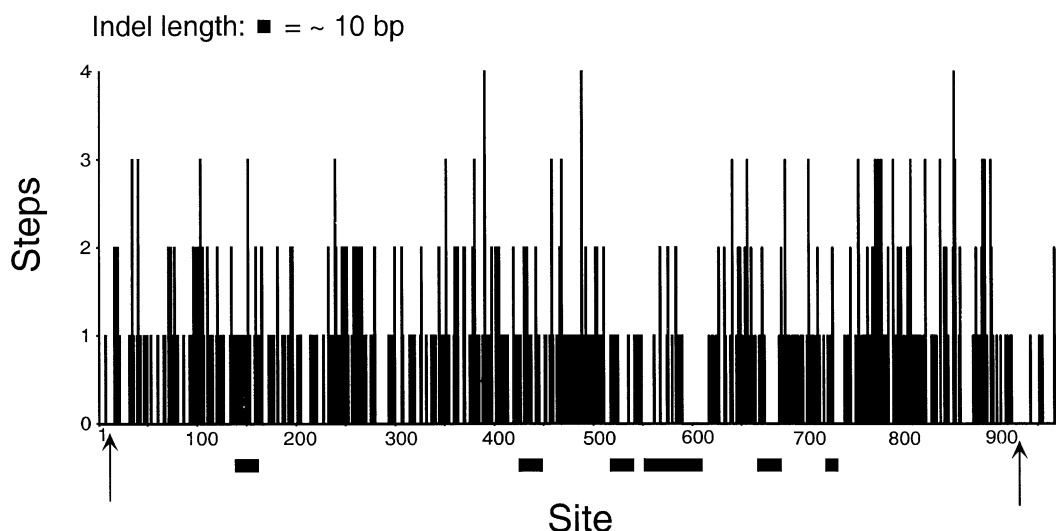


Fig. 2. Number of steps for each base position site in RDP1, calculated over the RDP1 maximum-likelihood tree (see below). Arrows indicate the borders between the intron and its flanking exon regions. There are several relatively conserved regions, including the areas around the exon boundaries and bp 203–230, 270–290, and 870–880. Most of the regions from bp 500 to 750 that appear to be conserved are partially an artifact produced by the presence of indels, which decrease the number of taxa being compared for those sequence areas. The position of all seven indels of ≥ 10 bp are indicated below the axis (the largest bar represents two indels).

nonphylogenetic χ^2 test for heterogeneity in base composition across taxa could not reject homogeneity ($\chi^2 = 18.0$, $df = 63$, $P = 1.0$). Similar results were obtained for base composition in ND2 ($\chi^2 = 34.9$, $df = 60$, $P = 1.0$). However, consistent with previous studies on animal mtDNA, base composition of ND2 was strongly skewed, with overall frequencies of 29.8% (A), 23.7% (T), 36.2% (C), and 10.2% (G).

For RDP1, 385 of 972 bp (39.6%) were variable and 270 (27.8%) were parsimony informative. ND2 had a larger percentage of variable sites (507 of 1072, or 47.3%); 406 (37.9%) sites were parsimony informative. RDP1 sequence divergence (uncorrected P) within the megapodes ranged from 0% among some *Megapodius* species to 6.9% for some intergeneric comparisons; for ND2, divergence for these comparisons ranged from 0.7 to 18.8%. RDP1 sequence divergence between ingroup and outgroup species was 24.1–27.0%, and for ND2 it was 18.8–25.2%. Overall, the number of reconstructed changes per site for those that had at least one substitution was 2.1 for ND2 vs 1.4 for RDP1.

Comparison of pairwise divergences for closely related taxa using a simple regression suggested that ND2 evolves about 6.4 times faster than RDP1 (Fig. 3). However, this slope decreased in more distant comparisons, suggesting that ND2 is more subject to homoplasy than RDP1, and that for both genes, transitions are more subject to homoplasy than transversions (Fig. 4).

As expected for mitochondrial vs nuclear DNA sequences, estimates of κ were higher (19.4 vs 4.3) and estimates of α lower (0.286 vs 1.19) for ND2 than for RDP1. Despite these differences, a partition homogeneity test (Farris et al., 1994, 1995) indicated that RDP1

and ND2 had congruent phylogenetic signal ($P = 0.84$) and were thus good candidates for combined phylogenetic analysis.

3.2. Phylogenetic analyses of RDP1

Both maximum-likelihood and parsimony bootstrap consensus analyses of RDP1 sequences produced trees that were remarkably well resolved and had identical topologies at the genus level (Fig. 5). However, the phylogenetic signal provided by RDP1 for comparisons among *Megapodius* species was weak. Although the maximum-likelihood tree provided some structure (not shown), none of these branches achieved greater than 50% bootstrap support, and are presented as unresolved (Fig. 5). Tree topology was not affected by the parsimony weighting scheme employed. However, the tree with the best support was obtained by using the heaviest of the three weighting schemes, which downweighted transitions by the estimated transition bias ($\kappa = 4.3$; Table 2). This weighting scheme produced bootstrap values of $\geq 75\%$ for six of seven higher nodes and the highest RC value (0.83) and combined bootstrap sum of any of the RDP1 analyses (Table 2).

3.3. ND2 phylogenies

Maximum-likelihood and parsimony also produced identical trees for ND2 (Fig. 5) except for a single *Megapodius* node (Fig. 6) that was collapsed in the majority-rule consensus tree. The ND2 sequences provided much more resolution than RDP1 at the intra-generic level, with 8–10 of 14 nodes supported at $\geq 75\%$

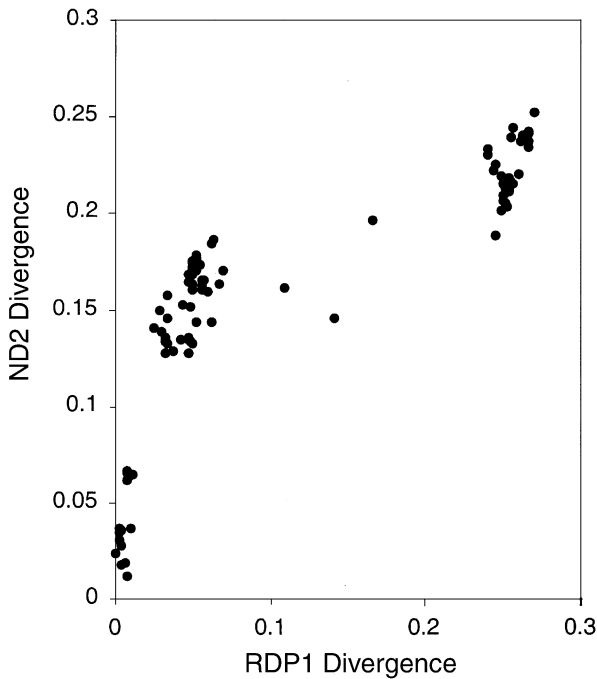


Fig. 3. Comparison of pairwise sequence divergences (uncorrected P) between RDP1 and ND2. Data points fell in distinct clusters based on taxonomic level of comparison: those in the bottom left corner of the graph include all comparisons within the genus *Megapodius* and a single intergeneric comparison (*Alectura* + *Aepyodius*); the group of points directly above includes all intergeneric comparisons within megapodes; the upper right-hand group includes all comparisons between outgroups and megapode species; the three points intermediate are comparisons among the three outgroup species (*Numida*, *Pavo*, and *Dendragapus*) common to both analyses. Using the function LINEST in Microsoft Excel and only those points with <10% divergence for ND2 (the taxonomic level of comparisons with the least evidence of homoplasy) we plotted a regression line that went through the origin (not shown) and calculated its slope as an estimate of the relative rates of substitution for these two genes.

bootstrap values (Table 2). In contrast to RDP1 analyses, weighting affected tree topology in addition to bootstrap support and RC values. A single node was affected: the “unweighted” (1:1) consensus tree topology was identical to RDP1, with *Leipoa* grouped more closely than *Talegalla* to the brush-turkey clade (*Alectura* + *Aepyodius*), whereas in analyses weighted by Ti/Tv or κ , the placement of the *Leipoa* and *Talegalla* branches was reversed (Table 2). The maximum-likelihood tree was identical to that produced by these two weighted parsimony schemes for generic-level comparisons. In addition, as in the RDP1 analysis, the weighted ND2 trees tended to have higher RC indices and overall higher bootstrap values. Consistent with several recent studies (Allard et al., 1999; Baker et al., 2001; Broughton et al., 2000; Källersjö et al., 1999), downweighting only third positions was clearly not a good choice for these data; it resulted in lower RC values and/or bootstrap support than the other analyses (Table 2).

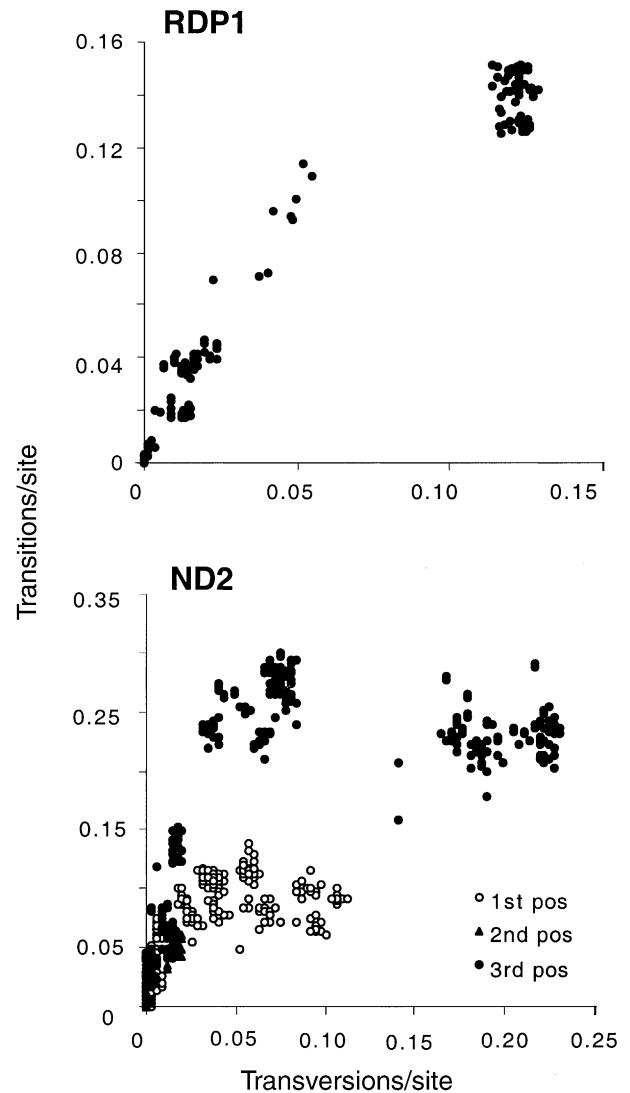


Fig. 4. Comparison of base substitution dynamics for RDP1 and ND2. Plots are observed numbers of transitions vs transversions per site for all pairwise comparisons of taxa for RDP1 and ND2, respectively. For ND2 data, differences are partitioned according to codon position.

Maximum-likelihood analyses excluding outgroups produced tree topologies identical to those including outgroups (Fig. 6). Midpoint rooting of these trees placed the root in the same position as for analyses using outgroups. Consistent with this result, RDP1 evolved in a clock-like manner for megapode taxa; differences of log-likelihood scores for unconstrained analyses and analyses with “molecular clock” enforced were not significant ($\text{Ln}_{\text{clock}} = 1858.5$, $\text{Ln}_{\text{noclock}} = 1847.9$, $\text{df} = 15$, $P = 0.13$). However, ND2 did not evolve in a clock-like manner ($\text{Ln}_{\text{clock}} = 4562.0$, $\text{Ln}_{\text{noclock}} = 4540.5$, $\text{df} = 21$, $P = 0.003$), which may limit its utility for predicting divergence times among some megapodes (Arbogast and Slowinski, 1998; Huelsenbeck and Rannala, 1997).

When information from the two genes was combined, the resulting tree reflected the resolving power of both

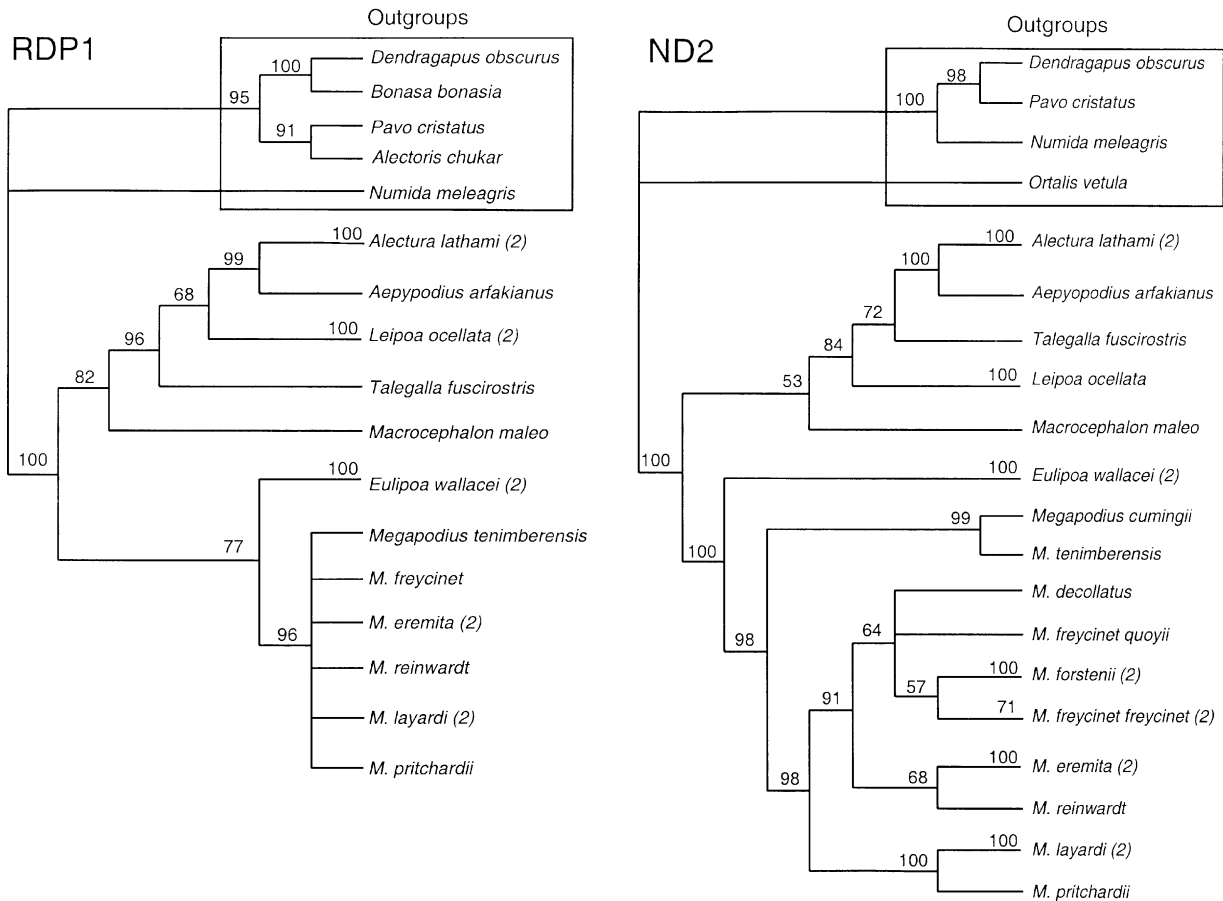


Fig. 5. Phylogenetic trees (cladograms) based on maximum-likelihood and parsimony bootstrap consensus analyses of RDP1 and ND2 datasets. Numbers above branches near nodes indicate bootstrap values (1000 rep) from the parsimony analysis. Numbers near branch ends indicate bootstrap support for taxa for which sequence from two individuals was available (noted in parentheses).

genes (Fig. 7). The combined maximum-likelihood tree was identical to the ND2 maximum-likelihood tree except for the reversal of the *Leipoa/Talegalla* branching order found by all RDP1 analyses and the unweighted ND2 parsimony analysis. In parsimony bootstrap consensus analyses, the combined tree produced under the Ti/Tv weighting was more strongly supported than any ND2 tree (Table 2). However, as with the ND2 parsimony consensus trees, the branching order of *Leipoa/Talegalla* was reversed in different weighting schemes.

4. Discussion

4.1. Phylogenetic utility of RDP1

Inclusion of nuclear sequence data remains uncommon in avian phylogenetic studies, despite the many advantages of complementing mtDNA with nuclear data. Although several recent authors have provided information on the phylogenetic utility of some nuclear

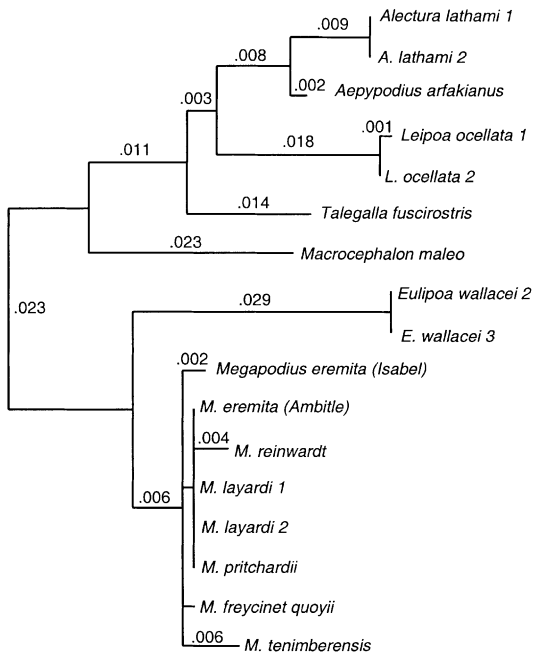
exons (e.g., Groth and Barrowclough, 1999; Hughes and Baker, 1999; Lovette and Bermingham, 2000), these sequences probably evolve too slowly for many comparisons at the species or generic level. Nuclear introns have the potential to fill this niche: they evolve relatively rapidly, are common and often of convenient size for PCR analysis, and can be readily amplified with primers designed in conserved flanking exon regions (Prychitko and Moore, 1997; Slade et al., 1994). Thus far only a single intron, β -fibrinogen 7, has been described with regard to phylogenetic utility for birds at the generic level (Johnson and Clayton, 2000; Prychitko and Moore, 1997; but see Congdon et al., 2000, for intraspecific studies). Although the RDP1 amplification primers used here were not specifically designed to be universal, they may prove useful for other avian taxa, including passerines (Andersson, personal communication). The conserved nature of the rhodopsin gene in vertebrates should provide information for designing additional primers specifically targeted at a larger range of both avian and nonavian taxa (Okano et al., 1992).

Table 2
 Bootstrap support for parsimony weighting schemes and datasets

Node	RDPI			ND2			ND2 3rd pos		(RDP and ND2)			Min	Max	Node description
	l	Ti/Tv	κ	l	Ti/Tv	κ	Ti/Tv	κ	l	Ti/Tv	κ			
A	96	99	99	100	100	100	100	100	100	100	100	96	100	<i>Aepyrodus</i> , <i>Alectura</i>
B	61 (L)	65 (L)	68 (L)	47 (L)	66 (T)	72 (T)	64 (T)	60 (T)	61 (L)	67 (T)	72 (T)	47	72	<i>Aepyrodus</i> , <i>Alectura</i> , (<i>Leipoa</i> or <i>Talegalla</i>)
C	92	96	96	85	87	84	64	51	98	90	86	84	98	<i>Aepyrodus</i> , <i>Alectura</i> , <i>Leipoa</i> , <i>Talegalla</i>
D	51	71	82	64	52	53	39	32	73	58	56	32	82	<i>Aepyrodus</i> , <i>Alectura</i> , <i>Leipoa</i> , <i>Talegalla</i> , <i>Macrocephalon</i>
E	100	100	100	100	100	100	100	100	100	100	100	100	100	All megapodes
F	92	83	77	100	100	100	100	100	100	100	100	77	100	<i>Eulipoa</i> , (all <i>Megapodius</i> species)
G	96	96	96	100	100	98	100	100	100	100	100	96	100	All <i>Megapodius</i> species
H	—	—	—	81	99	99	99	99	76	100	99	76	100	<i>Megapodius cumingii</i> , <i>M. tenimberensis</i>
I	—	—	—	100	100	98	100	100	100	100	100	98	100	All other <i>Megapodius</i> species
J	—	—	—	90	94	91	93	94	86	93	94	86	94	<i>Megapodius decollatus</i> , <i>M. freycinet</i> , <i>M. forstenii</i> , <i>M. eremita</i> , <i>M. reinwardt</i>
K	—	—	—	58	73	64	72	71	61	81	73	58	81	<i>Megapodius decollatus</i> , <i>M. freycinet</i> , <i>M. forstenii</i>
L	—	—	—	30	67	57	65	64	37	76	73	37	76	<i>Megapodius freycinet freycinet</i> , <i>M. forstenii</i>
M	—	—	—	82	71	68	74	74	78	67	71	67	82	<i>Megapodius eremita</i> , <i>M. reinwardt</i>
N	—	—	—	93	97	100	97	97	93	99	94	93	100	<i>Megapodius layardi</i> , <i>M. pritchardii</i>
BS sums	588	610	618	1130	1206	1184	1167	1142	1163	1231	1218	1047	1285	
Nodes with BS \geq 75%	5	5	6	10	9	9	8	8	10	11	9			
Tree CI	0.81	0.83	0.89	0.57	0.68	0.70	0.65	0.67	0.66	0.71	0.72			
Tree RI	0.91	0.93	0.94	0.72	0.79	0.81	0.77	0.78	0.79	0.82	0.83			
Tree RC	0.74	0.77	0.83	0.41	0.54	0.57	0.50	0.52	0.52	0.58	0.60			

Note. All numbers based on 1000 rep parsimony bootstrap consensus analyses. Min, Max = minimum and maximum bootstrap support for all analyses; CI, RI, RC = consistency index, retention index, and rescaled consistency index (CI \times RI), respectively.

RDP1 — .005 substitutions per site



ND2 — 0.01 substitutions per site

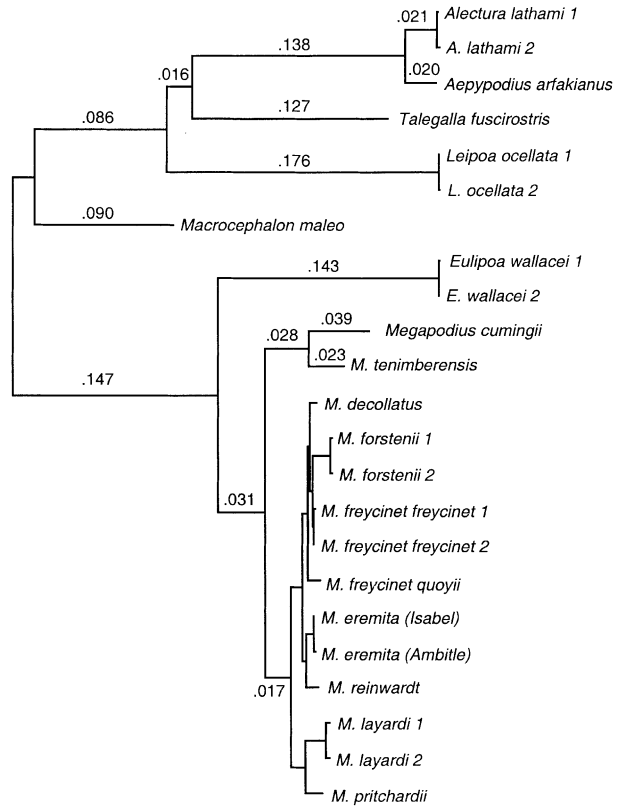


Fig. 6. Trees resulting from a maximum-likelihood analyses of megapode taxa only, with branch lengths indicated (>0.001 for RDP1; >0.015 for ND2). Trees were rooted at the midpoint.

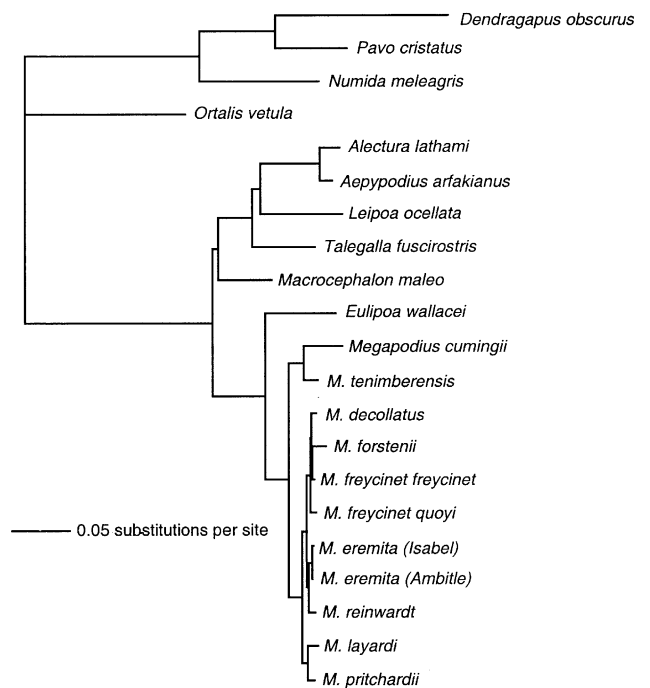
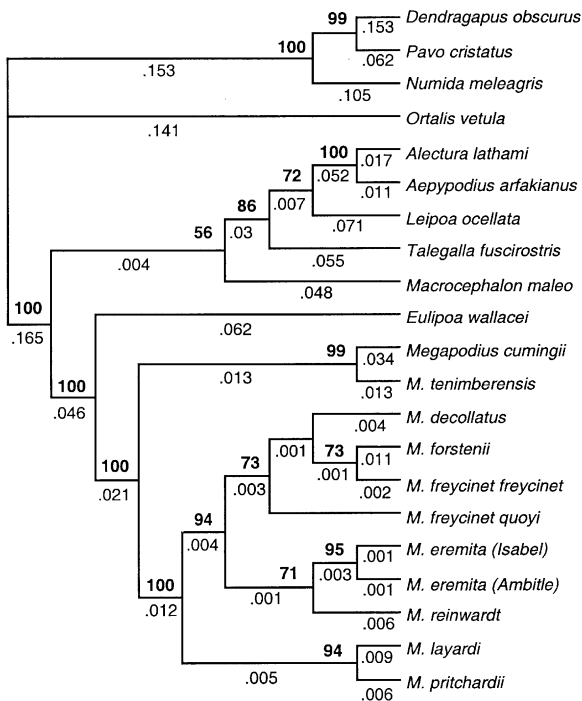


Fig. 7. Maximum-likelihood tree based on a combined dataset of RDP1 and ND2 sequences. Cladogram (left) shows bootstrap values $\geq 50\%$ (1000 rep; above branches) for weighted parsimony analysis, with each gene region weighted by the appropriate Ti/Tv; branch lengths (from maximum-likelihood analyses) are indicated below branches. Phylogram (right) provides a visual comparison of relative branch lengths.

RDP1's relatively slow rate of evolution (about six times slower than mitochondrial ND2) is consistent with previous studies indicating that introns evolve 5–10 times slower than mtDNA (Johnson and Clayton, 2000; Pritchko and Moore, 1997; Sheldon et al., 1999; Slade et al., 1994). In our study, sequence from RDP1 resolved intergeneric but not interspecific relationships. This may in part be due to recent speciation within the genus *Megapodius*, whose genetic distances are small even in comparisons of ND2 sequence. But we suspect that intron sequence will be most valuable to studies at the genus level or above. ND2 sequence provided resolution at the species level for *Megapodius*, where RDP1 evolved too slowly. Rapidly evolving sites in mitochondrial sequences can also provide valuable information for deep divergences, and recent studies have shown that this information may be less compromised by the confounding effects of homoplasy than previously thought (Baker et al., 2001; Björklund, 1999; Broughton et al., 2000; Källersjö et al., 1999; Saunders and Edwards, 2000; Wenzel and Siddall, 1999; Yang, 1998). However, in this study, RDP1 sequence provided stronger resolution for some deep nodes that had relatively weak support based on ND2 sequences alone (e.g., *Macrocephalon* + mound-building genera).

The minor conflict between the RDP1 and ND2 datasets over the branching order of *Leipoa/Talegalla* emphasizes how independent estimates of phylogeny can help expose particular weaknesses in the data and phylogenetic conclusions. This branching order may be resolved with additional information from missing taxa (see below), or alternatively, may indicate a real conflict between the datasets. Such conflict could arise if homoplasy diluted the true phylogenetic signal, which is more likely for the ND2 dataset. It could also arise if the gene tree was not congruent with the species tree, or from lack of nuclear monophyly in the RDP1 dataset due to the relatively long coalescent times of nuclear vs mitochondrial genes. Theoretically, one can determine the likelihood of such monophyly by using the “three-times rule” to compare tree branch lengths to sequence variation within species (Palumbi et al., 2001). However, this comparison requires multiple sequences from each species and is thus impractical for taxonomic groups such as megapodes, which include many species that are rare or geographically remote. But because it is so valuable to have the independent estimates of phylogenies that nuclear and mitochondrial datasets provide, it would be useful for future studies with larger sample sizes to evaluate the likelihood of non coalescence of nuclear alleles for a number of potential markers and taxonomic levels of comparison, to see if any reliable patterns emerge. If the likelihoods for some markers or taxonomic levels tend to be very low or very high, the nuclear data could be assumed to be more or less reliable, respectively.

4.2. Phylogenetic analyses

Despite large differences in sequence evolution (divergence rates, rate heterogeneity across sites, transition/transversion bias), the intron and mitochondrial sequences contained similar phylogenetic signal and were combined in a final tree that took advantages of the strengths of both genes (Huelsenbeck et al., 1996). Both maximum-likelihood and parsimony methods proved complementary in this study, though parsimony weighting schemes affected bootstrap support for nodes, estimates of homoplasy in the data (as measured by RC indices) and in one case, tree topology. Weighting by either Ti/Tv or κ provided better-resolved trees than weighting all character-states equally. In addition, for ND2 data weighting all sites improved tree scores compared to weighting third positions only. Consistent with other studies, weighting tended to influence tree robustness much more than tree topology (e.g., Saunders and Edwards, 2000).

4.3. Overview of phylogenetic results

Not surprisingly, there is overwhelmingly strong evidence for megapode monophyly, with bootstrap values of 100% for all analyses. There are also several intrafamilial relationships with strong support, including monophyly of all the mound-building genera (*Alectura*, *Aepyodius*, *Leipoa*, and *Talegalla*). Within this group, the strongest affinity is between the two brush-turkey species, *Alectura lathamii* (Australia) and *Aepyodius arfakianus* (New Guinea), which were always sister with bootstrap values of $\sim 100\%$. Based on estimated sequence divergence (uncorrected $P = 3.7\%$ for ND2), these are the two most closely related genera within the megapodes. In fact, this level of divergence is much smaller than for other intergeneric comparisons ($P = 11.1\text{--}18.8\%$) and is more consistent with intra-generic comparisons for *Megapodius* ($P = 0.1\text{--}7.7\%$).

Perhaps the most surprising affiliation is between the mound builders and the Maleo (*Macrocephalon*), which nests in burrows. Although the bootstrap support for this node varied more than most, it was $>50\%$ for most analyses, and the node was especially well supported by RDP1 data, which showed less homoplasy. Additionally, the clade consisting of *Eulipoa* and *Megapodius* diverged early from all other megapodes, with a relatively deep division between *Eulipoa* and *Megapodius*, which have been frequently combined into a single genus. *Megapodius* species form a monophyletic group with genetic distances from *Eulipoa* typical of intergeneric comparisons in this family ($P = 12.4\text{--}14.3\%$ for ND2), which supports *Eulipoa*'s current taxonomic status as a separate genus.

Most *Megapodius* species appear to be very closely related. Within *Megapodius*, there is an early split be-

tween (*M. cuningii*, *M. tenimberensis*) and all other *Megapodius* species analyzed ($P = 5.5\text{--}8.8\%$, vs $0.1\text{--}4.1\%$ for comparisons among *Megapodius* species outside this clade). This split is consistent with morphological studies, as is the one between the clade containing *M. pritchardii* and *M. layardi*, and the remaining *Megapodius* species (Roselaar, 1994). Affinities within the latter group indicate that *M. freycinet* may be paraphyletic: two subspecies that we sampled, *M. freycinet quoyii* (from the Moluccas) and *M. freycinet* (from Waigeu), clustered with other species rather than as sisters in the ND2 tree.

4.4. Previous studies and future directions

A few previous studies have provided hypotheses about phylogenetic relationships within the megapodes. The phylogenetic hypothesis currently most widely accepted is a generic-level phylogeny based on seven morphological characters (Brom and Dekker, 1992; Jones et al., 1995). In addition, Downie et al., (1993) did an exploratory study of six species based on a short sequence (300 bp) of cytochrome *b*, and Mey (1999) presented a phylogeny of some megapodes based on the relationships of their parasitic chewing lice. Several phylogenetic relationships proposed here are consistent with one or more of these studies, including the close association between the Australian and New Guinean brush-turkeys (*Alectura* and *Aepyodius*), and the Moluccan megapode (*Eulipoa*) and *Megapodius* (Brom and Dekker, 1992; Downie et al., 1993; Jones et al., 1995; Mey, 1999). The difficulty of resolving the branching order for the Malleefowl (*Leipoa*) and *Talegalla* is also consistent with earlier work (Jones et al., 1995; Mey, 1999). In contrast, by dividing megapodes into two major clades containing on the one hand all the mound-building genera and *Macrocephalon*, and on the other *Eulipoa* and *Megapodius*, our study revealed relationships that differed strongly from the Jones et al. (1995) phylogeny, which placed brush-turkeys (*Alectura* and *Aepyodius*) basally as a monophyletic clade, and *Macrocephalon*, *Megapodius*, and *Eulipoa* in derived positions. Overall, our phylogeny is more consistent with Mey's phylogeny based on parasitic chewing lice (1999).

Because most megapode species and all genera were included in this study, and because morphological evidence suggests excluded taxa were very closely related to those included, adding additional megapode taxa should not produce much change in tree topology at the generic level. However, as discussed above, the branching order of *Talegalla* and *Leipoa* differed among analyses, and adding the two missing *Talegalla* species and newly re-discovered *Aepyodius bruijnii* (Heij and Post, 2001) could help resolve this branching order. In addition, adding the four missing *Megapodius* species would al-

most certainly produce some changes in the topology of this complex genus. Several difficult-to-obtain species (most notably *M. nicobariensis* and *M. laperouse*) were missing from this study, and because *Megapodius* disperse so readily and tend to hybridize in some areas, much about relationships within this genus will be resolved only with intensive sampling from multiple populations of all species.

Acknowledgments

We are very grateful to the following individuals and institutions for their generous assistance in obtaining tissues and/or DNA samples: L. Christidis (Museum of Victoria, Melbourne), W. Boles (Australian Museum, Sydney), G. Barrowclough and P. Sweet (American Museum of Natural History), C. Wood and S. Drovetskiy (University of Washington Burke Museum), R. Dekker (Leiden Museum), C. Moeliker and C. Heij (Rotterdam Museum), R. Sinclair, G. Baker, E. Curio, A. Göth, U. Vogel, S. Rabinowitz, J. Kornegay, and D. Jones. In Vanuatu, E. Bani assisted with permits and K. Davis, R. Hills, T. Foster, and several individuals from Ambrym assisted with logistics in the field. We also thank H. Hoekstra, G. Voelker, M. Saunders, S. Drovetskiy, L. Wang, and J. Gasper for support in the lab, and R. Zink and an anonymous reviewer for helpful comments on an earlier version of this manuscript. This work was supported financially by a National Science Foundation Alfred P. Sloan Postdoctoral Fellowship to S.B., and University of Washington Royalty Research Grant (#1032) and NSF Grant #DEB-9419738 to S.E.

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