Molecular phylogeny of cuckoos supports a polyphyletic origin of brood parasitism

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Abstract

We constructed a molecular phylogeny of 15 species of cuckoos using mitochondrial DNA sequences spanning 553 nucleotide bases of the cytochrome b gene and 298 nucleotide bases of the ND2 gene. A parallel analysis for the cytochrome b gene including published sequences in the Genbank database was performed. Phylogenetic analyses of the sequences were done using parsimony, a sequence distance method (Fitch-Margoliash), and a character-state method which uses probabilities (maximum likelihood). Phenograms support the monophyly of three major clades: Cuculinae, Phaenicophaeinae and Neomorphinae-Crotophaginae. *Clamator*, a strictly parasitic genus traditionally included within the Cuculinae, groups together with *Coccyzus* (a nonobligate parasite) and some nesting cuckoos. *Tapera* and *Dromococcyx*, the parasitic cuckoos from the New World, appear as sister genera, close to New World cuckoos: Neomorphinae and Crotophaginae. Based on the results, and being conscious that a more strict resolution of the relationships among the three major clades is required, we postulate that brood parasitism has a polyphyletic origin in the Cuculiformes, with parasite species being found within the three defined clades. Evidence suggests that species within each clade share a common parasitic ancestor, but some show partial or total loss of brood parasitic behaviour.

Introduction

Cuckoos vary considerably in size and appearance, but the species are most often slender, long-tailed, medium sized (20–30 cm) birds with two toes directed forward and two backward. As a group, cuckoos are cosmopolitan with species on all tropical and temperate continents and on many islands. The species are most numerous in the tropics, especially in south-eastern Asia and Central Africa. The diversity of the Cuculiformes can be appreciated in every aspect of the biology of this group: from fruit-eating species to species feeding strictly on distasteful caterpillars, from resident to long-distance migratory species. Particularly, Cuculiformes are of special ecological and evolutionary interest because of the interspecific brood parasitism of some of the species, that is, the female lays the eggs in the nest of other species, the host, which will take care of the young parasite (Brooker & Brooker, 1990; Soler & Møller, 1990; Moksnes *et al.*, 1991; Lotem *et al.*, 1992; Takasu *et al.*, 1993). All the parasitic cuckoos seem to be capable of parasitizing different species of host, and most are regarded as generalists when considering host choice (Lack, 1968). However, in any one area, cuckoos parasitize a very few hosts, while in other areas the same species selects a different group of hosts (Brooker & Brooker, 1990). Species having this kind of strategy were defined by Fox & Morrow (1981) as 'local specialist'.

Avian brood parasitism has been proposed as an ideal system for the study of coevolution (Rothstein, 1990; Yamauchi, 1995). However, studies of the evolution of this

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life history strategy have been hindered by the lack of well-supported phylogenies for the taxa involved. Although obligate brood parasitism has been found in quite different groups of birds – the honey guides (Indicatoridae), two genera of finches (*Vidua* and *Anomalospiza*), five species of cowbirds (Icteridae), a duck (*Heteronetta atricapilla*) and two subfamilies of cuckoos (Cuculinae and Neomorphinae) (Payne, 1977) – slightly less than 1% of the species of living birds show brood parasitic behaviour; how counter-intuitive therefore it is that behaviour so rare among birds should arise twice in one family?

Divergence between the Cuculiformes and their sister groups probably occurred in the Cretaceous (Sibley & Ahlquist, 1990). Traditionally, turacos (Musophagidae) have been considered the closest relatives to the cuckoos, but recent studies on DNA hybridization (Sibley *et al.*, 1988; Sibley & Ahlquist, 1990) point out that cuckoos are the sister group of a large assemblage that includes more than half of the groups of living birds. They are roughly equally diverged from many of the nonpasserine groups.

The current systematic arrangement of cuckoos is based largely on breeding biology and geographical distribution rather than on details of morphology. Within several subfamilies the anatomy varies among species, and sometimes cuckoos within a subfamily are more different from each other than from cuckoos placed in different groups.

The currently most accepted classification of the cuckoos is that of Peters (1940), which divided the order into six subfamilies. The subfamily Cuculinae includes the brood parasitic cuckoos of the Old World. The subfamily Phaenicophaeinae groups nest-building cuckoos of the Old and New Worlds, and some species of facultative brood parasites. The subfamily Crotophaginae includes the anis and the guira cuckoo, group-living cuckoos from South America with territorial behaviour and sometimes communal nests. The subfamily Neomorphinae includes mainly terrestrial cuckoos of the New World, both nesting and parasitic species. The subfamily Couinae includes pigeon-sized nonparasitic cuckoos from Madagascar, often with bright colours and naked skin on the head. Finally the coucals, terrestrial cuckoos from the Old World that build a covered nest, are grouped in the subfamily Centropodinae.

A recent phylogeny, using behavioural and ecological characters (Hughes, 1996a) (Fig. 1a), differs from traditional classifications in two fundamental ways: the polyphyly of the Neomorphinae and that of the Phaenicophaeinae. According to Hughes the obligate parasite *Tapera* (and *Dromococcyx*, seen as its sister group) should be moved from the Neomorphinae to the Cuculinae. The subdivision of Peters' Phaenicophaeinae into at least two groups by transferring *Coccyzus* to the Cuculinae is also suggested. These results points in the same direction as those derived from the study on cuckoos' post-cranial skeleton by Seibel (1988), the first large-scale analysis of the group using the methods of phylogenetic systematics.

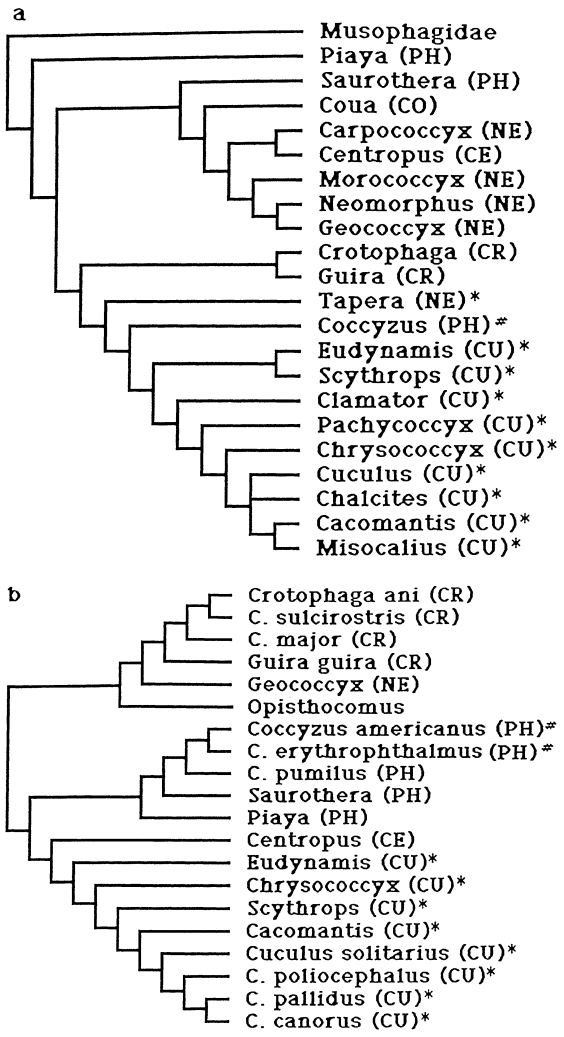

Fig. 1 Phylogenies proposed for Cuculiforms: (a) based on combined osteological, behavioural and ecological data (Hughes, 1996a); (b) based on DNA–DNA hybridization (Sibley & Ahlquist, 1990; branch lengths not shown). Capital letters in parentheses correspond to subfamilies in Peters' classification (1940): (CU) *Cuculinae*, (PH) *Phaenicophaeinae*, (CR) *Crotophaginae*, (NE) *Neomorphinae*, (CO) *Couinae*, (CE) *Centropodinae*. (*) Obligate parasites. (#) Facultative parasites.

The only published classification of Cuculiformes based on DNA data is that by Sibley & Ahlquist (1990) using DNA–DNA hybridization (Fig. 1b). The diversity within Cuculiformes is not completely represented by their results because of the absence of many genera. However, these limited comparisons support the conclusion that the group is diverse, and that classifications do not express this diversity.

Our aim in this study was to construct a molecular phylogeny of cuckoos using DNA sequences as characters in order to provide more evidence about the origin of brood parasitism. Reconstructed phylogenetic relation-

ships are basic for testing hypotheses of adaptation (Brooks & McLennan, 1991). Characters shared among related taxa cannot be treated as statistically independent if they are shared through common ancestry but may rather represent adaptive convergence (Frumhoff & Reeve, 1994).

We report sequences of three fragments of the mitochondrial genome: two fragments of the cytochrome b gene (cyt b), at the extremes 5′ and 3′ of the gene, and one fragment of the NADH dehydrogenase subunit 2 gene (ND2), following the idea that blocks of contiguous sites are less likely to lead to the whole-genome tree than samples composed of sites drawn individually from throughout the genome (Cummings *et al*., 1995). Several problems have been pointed out when using cyt b such as base compositional biases, early saturation of third codon positions, and limited variation in first and second codon positions resulting in little phylogenetic information for 'deep evolutionary events' (Meyer & Wilson, 1990; Irwin *et al*., 1991; Smith *et al*., 1992; Meyer, 1994). However, this gene is the most widely used for this kind of study in vertebrates, including birds (Edwards *et al*., 1991; Avise *et al*., 1994; Krajewski & Fetzner, 1994; Lanyon & Hall, 1994; Leeton *et al*., 1994; Ellsworth *et al*., 1996), because of the good knowledge of its structure and evolutionary rate (DeSalle *et al*., 1987; Harrison, 1989; Kocher *et al*., 1989; Irwin *et al*., 1991). ND2 is a less conserved gene suitable for the resolution of relatively shallow (less than 80 million years ago) divergences (Graybeal, 1994), and it has rarely been used for the study of bird phylogenies (Hackett, 1996; Roy *et al*., 1998).

Materials and methods

Species

The 15 species of cuckoos sequenced in this study represent four of the six subfamilies of Cuculiformes, according to Peters' classification: Cuculinae (*Cacomantis flabelliformis*, *Cercococcyx montanus*, *Chrysococcyx osculans*, *Clamator glandarius*, *Clamator jacobinus*, *Cuculus canorus*, *Cuculus poliocephalus*, and *Surniculus lugubris*), Phaenicophaeinae (*Phaenicophaeus superciliosus*, and *Piaya cayana*), Crotophaginae (*Guira guira*) and Neomorphinae (*Dromococcyx phasianellus*, *Geococcyx californianus*, *Neomorphus geoffroyi* and *Tapera naevia*). Information for the voucher specimens is given in Table 1. Samples from species of Couinae and Centropodinae were not available for study. Also included in a parallel reconstruction were published sequences of the homologous cyt b segment from five species of cuckoos (Avise *et al*., 1994): *Cuculus pallidus* (Cuculinae), *Phaenicophaeus curvirostris*, *Coccyzus americanus, Coccyzus erythrophthalmus* (Phaenicophaeinae) and *Crotophaga sulcirostris* (Crotophaginae).

The choice of an outgroup was made difficult by the lack of conclusive evidence concerning the nearest relatives of Cuculiformes (Sibley & Ahlquist, 1990). The Musophagidae, Peters' sister taxon of cuckoos, were considered as a candidate. A fragment of the ND2 gene and the segment at the 5′ end of the cyt b gene were amplified for the species *Tauraco persa* using the primers described below. A methodological problem arose when amplifying the segment at the 3′ end of the cyt b gene: nonspecific PCR amplification led to a double band pattern on the sequencing gel, even when using sets of internal primers deduced from a cuckoo sequence. Consequently, the *T. persa* sequence was used when possible together with the published sequence of *Gallus gallus* (Desjardins & Morais, 1990) for double outgrouping. For the missing segment, a published cyt b sequence from the nightjar *Caprimulgus longirostris* (Genbank, accession number X95777) was used together with that of chicken. Caprimulgiformes are one of the nonpasserine groups equally diverged from cuckoos according to Sibley & Ahlquist (1990).

Preparation of DNA

Analysed samples consisted of fresh blood preserved in 25% DMSO and 6 M NaCl, tissue samples frozen on dry ice and stored in 96% ethanol, or fresh feather tips in 70% ethanol (see Table 1). DNA was extracted from samples using standard methods of cell lysis, Proteinase K digestion, extraction with phenol and chloroform, and ethanol precipitation (Arctander, 1988). The method was modified for tissue and feather samples, adding a preincubation time of 1 h in 1% SDS and Proteinase K to a final concentration of 1 mg mL^{-1}; after preincubation the samples were disrupted using a pestle, followed by the normal extraction protocol.

Polymerase chain reaction and direct sequencing

Portions of the selected genes were isolated and amplified via the polymerase chain reaction (PCR) (Innis & Gelfand, 1990). Primers and their sources are given in Table 2. The primers span 553 base pairs (bp) of cyt b gene: 304 bp at the 5′ extreme and 249 bp at the 3′ end beginning adjacent to positions 14 993 and 15 704, respectively, according to the chicken mtDNA sequence (Desjardins & Morais, 1990), and 298 bp of the ND2 gene beginning at the 5241 position according to the chicken mtDNA sequence (Desjardins & Morais, 1990).

PCR amplifications of desired fragments (both cyt b and ND2 genes) were done in 20 μL volumes using 2 μL Taq-buffer (0.67 M Tris Cl pH 8.8, 0.02 M $MgCl_2$, 0.166 M NH_3SO_4 and 0.1 M β-mercaptoethanol), 8 μL dGATC-mix (0.5 μM per nucleotide), 2 μL of each primer in a 10 μM concentration, 4.8 μL ddH_2O, 1 μL DNA and 0.2 μL AmpliTaq DNA polymerase (1.0 U of enzyme). A thermal cycle began with 3 min at 94 °C for initial denaturation, followed by 30 cycles of denaturation (94 °C, 15 s), primer annealing (52 °C, 15 s) and polymerase extension (72 °C, 15 s). A final extension for

Table 1 Voucher specimens with institution, reference number and locality of sampling. Abbreviations: ZMC, Zoological Museum Copenhagen; LSUMNS, Louisiana State University Museum of Natural Science; AZ, Antwerpen Zoo.

Species	Inst.	Sample	Reference	Locality
Cacomantis flabelliformis (Fan-tailed cuckoo)	Wild	Blood	One individual	Gooseberry Hill, Australia
Cercococcyx montanus (Mountain long-tailed cuckoo)	ZMC	Blood	P823, P827, P833	Iringa, Tanzania
Chrysococcyx osculans (Black-eared cuckoo)	Wild	Blood	One individual	Murchison River, Australia
Clamator glandarius (Great spotted cuckoo)	Wild	Blood	Three individuals	Granada, Spain
Clamator jacobinus (Jackobin cuckoo)	Wild	Blood	Two individuals	South Africa
Cuculus canorus (Eurasian cuckoo)	Wild	Blood	Two individuals	Granada and Sevilla, Spain
Cuculus poliocephalus (Small cuckoo)	ZMC	Blood	P920, P921, P922	Malindi, Kenya
Dromococcyx phasianellus (Pheasant cuckoo)	LSUMNS	Tissue	B11245, 15335	Ucayali, Perú, Santa Cruz, Bolivia
Geococcyx californianus (Road-runner)	AZ	Feather	Three individuals	Bred in captivity
Guira guira (Guira cuckoo)	LSUMNS	Tissue	B6625	Santa Cruz, Bolivia
Neomorphus geoffroyi (Scaled ground-cuckoo)	LSUMNS	Tissue	B2319	Darién, Panama
Phaenicophaeus superciliosus (Rough-crested cuckoo)	ZMC	Blood	P954	Luzon, Philippines
Piaya cayana (Squirrel cuckoo)	LSUMNS	Tissue	B14529, B14956, B15093	Santa Cruz, Bolivia
Surniculus lugubris (Drongo cuckoo)	ZMC	Blood	P508, P953	Isabella and Luzon, Philippines
Tapera naevia (Striped cuckoo)	LSUMNS	Tissue	B9747, B12577, B15026	Pando and Velasco, Bolivia
Tauraco persa buffoni (Guinea turaco)	AZ	Feather	Two individuals	Bred in captivity

Name	Sequence	Source
Cytochrome b		
L14841	5′-CCATCCAACATCTCAGCCATGATGAAA-3′	(Kocher *et al.*, 1989)
L15564	5′-CCACACATTAAACCCGAATGATA-3′	(Edwards *et al.*, 1991)
H15149	5′-TGCAGCCCCTCAGAATGATATTTGTCCTCA-3′	(Kocher *et al.*, 1989)
H15915	5′-AACTGCAGTCATCTCCGGTTTACAAGAC-3′	(Edwards *et al.*, 1991)
ND2		
L5215	5′-TATCGGGCCCATACCCCGAAAAT-3′	(Hackett, 1996)
H5578	5′-CCTTGAAGCACTTCTGGGAATCAGA-3′	(Hackett, 1996)

Table 2 Primer sequences and sources. Numbers refer to the 3′ base of the primer referenced to the complete mtDNA sequence of *Gallus gallus* (Desjardins & Morais, 1990). 'H' and 'L' refer to primers located on the heavy and light strands of the mitochondrial genome, respectively.

5 min was included in order to minimize the number of partial strands. PCR products were purified by electrophoresis through a 2% low-melting agarose gel (NuSieve gel) stained with ethidium bromide. Agarose plugs with the correct size band were taken using disposable pipettes and stored in 50–500 μL 1 × TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA).

Plugs were melted at 65 °C and 1 μL was removed for asymmetric PCR (McCabe, 1990). The reaction volume was 50 μL, containing 5 μL Taq-buffer, 20 μL dGATC-mix, 5 μL of each primer (excess primer in 10 μM concentration and limiting primer in 0.1 μM concentration), 13.8 μL ddH_2O and 0.2 μL AmpliTaq DNA Polymerase. The thermal cycle conditions were identical to those given above. A 5-μL volume of each reaction was electrophoresed through 2% agarose gel and visualized by ethidium bromide staining to check for the presence of bands. Successful reaction was filtered using Millipore Ultrafree filters, DNA was washed with water, redissolved in 25 μL of ddH_2O and used for sequencing. Dideoxy

sequencing followed the protocol for the Sequenase enzyme system (United States Biochemical) using [^{35}S] – αdCTP. Six per cent denaturing Polyacrylamide gels were run in 1 × TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA pH 8.0). Gel drying and autoradiography followed standard protocols.

Tree building and analysis

DNA sequences were recorded using the SeqApp program (Gilbert, 1992). Sequences were aligned manually without difficulties because of their similarity, and no gaps were required to maintain the reading frame. No double band pattern was found in the analysed sample except for the segment at the 3′ end of the cyt b gene in *T. persa*.

Maximum Parsimony analyses of sequence data were performed with PAUP version 3.1.1 (Swofford, 1993). Heuristic search with a random taxon addition sequence (10 replications) and the tree-bisection-reconnection branch swapping algorithm was selected to search for trees with the smallest number of evolutionary events (i.e. nucleotide substitutions). All uninformative nucleotide positions were excluded from the data set prior to analysis. We constructed a majority-rule consensus tree from all equally parsimonious solutions produced by the various approaches. Confidence limits for particular clades were estimated by bootstrap analysis with 100 iterations (Felsenstein, 1985). Bootstrapping was executed on the original data matrix before reduction.

In mitochondrial DNA transversions accumulate more linearly than transitions at all codon positions (Moritz *et al*., 1987; Meyer, 1994), and consequently transitions should be down-weighted or excluded from the phylogenetic analysis among distantly related species since they can provide misleading information (Hackett, 1996). Whereas the elimination of all transitions from the data set may be too extreme because it sacrifices phylogenetically informative characters (Hackett, 1996), two independent analyses were conducted because of the transition bias: (1) transitions and transversions were weighted equally, and (2) transversion substitutions were preferentially weighted (2 : 1). Mitochondrial DNA also shows reduced levels of variation in first and second codon positions, where most nucleotide substitutions will cause amino acid changes, making these changes more reliable markers for distant relationships because back mutations are less likely (Moritz *et al*., 1987; Meyer, 1994). Given the short length of sequence analysed, the differential weighting of a few sites can exaggerate the random component of nucleotide substitution and obscure the phylogenetic signal present in the data (Ellsworth *et al*., 1996), and consequently equal weight was applied to all sites.

A phenogram based on sequence distance data was constructed using the Fitch-Margoliash procedure without assuming a molecular clock with the program FITCH in the PHYLIP package version 3.5c (Felsenstein, 1993). Genetic distance values were calculated using the Kimura two-parameter model (Kimura, 1980) in the program DNADIST in PHYLIP. The programs SEQBOOT and CONSENSE (PHYLIP) were used to obtain the confidence values of the branching pattern based on 100 replications. A second tree was calculated using a maximum likelihood method (program DNAML in PHYLIP), assuming a transition/transversion ratio of 2.0. No bootstrapping was performed using maximum likelihood because the program proved too time consuming.

We also used MacClade 3.0 (Maddison & Maddison, 1992) for reconstructions of character evolution.

Results

Sequences have been deposited in GenBank under accession numbers AF072590–AF072636. No intraspecific variation was found in the sequenced fragments (see Table 1 for the number of individuals analysed per species). Among the 553 nucleotide positions assayed for cyt b, 331 exhibited variation, and 208 of these were potentially informative in a cladistic sense, the majority located at the third codon position (147). For the ND2 gene, among 298 nucleotides, 186 exhibited variation, and 136 were potentially informative, 74 being located at the third codon position.

A parsimony analysis without weighting of the whole data set (total evidence analysis) using *Gallus gallus* as the outgroup produced a single most-parsimonious tree of length 1127, a consistency index (CI) of 0.469, and a retention index (RI) of 0.382. A single most-parsimonious tree of 1621 steps with an identical topology was obtained when giving double weight to transversions (bootstrap consensus tree in Fig. 2a). The results suggest the inclusion of *Clamator*, traditionally considered a Cuculinae within the Phaenicophaeinae, the monophyly of the rest of Cuculinae *sensu* Peters, and the position of the Neomorphinae and the Crotophaginae *sensu* Peters in the same clade. *Dromococcyx* and *Tapera*, the parasitic cuckoos from the New World, appear as sister taxa with a high bootstrap support. The low bootstrap values for the deeper branches do not support the emergent relationship among the three major clades: *Clamator* plus Phaenicophaeinae, the rest of the Cuculinae, and the clade formed by Neomorphinae and Crotophaginae.

Sequence divergences for both genes, using a Kimura two-parameter model (Kimura, 1980), are shown in Table 3. Phenetic analysis using the matrix of divergence values for the total sequenced fragment and the Fitch–Margoliash algorithm produced the phenogram shown in Fig. 2(b). The topology differs from the most parsimonious tree in (a) the relative positions of the three major clades (defined above), but once again the low bootstrap values do not support the suggested results, (b) the polyphyly of the genus *Cuculus* which is unlikely and weakly supported by the bootstrap values and (c) the

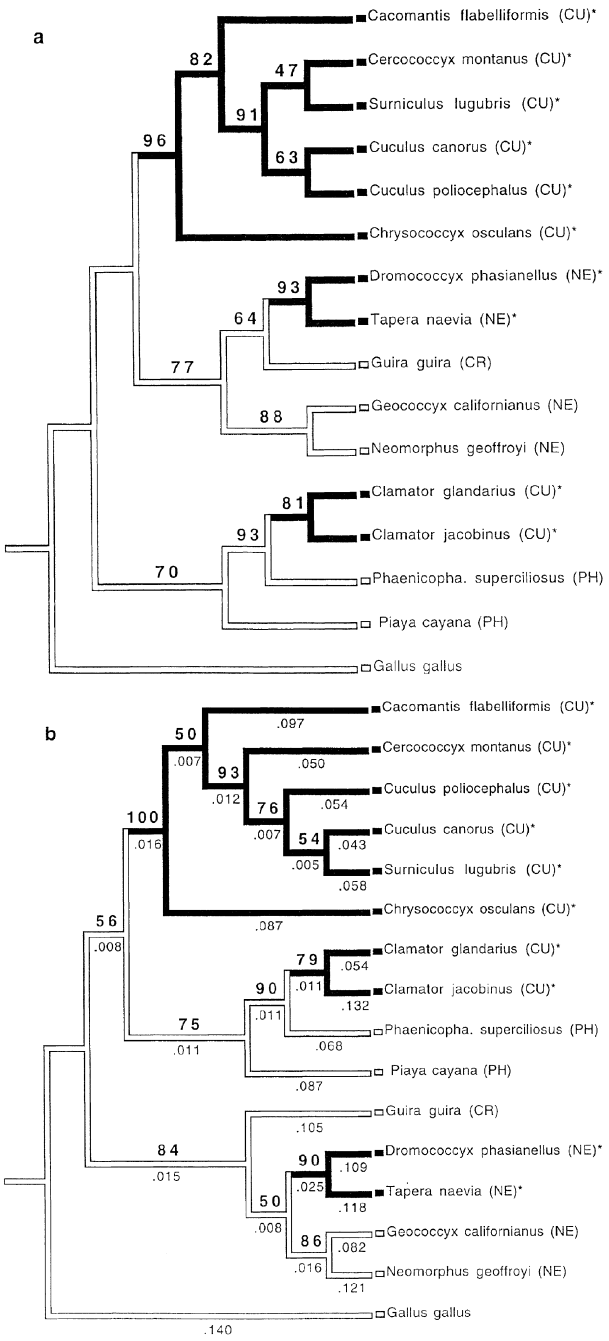

Fig. 2 Tree topologies based on total evidence. (a) Bootstrapped parsimony consensus tree, with relative weights of 2 : 1 of transversions with respect to transitions. (b) Fitch–Margoliash phenogram based on Kimura's two-parameter distances. Bootstrap percentages based on 100 pseudoreplicates indicated above nodes. Numbers below nodes (b) refer to branch lengths. Capital letters in parentheses are as in Fig. 1. (*) Obligate parasites. Character evolution: nesting (white), obligate parasitism (black).

relative position of *Guira* and the Neomorphinae, probably due to the absence of *Crotophaga*, the putative sister taxon of the guira cuckoo, in the analysed data set. A maximum likelihood search produced a tree identical to the most parsimonious tree (ln likelihood = −7039.7209, 422 trees examined).

Partial results from parsimony analysis for each gene are presented in Table 4. Although diverse topologies were generated by the different phylogenetic approaches and the different outgroups used (Table 5), the basic conclusions based on bootstrap support (data not shown) do not differ from the results described above: the genus *Clamator* groups with the Phaenicophaeinae *sensu* Peters, the rest of the Cuculinae form a monophyletic clade (*Cuculus* appears as a polyphyletic genus in reconstructed phylogenies on cyt b, but as a monophyletic one with a high bootstrap support in those based on ND2 sequence), and *Dromococcocyx* and *Tapera* are sister genera in mosts cases.

A parallel analysis was conducted for the cyt b sequence including fragments available from the Genbank database. The Fitch–Margoliash phenogram using *Caprimulgus longirostris* as the outgroup is shown in Fig. 3. The new species included groups within their putative clades: *Cuculus pallidus* within the Cuculinae, *Coccyzus erythrophthalmus*, *Coccyzus americanus* and *Phaenicophaeus curvirostris* within the Phaenicophaeinae, and *Crotophaga sulcirostris* arises as the sister species of *Guira guira* with a high bootstrap support. Topologies obtained when performing parsimony (both unweighted and giving double weight to transversions over transitions) and maximum likelihood are identical to that shown in Fig. 3 for the clade Cuculinae–Phaenicophaeinae. The Crotophaginae form a monophyletic clade with the parasitic Neomorphinae, being the sister clade of the nonparasitic Neomorphinae in parsimony analysis, and of the Cuculinae–Phaenicophaeinae in the maximum likelihood analysis.

Topologies derived from this analysis show the polyphyly of three genera: *Cuculus*, *Coccyzus*, and *Phaenicophaeus*, which is unlikely. Related to *Phaenicophaeus*, results may reflect the diversity of this genus with a large number of synonyms in different taxonomies. Specific names used in the present study are those proposed in the taxonomy of Sibley & Monroe (1990), but following Peters (1940) or Howard & Moore (1991) different synonyms should be used: *Rhamphococcyx curvirostris* and *Dasylophus superciliosus*. Bootstrap values within the clade Cuculinae are not sufficiently high for supporting polyphyly of *Cuculus*; however, the bootstrap values for polyphyly of *Coccyzus* are among the largest in the analysis.

In order to test the degree of repeatability between the fragments sequenced for the present study and the sequences already published in Genbank, one species, *Piaya cayana*, was included in both datasets. No intraspecific variation was found among the three sequenced

Table 3 Sequence divergence (substitutions per site) among taxa computed using Kimura's (1980) two-parameter method. The lower matrix corresponds to cyt b gene sequences (outgroup: *Caprimulgus longirostris*) and the upper matrix to ND2 gene sequences (outgroup: *Tauraco persa*).

		Taxon														
Taxon		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	C. flabelliformis	–	0.122	0.185	0.224	0.250	0.177	0.186	0.287	0.264	0.261	0.278	0.243	0.254	0.176	0.347	0.319
2	C. montanus	0.175	–	0.197	0.238	0.228	0.108	0.108	0.278	0.253	0.206	0.220	0.210	0.221	0.108	0.329	0.297
3	C. osculans	0.167	0.154	–	0.231	0.215	0.230	0.234	0.314	0.336	0.245	0.272	0.281	0.237	0.234	0.311	0.300
4	C. glandarius	0.205	0.117	0.165	–	0.152	0.239	0.247	0.337	0.292	0.291	0.301	0.188	0.179	0.247	0.375	0.297
5	C. jacobinus	0.316	0.278	0.312	0.205	–	0.221	0.239	0.308	0.269	0.265	0.288	0.160	0.161	0.252	0.349	0.321
6	C. canorus	0.170	0.117	0.128	0.148	0.272	–	0.049	0.311	0.245	0.228	0.221	0.237	0.201	0.112	0.359	0.335
7	C. poliocephalus	0.181	0.112	0.147	0.148	0.295	0.121	–	0.313	0.226	0.251	0.231	0.214	0.200	0.121	0.352	0.348
8	D. phasianellus	0.267	0.255	0.245	0.231	0.326	0.246	0.248	–	0.267	0.299	0.300	0.317	0.313	0.349	0.313	0.358
9	G. californianus	0.256	0.216	0.206	0.189	0.316	0.208	0.236	0.186	–	0.304	0.196	0.274	0.242	0.244	0.367	0.303
10	G. guira	0.234	0.200	0.214	0.196	0.295	0.201	0.199	0.208	0.193	–	0.283	0.279	0.239	0.264	0.347	0.353
11	N. geoffroyi	0.273	0.258	0.246	0.254	0.372	0.255	0.281	0.252	0.208	0.242	–	0.335	0.301	0.273	0.391	0.389
12	P. superciliosus	0.211	0.161	0.162	0.110	0.225	0.153	0.157	0.230	0.181	0.199	0.230	–	0.191	0.224	0.370	0.292
13	P. cayana	0.213	0.175	0.187	0.155	0.285	0.174	0.173	0.253	0.212	0.222	0.278	0.154	–	0.218	0.334	0.316
14	S. lugubris	0.160	0.115	0.139	0.162	0.283	0.095	0.132	0.255	0.216	0.225	0.269	0.178	0.204	–	0.399	0.307
15	T. naevia	0.266	0.226	0.224	0.211	0.332	0.226	0.224	0.187	0.218	0.191	0.246	0.213	0.240	0.260	–	0.445
16	Outgroup	0.264	0.234	0.251	0.201	0.316	0.239	0.255	0.226	0.191	0.227	0.275	0.198	0.250	0.254	0.210	–

Table 4 Most-parsimonious trees based on cyt b and ND2 genes sequence fragments. (Tv/Ts) Relative weight of transversions related to transitions. (*n*) Number of equally most-parsimonious trees. (L) Tree length. Species numerical code: (1) *Cacomantis flabelliformis*, (2) *Cercococcyx montanus*, (3) *Cuculus poliocephalus*, (4) *Cuculus canorus*, (5) *Surniculus lugubris*, (6) *Chrysococcyx osculans*, (7) *Clamator glandarius*, (8) *Clamator jacobinus*, (9) *Phaenicophaeus superciliosus*, (10) *Piaya cayana*, (11) *Guira guira*, (12) *Dromococcyx phasianellus*, (13) *Tapera naevia*, (14) *Geococcyx californianus*, (15) *Neomorphus geoffroyi*.

Gene Outgroup Tv/Ts; *n*; L	Tree topology
Cyt b	
Gallus gallus	
1/1; 8; 670	((((1,6)((2,3)(4,5)))(((7,8)9)10))((12(11,13))14,15))
2/1; 1; 964	((((1((2,3)(4,5)))6)(((7,8)9)10))((((12,13)11)14)15))
C. longirost	
1/1; 5; 688	(((((1((2,3)(4,5))6)10)((7,8)9))(12(11,13)))(14,15))
2/1; 1; 954	((((((1,6)((2,3)(4,5)))(((7,8)9)10))11)(14,15))(12,13))
ND2	
Gallus gallus	
1/1; 1; 442	(((((((1,2)5)(3,4))(6,11))10)(7(8,9)))(12,13))
2/1; 3; 639	(((((1((2,5)(3,4)))(14,15))(6,11))(((7,8)9)10))(12,13))
T. persa	
1/1; 3; 444	((((((1,2)5)(3,4))(14,15))((6,11)((7(8,9))10)))(12,13))
2/1; 2; 629	((((((1,2)5)(3,4))((6,11)((7,8,9)10)))(14,15))(12,13))

individuals specified in Table 1. However, a total of 18 base changes were detected between our sequence and that published by Avise *et al.* (1994), 10 in the first 304 bp (3.3% of sequence divergence, seven transitions and three transversions), and eight in the last 249 bp (3.2% of sequence divergence, three transitions and five transversions). Despite the differences, in every phylogenetic reconstruction both *Piaya cayana* sequences group together with a 100% bootstrap support (Fig. 3). The observed level of divergence may be expected within avian genera (Lanyon, 1994) and may be due to geographical differentiation among populations of a widespread species ranging from Mexico to Argentina (Sibley & Monroe, 1990). The absence of locality information in Avise *et al.* (1994) does not allow further discussion of this subject.

Discussion

To date just five genes, according to the Genbank database updated on 15 October 1997, have been partially sequenced in cuckoos: cyt b gene (Avise *et al.*, 1994; Chikuni *et al.*, 1996), NADH dehydrogenase subunit 6 (ND6) gene (Jones & Gibbs, 1997) and ribosomal RNAs 12S (Hedges *et al.*, 1995; Mindell *et al.*, 1997), 16S (Hedges *et al.*, 1995) and 18S (Chikuni *et al.*, 1996) genes. The analysed taxa include three subfamilies *sensu* Peters (Cuculinae, Phaenicophaeinae and Crotophaginae),

Table 5 Monophyly of the subfamilies of cuckoos according to the results derived from the different phylogenetic approaches (Parsimony, Fitch–Margoliash and maximum likelihood), sequence fragments (Cyt b and ND2) and outgroups used (*Caprimulgus longirostris*, *Tauraco persa*, and *Gallus gallus*). (Tv/Ts): relative weight of transversions related to transitions. (+) Monophyly supported by the tree topology. (−) Tree topology does not support monophyly. (C) Cuculinae *sensu* Peters excluding the genus *Clamator* (genera *Cuculus*, *Cercococcyx*, *Chrysococcyx*, *Cacomantis* and *Surniculus*). (P) Phaenicophaeinae *sensu* Peters including the genus *Clamator* (genera *Phaenicophaeus*, *Piaya* and *Clamator*). (D) Parasitic cuckoos from the Neomorphinae (genera *Dromococcyx,* and *Tapera*). (N) Nonparasitic cuckoos from the Neomorphinae (genera *Geococcyx* and *Neomorphus*). Relative position of *Guira* (Crotophaginae) is indicated on the tree topology. (*) Subfamilies defined as above but with one genus grouping outside the monophyletic clade (the genus is indicated on the tree topology).

	C	P	D	N	Tree topology
Cyt b					
Tv/Ts: 1/1					
Gallus	+	+	−	+	((C,P)((D+ Guira)N))
Caprimulgus	+	−	−	+	((((C,*Piaya*)P*)(D+ *Guira*))N)
Tv/Ts: 2/1					
Gallus	+	+	+	+	((((C,P)Guira)N)D)
Caprimulgus	+	+	+	−	((C,P)((D,*Guira*)N))
Fitch-Marg.					
Gallus	+	+	+	+	((C,P)((D,N)Guira))
Caprimulgus	+	+	+	+	((((C,P)*Guira*)N)D)
Maximum Likelihood					
Gallus	+	+	+	+	((C(Guira(N,D)))P)
Caprimulgus	+	+	+	+	((((C,P)Guira)N)D)
ND2					
Tv/Ts: 1/1					
Gallus	−	−	+	+	(((((C*,N)(*Guira*,*Chrysococcyx*)*Piaya*)P*)D)
Tauraco	−	+	+	+	(((C*,N)((*Guira*,*Ghrysococcyx*)P))D)
Tv/Ts: 2/1					
Gallus	−	+	+	+	((((C*,N)(Guira,Chrysococcyx))P)D)
Tauraco	−	+	+	+	(((C*((*Guira*,*Chrysococcyx*)P))N)D)
Fitch-Marg.					
Gallus	+	+	+	+	((C(Guira(N,D)))P)
Tauraco	−	+	+	+	(((C*(*Guira*(N,D)))*Chrysococcyx*)P)
Maximum Likelihood					
Gallus	−	+	+	+	(((C*(N,D))(Guira,Chrysococcyx)P)
Tauraco	+	+	+	+	(((C,P)Guira)(N,D))

eight genera (*Cacomantis*, *Chrysococcyx*, *Cuculus*, *Coccyzus*, *Phaenicophaeus*, *Piaya*, *Crotophaga* and *Guira*), and 13 species. The present study contributes sequences of a new gene (ND2), a new subfamily (Neomorphinae), seven new genera (*Cercococcyx*, *Surniculus*, *Clamator*, *Dromococcyx*, *Tapera*, *Geococcyx* and *Neomorphus*) and 11 new species.

Different attempts have been made to understand the evolutionary history of cuckoos. In reconstructions based on electrophoresis of feather keratins (Brush & Witt, 1983) and egg white proteins (Sibley & Ahlquist, 1972) the clustering patterns contained inconsistencies, presumably reflecting both limitations of these particular systems and inaccuracies in the accepted classification. Currently, phylogenies based on morphology (Seibel, 1988), DNA–DNA hybridization (Sibley *et al*., 1988) and ecological and behavioural characters (Hughes, 1996a) are available.

Although the results presented in this study basically agree with the classifications proposed by Peters (1940) and Sibley & Ahlquist (1990), several points should be discussed, especially the position of *Clamator* and the monophyly of the parasitic cuckoos from the New World and their paraphyly with respect to those from the Old World.

The subfamily Phaenicophaeinae *sensu* Peters has been considered as a 'catch-all' (Berger, 1952), grouping together species in the Old and the New World, including nesting species and species with parasitic behaviour (the nonobligate parasite *Coccyzus*). This heterogeneity leads to different taxonomic arrangements according to the criteria applied by the different authors (Sibley & Monroe, 1990; Howard & Moore, 1991). Our results increase this heterogeneity because, in both phylogenies, the 'Phaenicophaeinae clade' also includes the genus *Clamator*, the crested cuckoos, strictly parasitic species from the Old World traditionally included within the Cuculinae.

However, similarities between *Clamator* and *Coccyzus*, as well as divergences between *Clamator* and the rest of the Cuculinae, have already been highlighted in the literature. Verheyen (1956) grouped together *Clamator* and

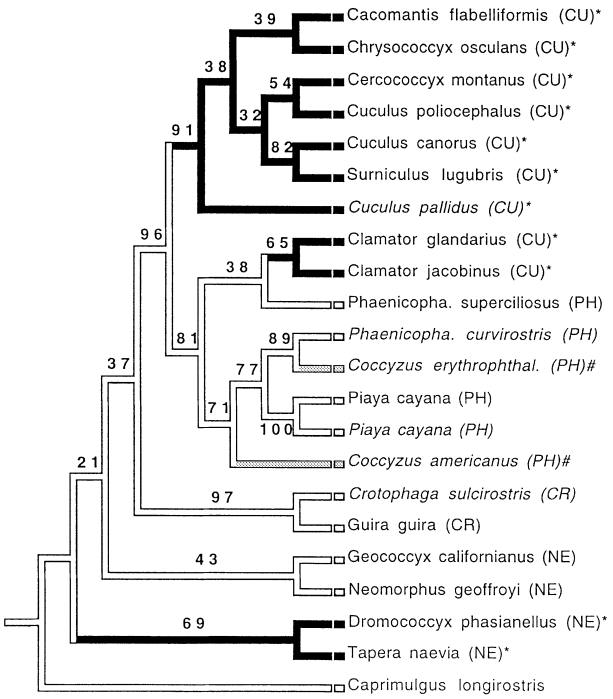

Fig. 3 Tree topology based on cyt b sequence, including published sequences in the Genbank database (italic), obtained when using the Fitch–Margoliash phenogram based on Kimura's two-parameter distances. Bootstrap percentages based on 100 pseudoreplicates are indicated above nodes. Capital letters in parentheses are as in Fig. 1. (*) Obligate parasites. (#) Facultative parasites. Character evolution: nesting (white), facultative parasitism (grey), obligate parasitism (black).

Pachycoccyx, both parasitic cuckoos, with *Coccyzus*, and Stresemann & Stresemann (1966) suggested an isolated position of *Clamator* based on the moult pattern of this genus which differs from that of *Cuculus*. The crested cuckoos have other similarities to the Phaenicophaeinae, by having only 13 cervical vertebrae (14 in the Cuculinae and in the other subfamilies of cuckoos) (Berger, 1960), and by having oval nostrils (the Cuculinae have tubular round nostrils) (Redondo, personal communication tested by S.A. at the collection of Estación Biológica de Doñana, Sevilla, Spain). Despite these differences, most authors have generally agreed that *Clamator* is just a primitive group in its particular subfamily, the Cuculinae (Friedmann, 1964).

Clamator and the Cuculinae not only differ in morphology, but also in parasitic behaviour. In the case of *Clamator*, the female lays more than one egg in the same host nest (one egg per nest in Cuculinae), and the nestlings do not eject the other young or eggs in the nest, but starve them in competition for food brought to the nest by the hosts (Lack, 1968; Soler, 1990). Ejection behaviour has been recorded in most Cuculinae genera: *Cuculus* and *Chrysococcyx* (Brooker & Brooker, 1989), *Pachycoccyx* (Fry *et al.*, 1988), and *Cacomantis*, *Chalcites* and *Penthoceryx* (Ali & Ripley, 1987), while for the rest of the genera little information is available.

Peters' Cuculinae also include two genera with a breeding biology similar to that of *Clamator*. *Scythrops*, an Australian cuckoo, often lays more than one egg per host nest and the young are not known to eject the contents of the nest, although usually any host young disappear within a week (Brooker & Brooker, 1989). The parasitic behaviour of the other genus, *Eudynamys*, has been studied in the species *E. scolopacea*, the koel, a species occurring from India to Australia. In the Indian populations, the female lays several eggs per nest and the young are reared with the host young (Ali & Ripley, 1987), whereas in Australia only one egg is found per host nest and the nestlings show ejection behaviour (Brooker & Brooker, 1989). In the phylogeny of Sibley & Ahlquist (1990) these two genera group within the Cuculinae. Unfortunately, samples were unavailable for the present study.

Within the Phaenicophaeinae clade emerging from our results *Clamator* is not the only genus of brood parasitic species. Some American cuckoos of the genus *Coccyzus* (*C. americanus* and *C. erythrophthalmus*) present occasional parasitic egg laying, apparently related to the abundance of food, toxic, hairy and spiny caterpillars (Nolan & Thompson, 1975). Although parasitism does not occur, anomalous reproductive features have been described: eggs from the same female may differ noticeably in size and colour, eggs are laid at irregular intervals, and clutch-size varies more than in many altricial birds (Nolan & Thompson, 1975). Data on the reproductive behaviour of the other genera included in the Phaenicophaeinae are scarce. Apart from *Coccyzus*, the rest are considered to be nesting cuckoos, and very long reproductive seasons have been described for *Phaenicophaeus pyrrhocephalus*, *Rhopodytes tristis* and *Taccocua leschenaultii* (Ali & Ripley, 1987).

The two genera of parasitic cuckoos from the New World, *Tapera* and *Dromococcyx*, always appear as sister genera in our DNA analysis, in agreement with the traditional taxonomies. Relationships of those genera with other cuckoos remain uncertain based on the presented phylogenetic reconstructions. Hughes (1996a) suggested the transfer of *Tapera* and *Dromococcyx* to the Cuculinae, based on behavioural and ecological characters as well as post-cranial osteological data (Seibel, 1988). We suggest, based on the total evidence analysis (Fig. 2), maintaining *Tapera* and *Dromococcyx* as sister genera included in the clade Crotophaginae–Neomorphinae, an association which is supported by high bootstrap evidence both using parsimony (Fig. 2a) and genetic distances (Fig. 2b). The behavioural and ecological similarity with the Cuculinae may arise from convergent evolution due to parasitism.

Terrestrial habits and geographical distribution have been the criteria used for grouping together the road-

runner-like cuckoos, including parasitic and nonparasitic species. However, many authors have questioned the relations among the Neomorphinae (Verheyen, 1956; Seibel, 1988; Hughes, 1996a). The reproductive biology of *Geococcyx* presents several peculiarities. Roadrunner nests contain three to six eggs, and clutches with as many as 12 eggs have been recorded. Eggs are laid at considerable time intervals and the development of the chicks is rapid (Bent, 1940). A possible case of interspecific parasitim has been suggested for this species. Greater roadrunner eggs have been found in the nests of the common raven (*Corvus corax sinuatus*) and northern mockingbirds (*Mimus polyglottos*) (Hughes, 1996b). The results presented here are not very conclusive because of the low level of resolution of the clades within Crotophaginae–Neomorphinae. In all but one of the proposed phylogenies *Geococcyx* appears as the sister genus of *Neomorphus*, but their association with *Tapera* and *Dromococcyx* (to form the Neomorphinae clade *sensu* Peters) is not evident.

Related to the association among the three major clades defined in the results: Cuculinae *sensu* Peters excluding the genus *Clamator*, Phaenicophaeinae *sensu* Peters including *Clamator*, and Crotophaginae–Neomorphinae *sensu* Peters, a better resolution is required before any hypothesis for the evolution of this avian order can be defended. However, for the evolution of brood parasitism, we found the inclusion of brood parasitic species in the three clades and, hence, a polyphyletic origin of such behaviour seems likely. This point of view differs from the conclusions of Seibel (1988) and Hughes (1996a): the position of *Coccyzus*, *Tapera* and *Dromococcyx* within a clade of obligate parasites implies that parasitism in cuckoos may have evolved just once.

The Phaenicophaeinae group includes strict parasites, nonobligate parasites and nesting species. We postulate that obligate parasitism in that group could be the ancestral state and therefore facultative brood parasitism is more likely to be a loss of such a habit rather than *de novo* evolution; loss may lead to the development of secondary nesting behaviour in cuckoos. This theory is also supported by Hughes (1996a) in the case of *Coccyzus*. In fact, the idea is quite old, as pointed out by Berger (1960, p. 80): 'Thus, if we are to place any value on morphological characters, we must assume either that parasitism has developed independently as many as four times in this one family (which seems highly unlikely) or that the parasitic habit (or tendency for it) developed in the primitive cuckoos …' In the case of the New World cuckoos, the monophyly of *Tapera* and *Dromococcyx* is supported, and their relations with *Geococcyx*, a nesting cuckoo with some 'parasite peculiarities', to some extent agree with the previously mentioned hypothesis: a clade of cuckoos sharing a common parasitic ancestor, some being parasites and others showing a progressive loss of this behaviour.

Only a small number of genera have been included in the present study because unavailability of samples. Consequently, conclusions presented here may have to be altered when data become available for the remaining genera of cuckoos. However, the most reasonable criterion for phylogenetic analysis must be congruence, and without any way of objectively measuring the accuracy of reconstruction, only agreement among data can be used to discriminate among competing hypotheses (Wheeler, 1995). Comparison of phylogenies based on different kinds of characters (molecular, morphological, ecological, behavioural) should be basic for a better knowledge of this diverse group of birds and for understanding of the evolution of brood parasitism.

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