

¹Departamento de Ecología Evolutiva, Instituto de Ecología, UNAM, México D.F., México; ²Departamento de Zoologia, Universidade de São Paulo, São Paulo, Brazil; ³Granja 'La Siberia', Vida Silvestre A.C. Av. Acozac s/n Ixtapaluca, Edo. de México, México; ⁴Unidad de Biología Molecular, Instituto de Fisiología Celular, UNAM, México D.F., México

Increased taxon and character sampling reveals novel intergeneric relationships in the Cracidae (Aves: Galliformes)

K. FRANK-HOEFELICH¹, L. F. SILVEIRA², J. ESTUDILLO-LÓPEZ³, A. M. GARCÍA-KOCH³, L. ONGAY-LARIOS⁴ and D. PIÑERO¹

Abstract

The Cracidae is one of the most endangered bird families in the World. Several studies have been published recently on the evolution and conservation of cracids. Phylogenetic analyses using a fragment of 661 bp of the mitochondrial *cytochrome b* gene for 39 different species of cracids corroborated most relationships found in previous studies. The present work attempts to refine the former phylogenetic hypothesis by increasing taxon sampling and combining molecular with osteological, integumentary and behavioural characters using Maximum Parsimony (MP) and Bayesian analyses. We present both separate and combined total evidence analyses with our molecular data, 152 osteological and 74 integumentary + behavioural characters. While supporting most aspects of the molecular-based hypotheses, the tree based on the combined matrix suggests several modifications of the generic composition for each of the two subfamilies: Penelopinae and Cracinae, and supports the merging of the genera *Pipile* with *Aburria* and *Mitu* with *Pauxi*. These results suggest that increased taxon and character sampling from a diversity of sources may be at least as important as increased sampling of only one type. Besides, of a total of 891 characters we had 437 parsimony-informative sites (almost half of the analyzable sites) proving the efficiency of a total-evidence approach.

Key words: Cracidae – taxon sampling – cytochrome *b* – morphology – behaviour – phylogeny

Introduction

The family Cracidae is a group of mainly Neotropical birds that comprises 11 genera with around 50 species and over 60 subspecies currently recognized (Dickinson 2003). Taxonomy of the Cracidae still presents numerous controversies in terms of the validity of species, the assignation of species to genera, and even the grouping of genera into tribes or subfamilies; Huxley (1867), Vuilleumier (1965), Vaurie (1968), Delacour and Amadon (1973) and Estudillo (1981) reached contradictory conclusions at all of these levels. Recently, Pereira et al. (2002), Pereira and Baker (2004) and Grau et al. (2005) produced molecular phylogenies that contributed to the clarification of some issues listed above. According to del Hoyo and Motis (2004), cracids are divided into two subfamilies, Cracinae and Penelopinae, and three easily identified natural groups: chachalacas, guans and curassows. Of these, the Horned-Guan (*Oreophaps derbianus* Gray, 1844) and the chachalacas (*Ortalis* spp.) are considered singular due to several remarkable morphological and behavioural modifications; the former presents a combination of characters associated with both subfamilies and representatives of the latter genus are considered to retain many of the primitive characters of the family (del Hoyo and Motis 2004). At present, the family Cracidae is composed by seven polytypic genera (*Ortalis* Merrem, 1786, *Penelope* Merrem, 1786, *Pipile* Bonaparte, 1856, *Chamaepetes* Wagler, 1832, *Crax* Linnaeus, 1758, *Mitu* Lesson, 1831 and *Pauxi* Temminck, 1813) and four monotypic genera (*Aburria* Reichenbach, 1853, *Penelopina* Reichenbach, 1862, *Nothocrax* Burmeister, 1856 and *Oreophaps*).

Cooper and Penny (1997) suggested that cracids survived across the Cretaceous-Tertiary boundary (65 Myr) and this age is consistent with a recent molecular study by Pereira and Baker (2005). Using a Bayesian approach, the latter authors estimated that within Galliformes the Cracidae branched off in

the late Cretaceous with a mean divergence time of 88 Myr and that the diversification of genera could have begun in the Eocene. In opposition to these arguments, Mayr (2005) sustains that the Paleogene (Paleocene–Oligocene) fossil record of birds does not support crown-group diversifications before the Oligocene, even if Galliformes are among the most basal taxa of neognathous birds. He argues that recent molecular studies dating diversification of galliform crown-taxa into the Middle Cretaceous are based on an incorrect interpretation of the fossil taxa used for molecular clock calibrations. This argument is sustained by the oldest fossils considered as cracids: *Procrax brevipes* (Tordoff and MacDonald, 1957), from South Dakota, North America and *Palaeonossax senectus* (Wetmore, 1956) from the Early and Late Oligocene, respectively.

With the rise of molecular systematics over the past decades, there have been expectations that molecular data could find the solution to longstanding taxonomic controversies and help to explain phylogenetic relationships. Although many problems indeed seem better resolved, molecular data have not automatically led to increased understanding of the phylogenetic history for many groups (Helm-Bychowski and Cracraft 1993; Jenner 2004; Wiens 2004). The availability of a great amount of molecular information has led to new areas of investigation. One such area relates to discrepancies in hypotheses of relationships depending on which taxa were included or excluded in a phylogenetic inference, regardless of quality and quantity of data used to describe a particular group (Nylander 2001). Therefore, the taxon sampling effect, a change in hypotheses caused by a change in representative taxa sampled, is one of the most important issues in contemporary systematics (Hillis 1998).

Pereira et al. (2002) published a robust molecular phylogeny for the family using 10 678 bp of both nuclear and mitochondrial markers of cracids using one species representing each

of the 11 genera recognized by del Hoyo et al. (1994), with a megapode (Megapodiidae) and two screamers (Anhimidae) as outgroups. Although undoubtedly an important contribution to cracids systematics, choosing only a single representative for each genus may bias the phylogenetic inference (Nylander 2001). Adding taxa as well as additional character types may lead to more reliable relationships (Farris et al. 1996).

Numerous phylogenetic studies of avian groups have been carried out using mitochondrial DNA (mtDNA) as a source of character variation; considered as a conserved molecule, it has a high rate of synonymous substitutions that increase the probability that it will contain synapomorphies that reveal recent periods of shared ancestry (Moore and DeFilippis 1997). Braun and Kimball (2002) have also provided evidence that there is a strong signal in mitochondrial genomes that is compatible with traditional views of bird evolution. Cytochrome *b* (cytb) not only works well for resolving relatively recent evolutionary history, it may be the best choice for birds because of their tendency to have low rates of genic divergence compared to any other vertebrate group (Moore and DeFilippis 1997). The cytb gene has been widely used as a standard molecular marker for avian systematics at specific, generic and familial levels (Edwards and Wilson 1990; Moore and DeFilippis 1997; Cicero and Johnson 2001).

The increased use of DNA sequence data for phylogeny reconstruction is inevitable and well founded, but crucial issues may be resolved by using data of other types of characters. Helm-Bychowski and Cracraft (1993) suggested that sequence data alone are not enough to specify a strongly defined cladistic signal and Wiens (2004) reasserts that morphological data contain useful phylogenetic information. Morphology has long been the basic source of character data in traditional taxonomic studies and comparative biology (Jenner 2004). As Poe and Wiens (2000) state 'much more morphologic variation could be included in phylogenetic analysis, than is used presently'. Morphological and molecular data are mutually complementary and equally important in guiding the overall strategy of phylogenetic inference (Donoghue and Sanderson

1992). The use of behavioural data is still controversial; it has been argued that they lack phylogenetic information, but a growing number of studies have found such characters to be at least as reliable as morphological and molecular ones (DeQueiroz and Wimberger 1993; McLennan and Mattern 2001; Birdsley 2002).

Grandcolas et al. (2001) recommended combining all kinds of data in a formal cladistic analysis, placing equal weight and confidence in each. Non-molecular characters may help to resolve nodes in family trees that are left unresolved by molecular data. Different types of characters may evolve at somewhat different rates at different times and in different levels of a lineage (Givnish and Systma 2000).

An analysis of different sources of data, including molecular and several kinds of morphological characters, as well as behavioural traits with a denser taxon sampling, is an important step towards a more integrative understanding of the biology of this family (Hillis 1998; Grandcolas et al. 2001). A robust phylogeny is essential for a better understanding of this family of threatened Latin American birds and for guiding conservation and reintroduction programmes, because taxonomy and species conservation are interdependent activities that address the biodiversity crisis (Mace 2004).

Materials and Methods

Taxon sampling

Taxa included in our analyses and GenBank accession numbers are listed in Table 1. We sampled 28 species of cracids and one megapode (*Alectura lathami* Gray, 1831), for outgroup comparison following Sibley and Ahlquist (1990). All samples were taken from captive individuals from the aviary of Jesus Estudillo-López near Mexico City. Since cracids can easily hybridize in captivity we were cautious to avoid any uncertainty of pure parentage. To increase taxonomic sampling, we obtained from GenBank 11 cracid sequences of mitochondrial cytb and six other sequences from four different families of Galliformes (Numididae, Phasianidae, Tetraonidae and Megapodiidae), plus two species of Anhimidae (Anseriformes). The overall sampling of the study includes 39 species of about 54 currently

Table 1. Species sampled for this study, with their respective GenBank accession numbers

Chachalacas:
<i>Ortalis motmot</i> (Linnaeus, 1766) (AY659778), <i>Ortalis leucogastra</i> (Gould, 1843) (AY659779), <i>Ortalis garrula</i> (Humboldt, 1805) (AY659780), <i>Ortalis ruficauda</i> Jardine, 1847 (AY659781), <i>Ortalis guttata</i> (Spix, 1825) (AY659782), <i>Ortalis canicollis pantanalensis</i> Cherrie & Reichenberger, 1921 (AY659783), <i>Ortalis poliocephala</i> (Wagler, 1830) (AY659784), * <i>Ortalis vetula</i> (Wagler, 1830) (L08384) and * <i>Ortalis canicollis</i> (Wagler, 1830) (AF165472).
Curassows:
<i>Mitu salvini</i> Reinhardt, 1879 (AY659785), <i>Mitu tomentosum</i> (Spix, 1825) (AY659787), * <i>Mitu mitu</i> (Linnaeus, 1766) (AY141926), * <i>Mitu tuberosum</i> (Spix, 1825) (AY141926 and AY354484), <i>Pauxi unicornis</i> Bond & Meyer de Schauensee, 1939 (AY659786), <i>Pauxi gilliardi</i> Wetmore & Phelps, 1943 (AY659788), * <i>Pauxi pauxi</i> (Linnaeus, 1766) (AY354486 and AY354486), <i>Crax alector</i> Linnaeus, 1766 (AY659789), <i>Crax fasciolata</i> Spix, 1825 (AY659790), <i>Crax blumenbachii</i> Spix, 1825 (AY659791), <i>Crax daubentoni</i> Gray, 1867 (AY65978592), <i>Crax rubra</i> Linnaeus, 1758 (AY659793), <i>Crax alberti</i> Fraser, 1852 (AY659794), * <i>Crax globulosa</i> Spix, 1825 (AY141924) and * <i>Nothocrax urumutum</i> (Spix, 1825) (AY354488 and AY354488).
Guans:
<i>Chamaepetes goudotti</i> (Lesson, 1828) (AY659795), <i>Chamaepetes unicolor</i> Salvin, 1867 (AY659796), <i>Pipile grayi</i> (Pelzeln, 1869) (AY659797), <i>Pipile cumanensis</i> (Jacquin, 1784) (AY659798), <i>Pipile cujubi</i> (Pelzeln, 1858) (AY659799), * <i>Pipile jacutinga</i> (Spix, 1825) (AF165476), * <i>Aburria aburri</i> (Goudot, 1828) (AY354489 and AY354489), <i>Penelope purpurascens</i> Wagler, 1830 (AY659800), <i>Penelope jacquacu</i> Spix, 1825 (AY659801), <i>Penelope montagnii</i> (Bonaparte, 1856) (AY659802), <i>Penelope argyrotis</i> (Bonaparte, 1856) (AY659803), <i>Penelope supercilialis</i> Temminck, 1815 (AY659804) (AY659795–AY4659804), * <i>Penelope obscura</i> Temminck, 1815 (AF165474), * <i>Penelopina nigra</i> (Fraser, 1852) (AY354492 and AY354492) and <i>Oreophasis derbianus</i> Gray, 1844 (AY659805 and AY354494).
Outgroups:
* <i>Numida meleagris</i> (Linnaeus, 1758) (L08383), * <i>Rheinardia ocellata</i> (Elliot, 1871) (AF330060), * <i>Tetrao urogallus</i> Linnaeus, 1758 (AB120132), * <i>Megapodius reiwaldi</i> Dumont, 1823 (AF165465), <i>Alectura lathami</i> Gray, 1831 (AY659806), * <i>Anhima cornuta</i> (Linnaeus, 1766) (AY140735) and * <i>Chauna torquata</i> (Oken, 1816) (AY140736).

The species marked with an asterisk were obtained from GenBank.

recognized in the family, plus seven outgroups. Around 14 species are missing from our total-evidence final tree [*Pipile pipile* (Jacquin, 1784), *Penelope albipennis* Taczanowski, 1877, *P. perspicax* Bangs, 1911, *P. ortoni* Salvin, 1874, *P. marail* (Muller, 1776), *P. ochrogaster* Pelzeln, 1869, *P. pileata* Wagler, 1830, *P. dabbeni* Hellmayr & Conover, 1942, *P. jacucaca* Spix, 1825, *P. barbata* Chapman, 1921 (10 guans); *Ortalis wagleri* Gray, 1867, *Ortalis cinereiceps* Gray, 1867, *Ortalis erythroptera* Sclater & Salvin, 1870 and *Ortalis superciliaris* Gray, 1867 (four chachalacas)].

Molecular data

Blood was taken from the axial wing vein of individuals of 29 species mentioned above and added in a proportion 1 : 10 ml to a lysis buffer of 100 mM Tris-HCl (pH 8), 100 mM EDTA (pH 8), 10 mM NaCl and 2% SDS; the samples were aliquoted and stored at -70°C . Total genomic DNA was isolated and purified with the Genomic DNA Purification Kit (PuregeneTM; Gentra, Minneapolis, MN, USA).

Cytochrome b

From total genomic DNA, we obtained a mitochondrial fragment of the cytb gene via polymerase chain reaction amplifications with the primers L14990 and H15696 (Kornegay et al. 1993). PCR was carried out in 50 μl reaction volumes using the PCR Core Kit (Boehringer, Ingelheim, Germany) with the following thermocycler profiles: 5 min at 94°C , then 50 cycles of: 1 min at 94°C , 45 s at 50°C , and 2 min at 72°C , with a final extension of 10 min at 72°C (Kocher et al. 1989). PCR products were gel-purified and extracted from agarose with Concert Gel Extraction Systems (Gibco Life TechnologiesTM, Gaithersburg MD, USA). PCR fragments were sequenced directly in an Applied Biosystems Automatic DNA Sequencer ABI Prism 3100 (Foster City, CA, USA), using a BigDye Terminator Cycle Sequencing Reaction Kit (Foster City). We obtained 29 sequences of 661 bp (GenBank accession numbers AY659778–AY659806).

Since it is known that mitochondrial genes have nuclear homologues in avian taxa (Sorenson and Quinn 1998), we assured that our amplifications were of mitochondrial DNA origin by obtaining clean and unambiguous electropherograms and by comparing our sequences with other cracid mitochondrial cytb genes from GenBank. For a further verification of our data, we also compared with the amino acid translation of the fragment done with MACCLADE 4.0 (Maddison and Maddison 2000); we had 219 amino acids that match positions 44 through 263 in a complete sequence of mtDNA cytb of two cracids, *Crax rubra* Linnaeus, 1758 (accession number AY274029) and *Ortalis vetula* (Wagler, 1830) (accession number L08384).

To obtain multiple sequence alignment formats ready to be exported as a NEXUS file, the sequences were edited and aligned using PILEUP and LINEUP with the Wisconsin Package Version 9.1 [Genetics Computer Group (GCG) 1997]. The sequences were checked and edited manually in MACCLADE 4.0 (Maddison and Maddison 2000). The final alignment included 661 bp fragments of cytb sequences for 46 species.

Morphological and behavioural data

We obtained a total of 152 osteological and 74 integumentary + behavioural characters. We used the binary coding system (Pleijel 1995) for most of our characters unless there was a compelling reason to use multistate coding; cases when there were clearly three different states of a character (multistate); when one or more taxa lack a component of the character definition (inapplicable); or when the information was not available (missing) following Peterson and Eernisse (2001).

We are treating the osteological and integumentary + behavioural matrices separately because they are frequently regarded as different characters-systems in the ornithological literature (Chu 1998) and because we had 43 terminals for the osteological matrix and only 39 for the integumentary + behavioural one. Osteological evidence is particularly useful for higher-level groups, whereas integumentary + behaviour evidence is important for resolving lower level

relationships (Chu 1998). Besides, separate analyses are useful to identify disagreements between data sets. The polarity of the character state transformation was assumed by using outgroup comparison.

The conventional view based on morphology (Cracraft and Clarke 2001; Silveira 2003) suggests that Megapodiidae is the sister taxon of the remaining Galliformes, whereas Cracidae is sister to Phasianidae, Numididae and Tetraonidae. This is also an emerging consensus with molecular markers (Sorenson et al. 2003; Pereira and Baker 2005), which differs from DNA–DNA hybridization results where cracids and megapodes were considered sister taxa (Sibley and Ahlquist 1990). To further investigate this discrepancy, we increased the outgroup sampling by adding four taxa from their sister group.

Osteological

We analysed 73 skulls and 283 skeletons belonging to 43 species of the 11 genera currently recognized within the cracids and 78 specimens of 26 species representing other Galliformes and Anseriformes to be used as outgroups (Silveira 2003). We codified 152 characters following Livezey (1997), 13 of them scored as multistate and the rest as binary. A description of the characters and character states, along with a data matrix, is presented in Appendix 1. The voucher specimens are deposited in natural history museums or osteological collections (see Acknowledgements).

Integumentary + behavioural

We examined and scored 74 integumentary + behavioural characters, for 39 cracids and seven outgroups represented by five Galliformes [Numididae: *Numida meleagris* (Linnaeus, 1758), Phasianidae: *Rheinardia ocellata* (Elliot, 1871), Tetraonidae: *Tetrao urogallus* Linnaeus, 1758 and Megapodiidae: *Alectura lathami* Gray, 1831 and *Megapodius reinwardt* Dumont, 1823] and two Anseriformes [Anhimidae: *Anhima cornuta* (Linnaeus, 1766) and *Chauna torquata* (Oken, 1816)]. The matrix includes gross external characteristics, several behavioural traits such as courtship and descriptive vocalizations, as well as tracheal modifications. Sixty-five characters were coded as binary and nine as multistate. This information was obtained directly from birds in captivity and from published literature and photographs (Vaurie 1968; Delacour and Amadon 1973; Sick 1984; del Hoyo et al. 1994; del Hoyo and Motis 2004) under the surveillance of Estudillo-López. A description of the characters and character states, along with a data matrix, is presented in Appendix 2.

Phylogenetic analyses

The molecular phylogeny with 42 terminals was reconstructed using equally weighted parsimony (MP) with PAUP* (Swofford 2001) version 4.0b10 for Macintosh (PPC). The analysis included all codon positions of the cytb mitochondrial coding-protein fragment because the highly divergent third codon positions appeared to contain phylogenetic information and produce better estimates (Yoder and Yang 2000). Their exclusion produces poorly resolved trees within cracids (Pereira et al. 2002). All characters were unordered. For the molecular data, the phylogenetic relationships between all taxa sampled were inferred by choosing a megapode, *Megapodius reinwardt*, and two screamers, *Anhima cornuta* and *Chauna torquata*, for outgroup rooting following Pereira et al. (2002), to allow comparisons with our results. We performed parsimony analyses using a heuristic search algorithm via stepwise addition, with 1000 random additional sequence replicates saving 100 trees per replicate and tree-bisection and reconnection (TBR) branch swapping. To assess branch support, we obtained bootstrap values with 1000 replicates using simple addition sequence and TBR swapping with 100 trees saved per replicate.

The transformation series for both data sets of non-molecular characters were analysed separately with PAUP, using equally weighted parsimony (MP) under the same parameters used for the molecular data. All characters were parsimony-informative.

For a total-evidence approach, we combined all our data in a single matrix. We included a total of 887 characters for 39 terminals, maintaining all taxa that had at least molecular, osteological, and

integumentary + behavioural characters. We included the same seven outgroup taxa since we had information of all character types. We analysed this combined data set with PAUP under the same parameters used in the molecular analysis.

A Bayesian inference approach as implemented in the program MR.BAYES v3.0b4 (Huelsenbeck and Ronquist 2001) was performed with the combined matrix. Searches were based on five data partitions: three codon positions, the osteological and the integumentary + behavioural characters. We ran 3 000 000 generations with one cold and three heated chains, a temperature of 0.5, while trees were sampled every 100th generation, starting from random trees and a burn-in of 1000 trees. The log-likelihood scores were plotted against generation time and the Markov chains were assumed to be stationary when the likelihood value had converged (around 50 000 generations). Sample points prior to stationary chains were discarded as burn-in values and the remaining values were used to generate a 50% majority consensus tree.

The program MODELTEST 3.06 (Posada and Crandal 1998) with the Akaike Information Criterion (AIC) chose the general time-reversible model with invariant sites and a gamma correction (GTR + I + Γ) as the best-fit evolutionary model of DNA substitution for our molecular data. For the discrete morphological data, we used a Mk generaliza-

tion of the Jukes-Cantor model (1969: JC69) assuming a discrete gamma distribution for the relative rates, where 'M' stands for Markov and 'k' refers to the numbers of states observed ($k \geq 2$) with no state considered plesiomorphic or apomorphic *a priori* (Yang 1994; Lewis 2001). The prior assumption of overall rate heterogeneity across partitions was set at variable to allow our partitions to evolve under different rates. The MCMC sampling was run three times to verify that the same topology was recovered for each.

Results

Cladistic analyses

Molecular tree

We found two most-parsimonious trees of 807 steps with a consistency index (CI) of 0.437 and a retention index (RI) of 0.676 (Fig. 1a); 209 sites were parsimony-informative. Our consensus tree is based on 42 terminals, 39 cracids plus the same three outgroups used by Pereira et al. (2002). Although the cytb tree (Fig. 1a) had lower bootstrap values, Fig. 1b,c also have just about the same nodes with support of < 50% for

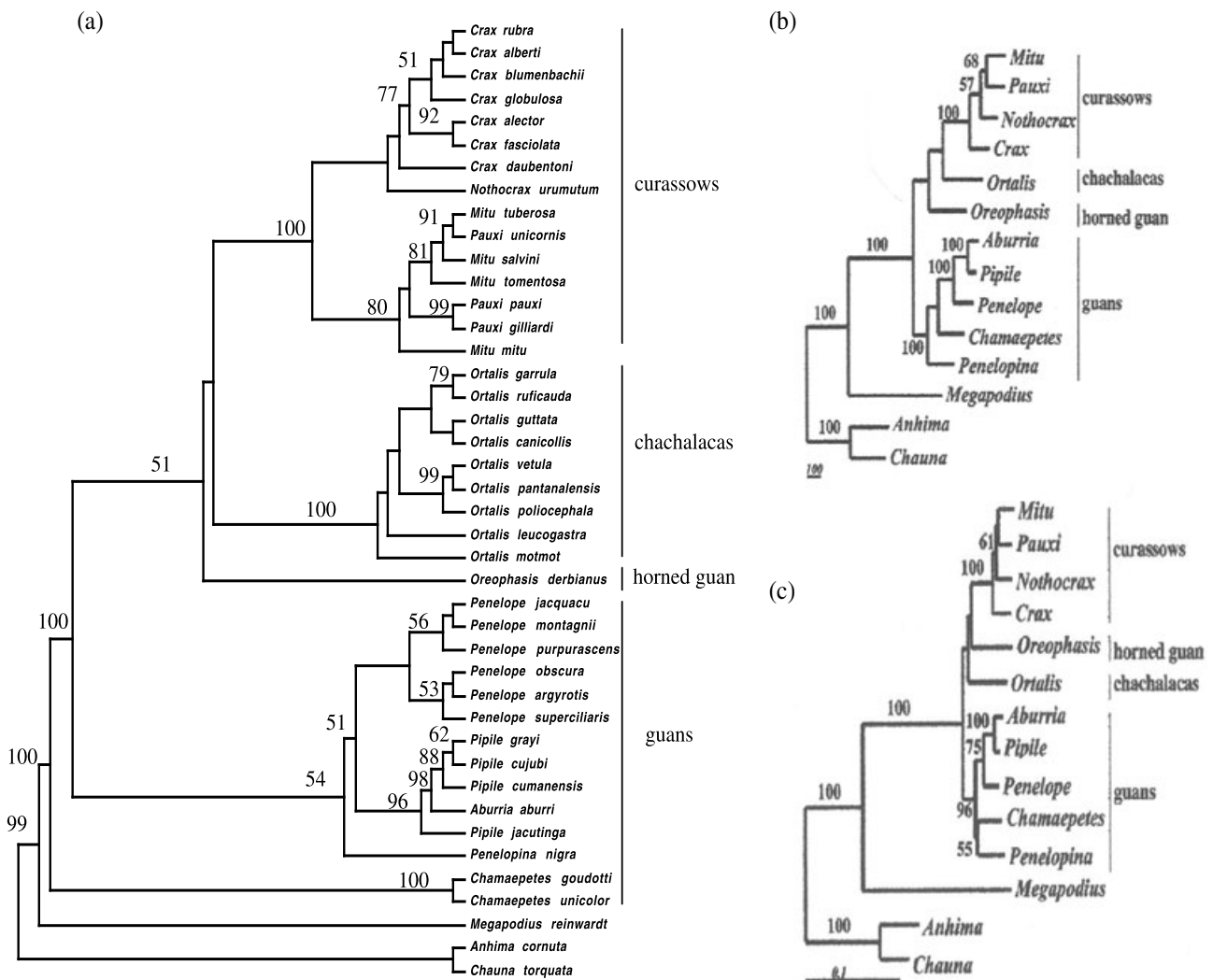


Fig. 1. (a) Strict consensus of two most parsimonious trees obtained with 661 base pairs of mitochondrial cytochrome b gene from 39 species representing all recognized genera of Cracidae. (b and c) MP and ML trees obtained by Pereira et al. (2002) with 10 678 base pairs of both mitochondrial and nuclear markers (reproduced for comparisons); one species represents each of the recognized genera of the family. The numbers along the branches are bootstrap values.

the relationship among *Oreophasis* and *Ortalis* to the curassows, and for the positions of *Penelopina* and *Chamaepetes*.

Huxley (1867) subdivided the Cracidae into two subfamilies: Penelopinae which included the genera *Oreophasis*, *Penelopina*, *Aburria*, *Pipile*, *Penelope*, *Ortalis*, and *Chamaepetes* while Cracinae included *Crax*, *Mitu*, *Pauxi*, and *Nothocrax*. Our results partially support the hypothesis proposed by Pereira et al. (2002) who identified these same two clades with *Oreophasis* and *Ortalis* within Cracinae. Inside the Cracinae the cytb tree shows *Ortalis* as sister group to the curassows, and *Oreophasis* as sister to the whole clade, matching Fig. 1b. Within the Penelopinae, the genus *Penelopina* is sister group to *Pipile* (including *Aburria*) and *Penelope*. With these data *Chamaepetes*, the sickle-winged guans, appears as sister group to all the other cracids. The availability of molecular data for almost all species within *Mitu*, *Pauxi*, *Pipile* and the monotypic species of *Aburria* confirms the inclusion of *Pipile* within *Aburria* (Grau et al. 2005) and the paraphyly of *Mitu* and *Pauxi* (Pereira and Baker 2004). *Mitu mitu*, though not well resolved with these data, is sister group to the *Mitu* + *Pauxi* clade and therefore could be also included within *Pauxi*.

Morphological trees

Osteological On the basis of the osteological data set, we found eight most-parsimonious trees with 343 steps, a CI = 0.477 and a RI = 0.888, all characters were parsimony informative (Fig. 2). The strict consensus tree obtained for these data shows both subfamilies represented by the same genera proposed by Huxley (1867). Within the subfamily Penelopinae, *Ortalis* and *Chamaepetes* are monophyletic sister groups, and in turn are a sister group of *Penelope*; *Aburria* + *Pipile*, *Penelopina* and *Oreophasis*, form successive sister taxa. Within the subfamily Cracinae, *Crax* is sister group of *Pauxi* + *Mitu* and *Nothocrax* is sister group to *Crax*, *Pauxi* and *Mitu*. As in the molecular analysis, the osteological hypothesis also supports the merging of *Mitu* with *Pauxi* and *Pipile* with *Aburria*. The tree shows some fairly robust bootstrap values, particularly for the Cracinae, but values are lower for the intergeneric relationships within Penelopinae.

The osteological characters include eight synapomorphies supporting the clade proposed as subfamily Cracinae (e.g. a well-developed apophysis furculae, character no. 101, Fig. 7 and Appendix 1), four synapomorphies (e.g. a small lacrimal process, character no. 3 and characters no. 2, 52 and 59,

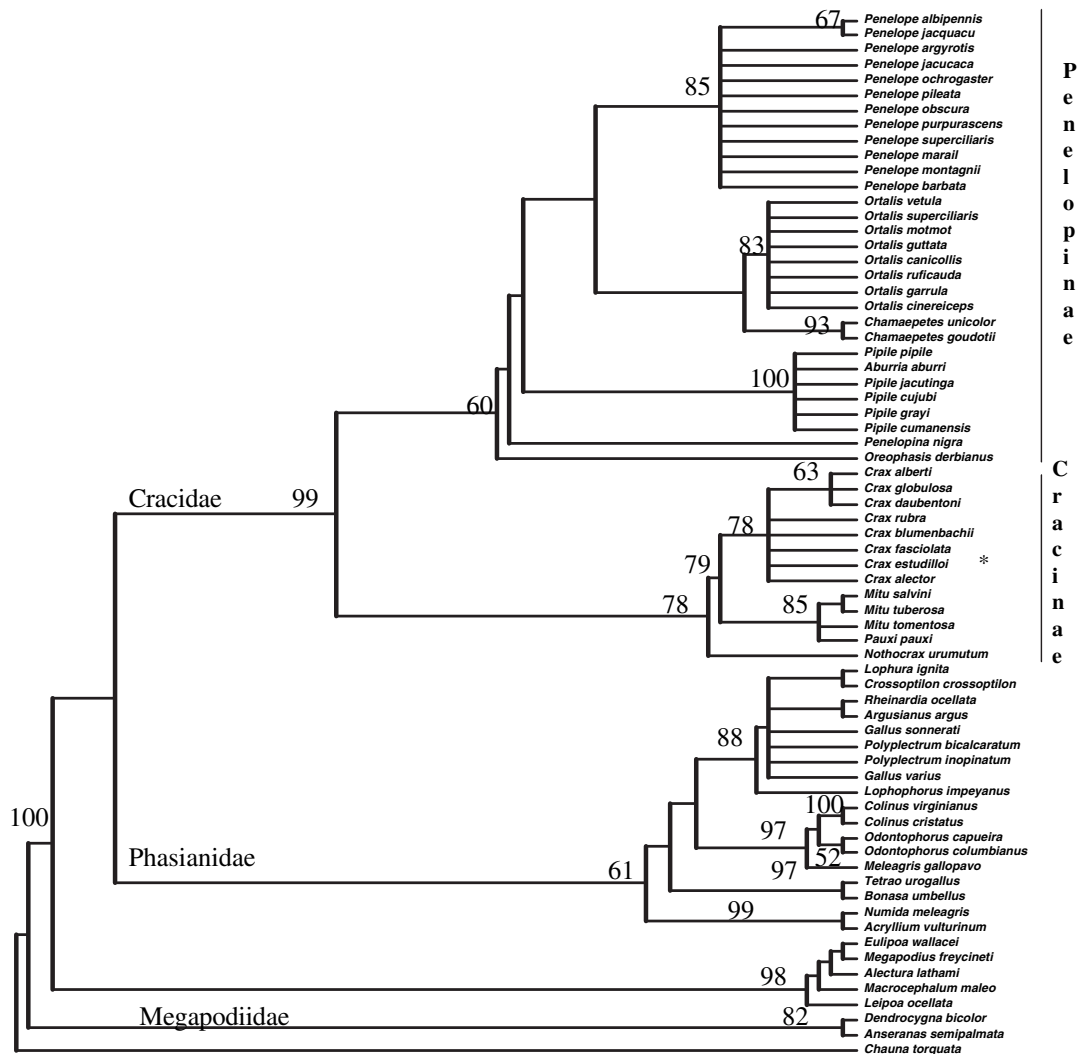


Fig. 2. The validity of the species *Crax estudilloi** was recently questioned (Joseph et al. 1999). One of us (Silveira) located the holotype specimen in the Museum of Natural Sciences, Louisiana State University, USA; further studies are necessary. Strict consensus of eight most-parsimonious trees based on 151 osteological characters. The numbers along the branches are bootstrap values

Chamaepetes is related to; *Pipile* may be derived from within *Aburria* and *Mitu* from within *Pauxi*. We thus decided to use a total-evidence analysis to address these issues.

Combined trees With the partition-homogeneity test as implemented in PAUP* (Swofford 2001), we found a p-value > 0.05 implying that there is no statistically significant incongruence, so we can combine the three data sets. The total-evidence analysis yielded 12 most-parsimonious trees of 1593 steps, a CI = 0.410 and a RI = 0.690 (Fig. 4); 413 characters were parsimony-informative. The strict consensus tree shows two subfamilies: Penelopinae represented by all the guans (excepting the Horned-Guan, *Oreophasis*) and chachalacas, and Cracinae containing the curassows and the Horned-Guan at the base of the clade. This tree has general higher bootstrap values for almost every intergeneric node, although the two main clades are still supported by relatively low values.

Bayesian inference The use of model-based optimality criteria was restricted primarily to molecular data, with MP being the only criterion applied to both discrete morphological and molecular data. The advances in Bayesian inference methods have recently incorporated using models for discrete morphological character data for the purpose of inferring phylogenies (Lewis 2001).

Considering Bayesian analysis (BA) as a valuable tool to assess the uncertainty in a phylogeny, or to corroborate its topology we built a total-evidence phylogenetic tree following the parameters previously explained. The 50% majority consensus tree, generated from the trees after the log-likelihood values reached a stable equilibrium, recovered the same intergeneric relationships as with MP but with higher reliability for several branches via posterior probability (p.p.). Within Penelopinae, *Chamaepetes* is the sister group of

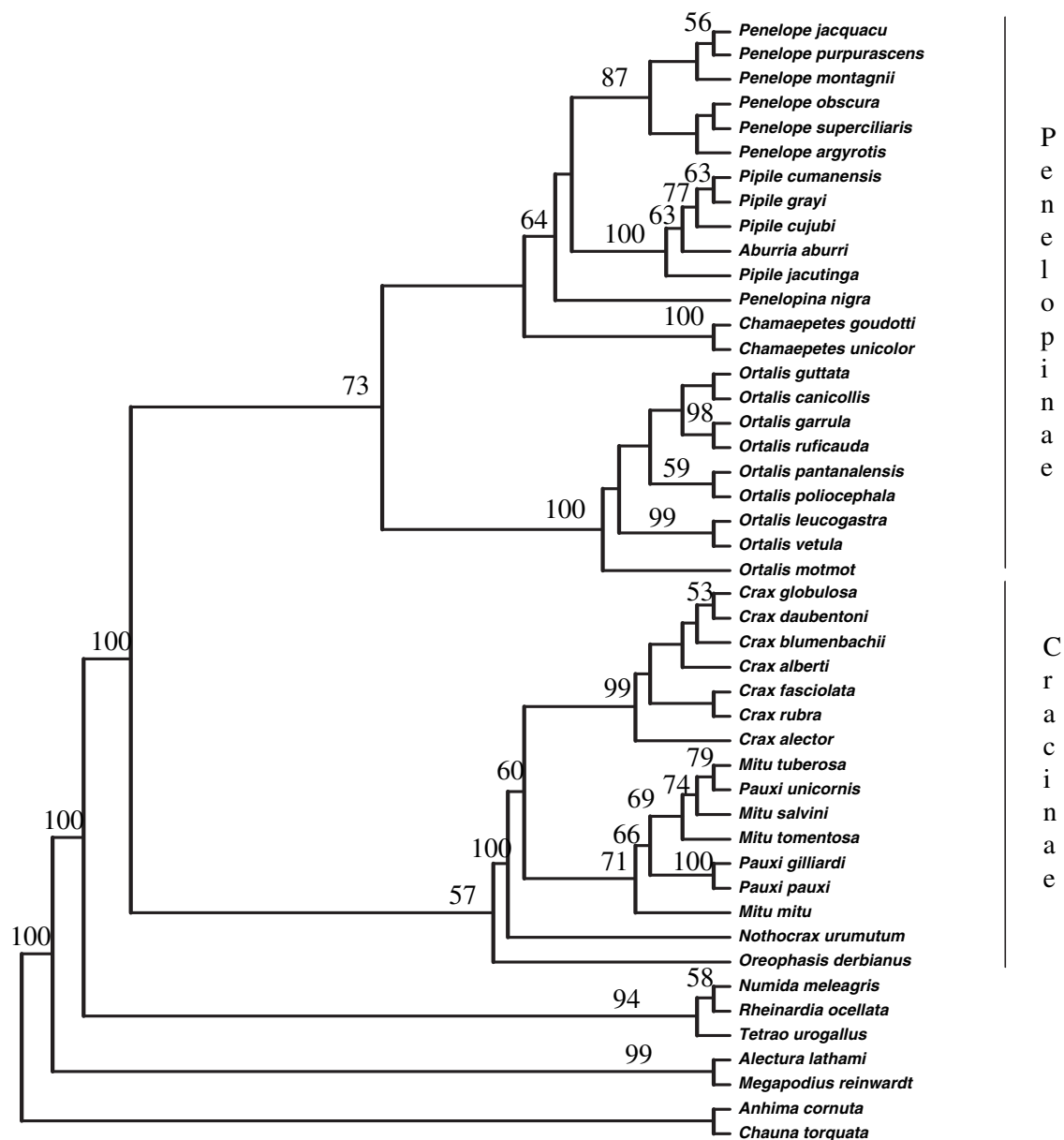


Fig. 4. Combined analysis. Strict consensus of 12 most-parsimonious trees combining 661 molecular characters with 151 osteological and 74 morphological and behavioural characters. The numbers along the branches are bootstrap values

Penelopina, *Penelope* and *Aburria* + *Pipile* with a p.p. of 0.98; *Penelopina* is the sister group of *Penelope*, *Pipile* + *Aburria* with a p.p. of 1.00 and with a p.p. of 1.00, *Pipile* is within the *Aburria* clade. Inside Cracinae, *Nothocrax* is the sister group of *Crax*, *Pauxi* + *Mitu* with a p.p. of 1.00; *Crax* is the sister group of *Pauxi* + *Mitu* with a p.p. of 0.99 and with a p.p. of 1.00, *Mitu* is within the *Pauxi* clade. Both clades comprising each subfamily have fairly low posterior probabilities: Penelopinae with 0.78 and Cracinae with 0.82 (Fig. 5).

The inclusion of *Ortalis* within Penelopinae and *Oreophasis* within Cracinae seem to affect the overall robustness of each clade. By running a BA without these taxa we found a posterior probability of 1.00 for both subfamilies; Cracinae with *Crax*, *Pauxi* + *Mitu* and *Nothocrax*; and Penelopinae with *Penelope*, *Aburria* + *Pipile*, *Penelopina* and *Chamaepetes* (Fig. 6).

With a total evidence approach with both Maximum Parsimony and a Bayesian inference, we propose a new phylogenetic hypotheses for the Cracidae: confirming the

inclusion of *Pipile* inside *Aburria* (Grau et al. 2005) and *Pauxi* within *Mitu* (Pereira and Baker 2004); reasserting the position of *Nothocrax* as sister to *Crax*, *Pauxi* + *Mitu* and *Oreophasis* as a sister lineage to the remaining members of the Cracinae; and *Chamaepetes* as sister group to *Penelopina*, *Penelope*, *Aburria* + *Pipile* and *Ortalis* as sister group of these four genera, in subfamily Penelopinae.

Discussion

Phylogeny

Expanded sampling of both taxa and different types of characters almost always result in more accurate estimates of phylogeny (Zwickl and Hillis 2002; Flynn et al. 2005). The validity of the conclusions drawn from a phylogeny can depend critically on which taxa are included and the relationships among a subset of ingroup taxa can be altered if another taxon is added to the analysis (Givnish and Systma 2000). To reconstruct phylogenetic relationships with increased

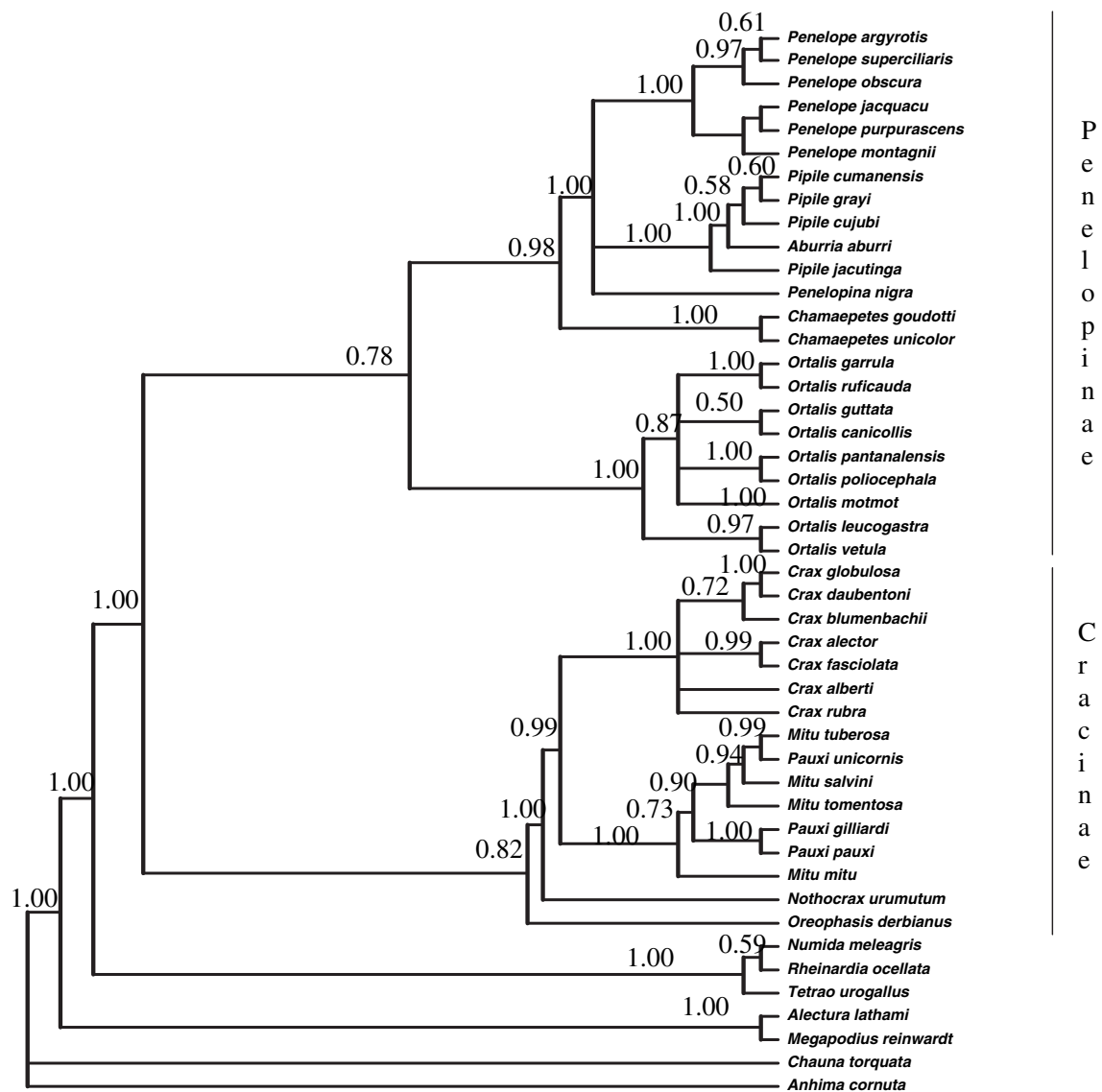


Fig. 5. Final Bayesian tree combining 887 molecular, osteological, morphological and behavioural characters of 39 species plus seven outgroups. The numbers at the interior branches indicate posterior probabilities

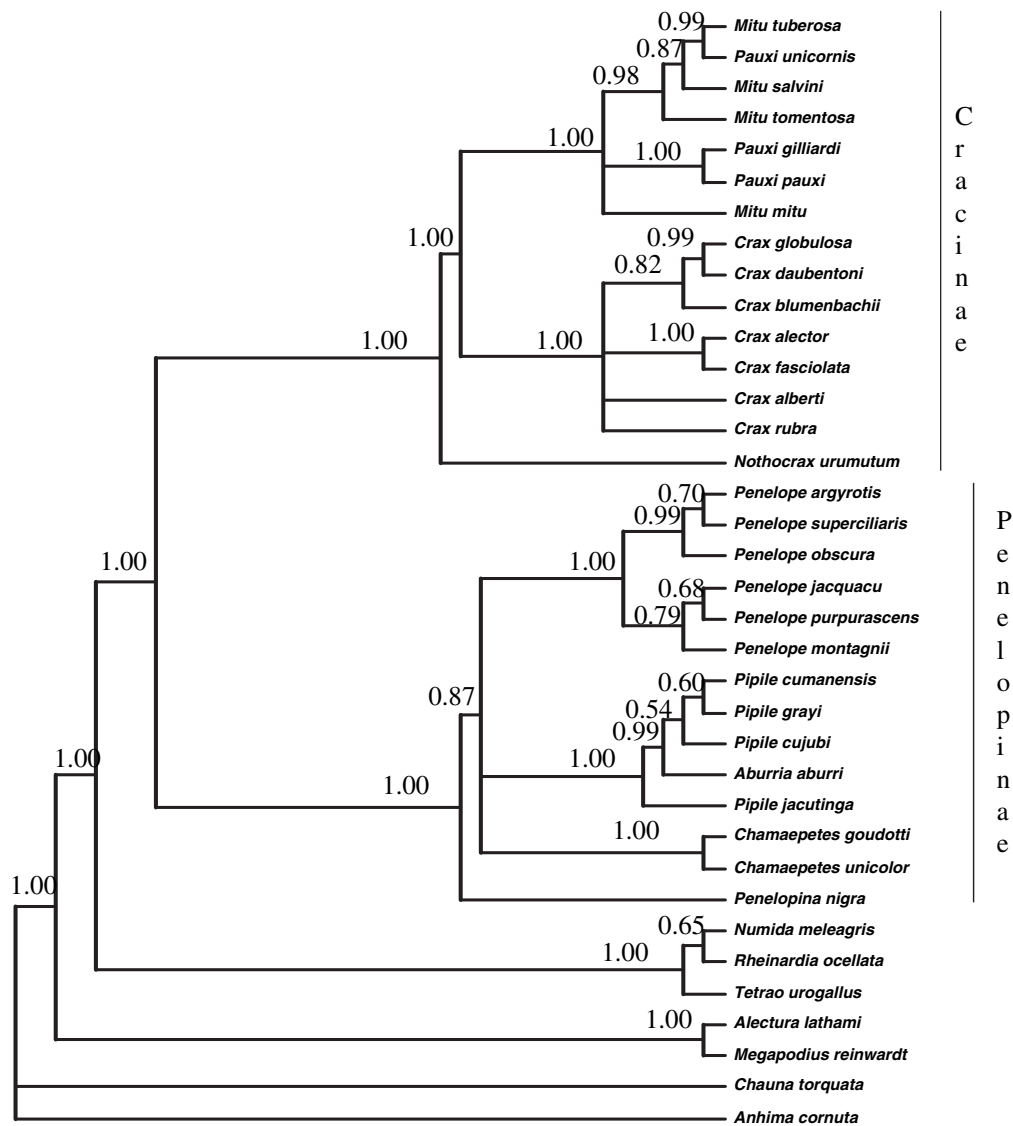


Fig. 6. Bayesian tree excluding *Oreophasis derbianus* and *Ortalis* spp. combining 887 molecular, osteological, morphological and behavioural characters of 29 species plus seven outgroups. The numbers at the interior branches indicate posterior probabilities

resolution and most robust support for various nodes as much characters as possible should be included in the analysis (Grandcolas et al. 2001).

It has been shown that larger molecular data sets outperform smaller ones, but it remains to be demonstrated whether any amount of sequence data will be able to resolve certain phylogenetic problems if taxon sampling is limited (Cummings et al. 1995). With a denser taxon sampling and a fragment of 661 bp of *cytb*, we obtained very similar trees to the ones found by Pereira et al. (2002), that used > 10,000 bp and one species for each of the 11 genera currently recognized in the family, plus three outgroups. Although they analysed 16 times more DNA data than the present study, relationships among all the genera were not entirely resolved. Several bootstrap values and two clades changed depending on the inference method used. It is noteworthy that of 10 678 analysable sites used in the former study only 1869 were parsimony-informative, while in our combined analysis with three different sets of data we had 437 parsimony-informative sites of a total of 891 characters, constituting almost half of the analyzable sites.

In this case, the total-evidence approach turns out to be more efficient.

The previous molecular phylogeny reassesses the taxonomic status of the 11 genera currently recognized in the family. Pereira and Baker (2004) analysed several mitochondrial genes for all species within curassows and determined that *Pauxi* and *Mitu* are not reciprocally monophyletic. In 2005, Grau et al. (2005) examined the entire genus *Pipile* and the monotypic *Aburria*, proposing a merging of both into a single genus, with additional support of osteological data (see also Silveira 2003).

The apparently rapid radiation of many avian orders near the K-T boundary has complicated the resolution of their relationships using only molecular data. The rapid radiations are characterized by a considerable amount of morphological evolution that could lead to short-branch length estimates from molecular data, when morphological branch lengths are actually long (Lee et al. 1997; Lewis 2001). A set of osteological and integumentary + behavioural synapomorphies can arise to characterize a clade, whereas too little molecular change will have accumulated during the time interval between

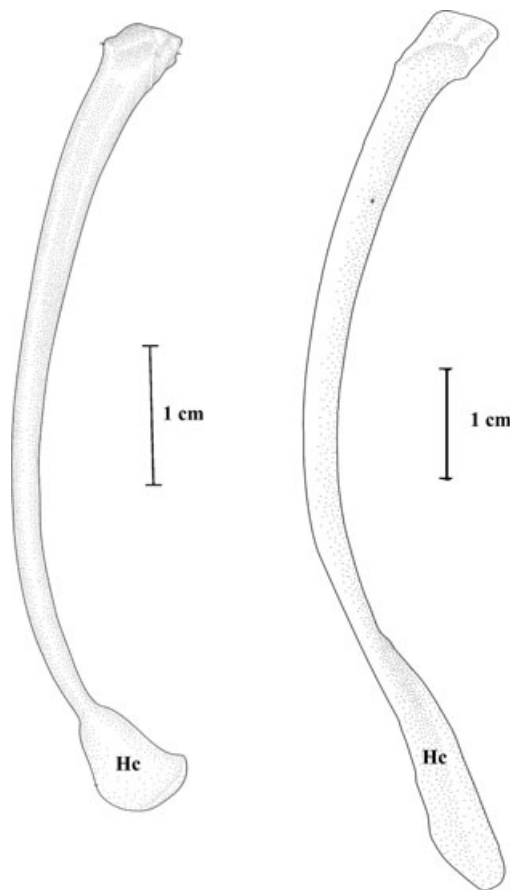


Fig. 7. Lateral view of left clavicle of *Penelope obscura* (MHNT 594, left) and *Crax fasciolata* (MHNT 727, right, character no. 101). Hc, Hypocleideum

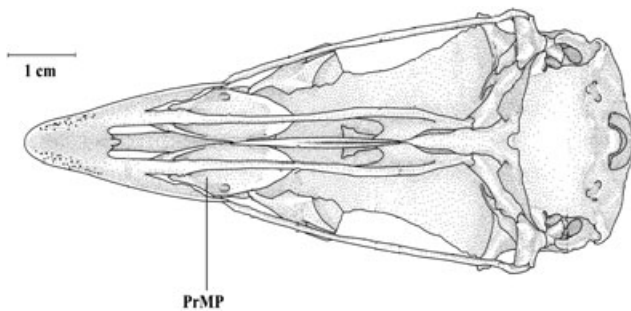


Fig. 8. Ventral view of *Penelope obscura* (MHNT 600) showing the large fossa in the processus maxillopalatinus (character no. 50). PrMP, Processus maxillopalatinus

Fig. 9. Artistic drawing representing nine species of the nine genera recognized in family Cracidae. From left to right: *Ortalis wagleri*, *Oreophasis derbianus*, *Aburria aburri*, *Penelopina nigra*, *Penelope purpurascens*, *Pauxi tomentosa*, *Chamaepetes unicolor*, *Crax globulosa* and *Nothocrax urumutum*



branching events. We are providing the first cladistic analyses of cracids based on osteological and integumentary + behavioural characters. Additionally, the current study greatly expands the taxonomic coverage compared with previous studies based on molecular data.

Regardless of methodological shortfalls, hierarchical classifications have been constructed on the basis of morphology and it seems that these classifications reflect and are congruent with several actual phylogenetic nodes (Jenner 2004). As mentioned before, it has also been widely demonstrated that behavioural data may produce well resolved trees (DeQueiroz and Wimberger 1993; McLennan and Mattern 2001; Birdsley 2002). But low bootstrap values and an increased number of most parsimonious trees are common when there are few characters, a typical case of morphological and behavioural analyses. As Magallón (2002) pointed out, there is an evident need for a different measure than bootstrapping for these kinds of data. Integumentary + behavioural characters contribute to combined analyses in the form of hidden clade support that becomes apparent only by joining different data sets. The descriptive efficiency and explanatory power of the data are thus potentially increased (Farias et al. 2000).

With an increased taxon sampling, we have collected and analysed molecular, osteological and integumentary + behavioural data separately and after corroborating that they yield similar phylogenetic hypotheses and have similar levels of consistency, we have analysed cracid relationships based on the combined data sets. When used separately, none of the three data sets provide a clear resolution of their relationships. Both Maximum Parsimony and Bayesian analysis including all genera with our three kinds of data provided increased resolution and a more robust support for various nodes compared to any of the previous separate analyses available for cracids. Our data rendered complementary information for each subfamily and with our total evidence-approach we were clearly able to infer only nine genera (Fig. 9).

The disagreement on which genera belong to each of the two subfamilies currently recognised has been a longstanding controversy. Contradictory results on the taxonomy of cracids have been obtained from several sources resulting from separate analyses. Pereira et al. (2002) estimated the divergence times of cracid genera, and both *Oreophasis* and *Ortalis* are among the oldest (31.1 and 30.9 Myr, respectively). Perhaps the Horned-Guan preserved several primitive characters such as overall coloration, the bare horn and the feathered base of the bill concealing the nostrils, as a result of its isolation. Our separate analysis supports the proposal by del Hoyo and Motis (2004) of some sort of link between both subfamilies. On the other hand, the chachalacas, while still within Penelopinae, also have peculiar characteristics that

distinguish them from the typical guans: their calls, display and ecology.

Cracinae

With integumentary + behavioural characters five synapomorphies support a clade including *Oreophasis*, *Nothocrax*, *Crax* and *Pauxi* + *Mitu*: they have robust and hooked bills and are heavier and larger than guans (excluding two species of *Penelope*); they also share elaborated ground display courtship, with males feeding females. *Crax* is the only genus within this subfamily with sexual dimorphism and only *Crax alector* Linnaeus, 1766 shows a less evident sexual dimorphism, which may be a primitive feature of this species. However, there is no discernible difference with the other curassows in courtship and general behaviour including males 'mooing' call. This call was attributed to a greatly lengthened trachea in males of *Nothocrax* and *Pauxi* + *Mitu*; but *Crax* and *Oreophasis* also present this vocalization although they lack the tracheal modification.

Pereira and Baker (2004) argued, among other things, that patterns of overall coloration of curassows could be used as evidence that *Nothocrax* is more closely related to *Mitu* and *Pauxi* than to *Crax*. But plumage coloration varies among Cracinae, *Nothocrax* has a reddish colour in both sexes, while *Mitu* is always glossy black with practically no sexual dimorphism and *Pauxi* could be both black and reddish; *Crax* males are always black, but several females can be as reddish as *Nothocrax*. Besides, curassows have a fully feathered throat while the Horned-Guan has a small naked area, also present in chachalacas, suggesting a plesiomorphic state.

Penelopinae

Five integumentary + behavioural synapomorphies support a clade including *Ortalis*, *Penelope*, *Penelopina*, *Aburria* + *Pipile* and *Chamaepetes*: they have thin and straight bills with no ornaments; medium size bodies and shorter-incubation time periods. *Penelopina* is the only genus with sexual dimorphism and the only female larger than the male. Only *Penelope* has tracheal modifications in both sexes and in various degrees of length (with two species lacking any loop), while *Ortalis* shows this modification exclusively in males, like some members of Cracinae. An elaborate flight display courtship can be seen in *Chamaepetes*, *Penelopina* and *Aburria* + *Pipile* and less complex in *Penelope*.

As with the curassows, plumage coloration varies within Penelopinae: black in *Aburria* + *Pipile*, *Chamaepetes unicolor* Salvin, 1867 and the males of *Penelopina*; reddish brown in *Penelope*, *Chamaepetes goudotii* (Lesson, 1828) and females of *Penelopina*; and duller colours in *Ortalis*. Wattles are notorious in *Penelopina*, *Aburria* + *Pipile* and *Penelope*, a naked area in the throat is present in *Ortalis* and *Chamaepetes* has a fully feathered throat.

Synonymy of taxa

Following the Principle of Priority of the International Code of Zoological Nomenclature, the genus *Mitu* Lesson, 1831 should be considered a synonym of *Pauxi* Temminck, 1813; and we can confirm that the genus *Pipile* Bonaparte, 1856 should be a synonym of *Aburria* Reichenbach, 1853, as proposed by Grau et al. (2005). All the taxa currently considered in the genus *Mitu* must be transferred to *Pauxi* and all the species of *Pipile* must be reassigned to the genus *Aburria*. So, following the taxonomy adopted by del Hoyo

et al. (1994) within the genus *Pauxi* (feminine) we have six species: *Pauxi pauxi* (Linnaeus, 1766); *Pauxi tomentosa* (Spix, 1825); *Pauxi tuberosa* (Spix, 1825); *Pauxi salvini* (Reinhardt, 1879); *Pauxi unicornis* Bond and Meyer de Schauensee, 1939 and *Pauxi mitu* (Linnaeus, 1766). In the genus *Aburria* (feminine), we consider five species: *A. aburri* (Lesson, 1828); *A. kujubi* (Pelzeln, 1858); *A. cumanensis* (Jacquin, 1784); *A. pipile* (Jacquin, 1784); and *A. jacutinga* (Spix, 1825) following Grau et al. (2005).

We believe that molecular data are extremely important, particularly when there is no other kind of information, but a multiple criteria approach can be significantly more efficient in terms of time and costs and provide a more balanced analysis.

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Resumen

Un incremento en el muestreo taxonómico y de caracteres revela nuevas relaciones intergenéricas en los cracidae (aves: galliformes)

La familia Cracidae pertenece a uno de los grupos más amenazados del mundo. Recientemente se han publicado diversos estudios sobre su evolución y conservación. Los análisis filogenéticos con un fragmento de 661 bp del citocromo b mitocondrial para 39 diferentes especies de crácidos, corroboran la mayoría de las relaciones obtenidas en estudios anteriores. Este trabajo intenta refinar las hipótesis filogenéticas anteriores mediante un incremento en el muestreo taxonómico y la combinación de datos moleculares con caracteres osteológicos, integumentarios y conductuales mediante análisis de máxima parsimonia (MP) y Bayesianos. Con nuestros datos moleculares, 152 caracteres osteológicos y 74 caracteres integumentarios y conductuales, realizamos análisis por separado y combinados con evidencia-total. Aunque nuestros resultados favorecen la mayoría de las relaciones propuestas

por la hipótesis molecular previa, el árbol obtenido de la matriz combinada sugiere varias modificaciones en los géneros que constituyen a cada una de las dos subfamilias: Penelopinae y Cracinae, y apoya la fusión del género *Pipile* con *Aburria* y de *Mitu* con *Pauxi*. Estos resultados sugieren que un incremento en el muestreo taxonómico y la inclusión de diferentes tipos de caracteres puede ser tan importante como un gran muestreo de un solo tipo. Además, de un total de 891 caracteres, obtuvimos 437 sitios parsimoniosamente informativos (casi la mitad de los sitios analizables) probando la eficacia de un enfoque de evidencia total.

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Authors' addresses: Katya Frank-Hoeflich (for correspondence), Daniel Piñero, Departamento de Ecología Evolutiva, Instituto de Ecología, UNAM, 04510, México D.F., México. E-mail: katya@miranda.ecologia.unam.mx; pineromx@yahoo.com.mx; Luís Fabio Silveira, Departamento de Zoologia, Universidade de São Paulo, São Paulo, Brazil. E-mail: lfsilvei@usp.br; Jesus Estudillo-López, Ana María García-Koch, Granja 'La Siberia', Vida Silvestre A.C. Av. Acozac s/n Ixtapaluca, Edo. de México, México. E-mail: crax@prodigy.net.mx; anamagk@hotmail.com; Laura Ongay-Larios, Unidad de Biología Molecular, Instituto de Fisiología Celular, UNAM, México D.F., México. E-mail: longay@ifc.unam.mx

Supplementary Material

The following supplementary material is available for this article online.

Appendix S1. List of osteological characters

Appendix S2. List of integumentary and behavioural characters

This material is available as part of the online article from <http://www.blackwell-synergy.com>