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Evolution of the Mitochondrial DNA Control Region and Cytochrome *b* Genes and the Inference of Phylogenetic Relationships in the Avian Genus *Lophura* (Galliformes)

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The entire mitochondrial DNA control region (mtDNA CR) and cytochrome *b* (cyt *b*) genes were sequenced in 10 of the 11 extant species of gallopheasants (*Lophura*). The cyt *b* from *L. diardi* and *L. ignita* showed unusual leucine-coding codons at the expected terminal 3′ end of the gene. Presence of conserved functional motifs in the inferred amino acid sequences, conserved secondary structures of the flanking tRNA^{Pro} and tRNA^{Thr}, and Southern hybridization concordantly suggest that these cyt *b* represent functional mitochondrial genes and not nuclear transpositions. Functional stop codons can be generated by RNA editing of the primary transcripts from these sequences. Despite strong site and domain substitution rate heterogeneity, CR and cyt *b* diverged at similar rates, on average, and expressed congruent phylogenetic signals. Phylogenetic analyses of the concatenated sequences split *Lophura* into five clades including (1) *L. bulweri,* (2) *L. diardi–L. ignita,* (3) *L. erythrophthalma–L. inornata,* (4) *L. leucomelanos–L. nycthemera,* and (5) *L. swinhoii–L. edwardsi–L. hatinhensis.* Basal relationships among these clades, which include species distributed in continental South East Asia and the Sundaland archipelago, were weakly resolved, suggesting the occurrence of rapid cladogenic events in the early evolutionary history of *Lophura.* A conventional calibration of mtDNA sequence divergence indicates a mid to late Pliocene evolution of the main clades in *Lophura,* which could have diversified in allopatry in continental South East Asia. Sundaland could have been colonized lately and independently by the different clades. Consequently, cyclic changes in late Pleistocene climate and landscape might not have increased rates of speciation in genus *Lophura* in Sundaland.

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INTRODUCTION

The *Lophura* gallopheasants (Galliformes; Phasianidae) are distributed across a wide range of lowland to highland, mainly forested habitats in South East Asia and Sundaland (Fig. 1). These regions support the highest number of pheasant species and, according to Johnsgard (1986), are considered centers of origin and major biodiversity of the entire family Phasianidae. Some of these species are threatened with extinction by severe population decline due to rampant deforestation in South East Asia (Jenkins, 1993). A reliable taxonomy, based on a supported phylogenetic framework, is urgently needed to implement conservation strategies for the endangered taxa in the genus *Lophura* (Hennache, 1997).

Lophura currently is split into 10–11 species and up to 32 subspecies, making this group the most taxonomically diverse group of pheasants (Johnsgard, 1986; McGowan, 1994). The pheasants in *Lophura* are sexually dimorphic, with inconspicuous and mimetic females, although, in some species, males also show cryptic plumages. Their reproductive displays are simpler than most other pheasants (Johnsgard, 1986; McGowan, 1994). Plumage colors, sexual dichromatism, specialized ornaments, courtship displays, and other anatomical and osteological traits have been used to infer evolutionary relationships and taxonomy of the pheasants (Verheyen, 1956; Delacour, 1977; Johnsgard, 1986). However, most of these phenotypic traits are not diagnostic and might have evolved repeatedly and independently among pheasants (Kim-

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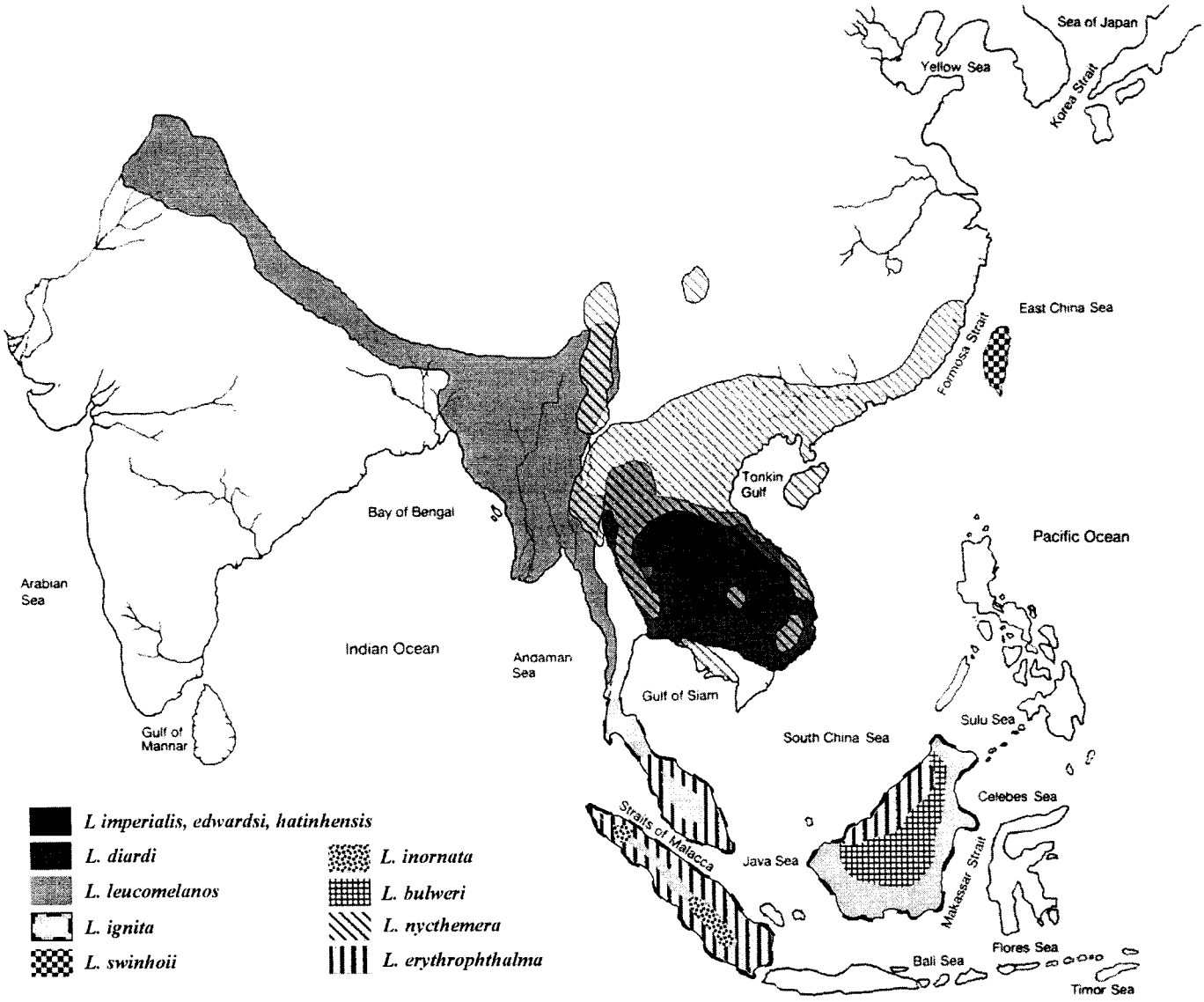

FIG. 1. Distribution map of the extant species of *Lophura* (adapted from Johnsgard, 1986; McGowan, 1994).

ball *et al.*, 1999). Thus, it is not surprising that the pheasants in *Lophura* include some taxonomic puzzles, such as the question of the rank of the endemic lowland Annamese taxa (*L. edwardsi, L. imperialis,* and *L. hatinhensis;* Scott, 1997; Randi *et al.*, 1997; Hennache *et al.*, 1998) and the interrelationships among nominal subspecies of *L. leucomelanos* and *L. nycthemera* (McGowan, 1994; McGowan and Panchen, 1994).

Estimating the phylogenetic relationships and extent of genetic divergence among evolutionary clades might help to unravel the biogeography of the pheasants in *Lophura* and thus contribute to the knowledge of South East Asian and Sundaland biogeography. The pheasants in *Lophura* include species widespread in continental South East Asia (i.e., *L. leucomelanos* and *L. nycthemera*), and island endemics (i.e., *L. bulweri, L. inornata,* and *L. swinhoii,* endemic to Borneo, Sumatra, and Taiwan, respectively; Fig. 1). Rates of molecular divergence have been used to date episodes of avian diversification in the Nearctic and Palearctic regions (Zink and Slowinski, 1995; Blondel and Mourer-Chauviré, 1998), raising controversial issues about the effect of climatic oscillations in the Pleistocene on avian evolution (Klicka and Zink, 1997, 1998; Avise and Walker, 1998; Avise *et al.*, 1998; Holder *et al.*, 1999). Avian biogeography in South East Asia is still at its infancy. We do not know whether the dramatic Pleistocenic climate changes which deeply affected faunal turnover in the northern hemisphere also influenced biodiversity in the Asian tropics (Heaney,

1986; Meijaard, 1999). So, it would be relevant to know whether (1) the species in *Lophura* diversified and speciated mainly in continental South East Asia and more recently colonized the Sundaland region through interisland land bridges exposed during Pleistocene glacial maxima or (2) population fragmentation in the Indonesian Archipelago, consequent to the mid Pleistocene eustatic sea level fluctuations, increased speciation rates in Sundaland and fostered the origins of extant island endemics. In the first scenario, the pheasants in *Lophura* would have diverged mainly before the Pleistocene, whereas in the second case, the phylogenetic trees should reveal the existence of at least some very recent clades including the island endemic species.

Biomolecular information is used extensively in current phylogenetic studies (Avise, 1994). Mitochondrial DNA sequences (mtDNA), in particular the control region (CR) and the cytochrome *b* (cyt *b*) genes, have been applied to study avian genetic diversification (Baker and Marshall, 1997; Moore and DeFilippis, 1997; Johns and Avise, 1998). Substitution rates vary at the different cyt *b* and CR nucleotide positions and structural domains (Moore and DeFilippis, 1997; Griffith, 1997; Randi and Lucchini, 1998), so these genes can provide phylogenetic information at different levels of evolutionary divergence (Baker and Marshall, 1997; Kidd and Friesen, 1998; Zink and Blackwell, 1998). A "conventional" mtDNA substitution rate of 2% per million year (Avise and Walker, 1998) has been used to date recent speciation events and to assess the effects of events in the Pliocene and Pleistocene on genetic diversification of vertebrates (Klicka and Zink, 1997; Avise and Walker, 1998; and references therein), despite the well-known debates concerning the existence of a universal constant molecular clock (see: notes 10 and 11 in Klicka and Zink, 1997).

In this paper we analyze nucleotide sequences from complete mtDNA cyt *b* and CR genes of the pheasants in *Lophura* to (1) evaluate the comparative patterns and rates of sequence divergence of these two genes, (2) produce a molecular phylogeny of the extant species of *Lophura*, (3) correlate the inferred phylogenetic relationships with Plio–Pleistocene biogeographical scenarios in South East Asia, and (4) propose hypotheses on the evolution of *Lophura* pheasants.

Sequence data revealed the presence of a leucine codon at the expected stop codon position at the 3′ end of the cyt *b* gene from *L. diardi* and *L. ignita*. Therefore, we have performed a set of experimental and analytical controls to determine whether these sequences represent true mitochondrial genes or nuclear transpositions (Sorenson and Fleischer, 1996; Quinn, 1997; Sorenson and Quinn, 1998).

MATERIALS AND METHODS

DNA Extraction, Amplification, and Sequencing

Total DNA was extracted from 95% ethanol-preserved tissue or feather root samples, using procedures described by Kimball *et al.* (1997) and by Randi and Lucchini (1998), and from blood samples, using the Puregene DNA isolation kit (Gentra Systems). The entire mtDNA CR and cyt *b* were PCR-amplified and sequenced as previously described (Randi and Lucchini, 1998; Kimball *et al.*, 1997, 1999). The additional primer H16417 (5′-GGGTTGGATTTGTTTTGG-3′), mapping on the heavy strand of the mtDNA and numbered according to the *Gallus* mtDNA sequence (Desjardins and Morais, 1990), was used to amplify and sequence the region downstream of cyt *b* in some samples.

Complete cyt *b* and CR sequences were obtained from 10 of the 11 extant species of *Lophura* and two closely related outgroups, *Phasianus colchicus* (ring-necked Pheasant) and *Crossoptilon crossoptilon* (white-eared Pheasant). Species names, origin of the studied specimens, and GenBank accession numbers of the individual sequences are listed in Table 1.

Criteria Used to Exclude Amplification and Sequencing of Nuclear Copies of the mtDNA Genes

In all the sequenced samples of *L. diardi* and *L. ignita* we have observed a leucine codon in place of the expected stop codon at the end of cyt *b* (see Results). Nuclear-mitochondrial-transposed sequences (numts) with homology to CR and cyt *b* exist in some avian and mammalian species (Sorenson and Fleischer, 1996; Quinn, 1997; Sorenson and Quinn, 1998). To check for the presence of numts in the genomes of *Lophura* and to validate the mtDNA origin of our sequences, we have performed the following analyses.

(1) Authenticity of cyt *b* sequences from *L. diardi*, *L. ignita*, *L. nycthemera*, and *P. colchicus* was controlled by Southern hybridization, using 5 μg of total DNA from one individual of each species, which was digested overnight with the restriction enzymes *Eco*RI, *Hin*dIII, *Mbo*I, and *Msp*I. All digested DNAs were obtained from blood samples, which are relatively poor in mitochondria; thus, nuclear and mitochondrial copies would be present in roughly equal amounts (Quinn, 1997). Digested DNA was separated on an 0.8% agarose gel, transferred to Hybond N membranes (Amersham) under alkaline conditions, and hybridized in phosphate–SDS buffer at 60°C with a probe (corresponding to the C-terminal region of cyt *b* amplified from *Gallus gallus* using primers L15662 and H16065; Kimball *et al.*, 1999) labeled with [α^{-32}P]dCTP. If there had been an insertion of either all or at least the C-terminal region of the mitochondrial cyt *b* gene into the nuclear genome of *L. diardi* and *L. ignita*, Southern hybridization

TABLE 1

List of the Studied Species of *Lophura*, with Indication of Their Natural Distributions and Prevalent Habitat Types (after McGowan, 1994)

Species	Common name	Distribution	Habitat	Accession Nos.
L. bulweri	Bulwer's pheasant	Borneo, Bangka Island (Indonesia)	Mountain forest	AF314637 AJ300146
L. inornata	Salvadori's pheasant	Sumatra	Mountain forest	AF314642 AJ300152
L. erythrophthalma	Crestless fireback	Peninsular Malaysia, Sumatra, North Borneo	Lowland forest	AF314639 AJ300149
L. diardi	Siamese fireback	Thailand, Indochina	Lowland forest	AF028797 AJ300147
L. ignita	Crested fireback	Peninsular Malaysia, Sumatra, Borneo	Evergreen lowland and mountain forest	AF314641 AJ300151
L. leucomelanos	Kalij pheasant	Himalayas, Burma, Penninsular Thailand	Lowland and mountain forest	AF314643 AJ300153
L. nycthemera	Silver pheasant	Southeastern China, Burma, Thailand, Indochina	Forested hills and mountains	L08380 AJ300154
L. swinhoii	Swinhoe's pheasant	Taiwan	Forest	AF314644 AJ300155
L. edwardsi	Edward's pheasants	Central Annam (Vietnam)	Evergreen lowland forest	AF314638 AJ300148
L. hatinhensis	Vietnamese pheasant	North of Central Annam and Vietnam	Lowland forest	AF314640 AJ300150
Outgroups				
Phasianus colchicus	Ring-necked pheasant	Caucasus		AJ298920 AF028798
Crossoptilon crossoptilon	White eared-pheasant	China		AJ298921 AF028794

Note. GenBank Accession Nos. of the cytochrome *b* (cyt *b*, AF numbers and L08380) and control region (CR, AJ numbers) sequences are reported. The tissue samples were collected from pheasants reared at Parc Zoologique de Clères, Clères, France, except for the Bulwer's pheasant (from Antwerp Zoological Garden, Antwerp, The Netherland) and the Vietnamese pheasant samples (from Hanoi Zoological Garden, Hanoi, Vietnam).

should indicate the presence of two copies of this region (Sorenson and Quinn, 1998). We have detected numts with this technique in other avian taxa (R. T. Kimball, J. L. Bollmer, and P. G. Parker, unpublished data).

(2) Authenticity of CR sequences from two representative species of *Lophura* (*L. edwardsi* and *L. nycthemera*) and *P. colchicus* was controlled by PCR amplification and sequencing of the DNA obtained from mitochondrial purifications through alkaline lysis of liver samples (Palva and Palva, 1985).

(3) Moreover, we have sequenced the two tRNAs ($tRNA^{Thr}$ and $tRNA^{Pro}$) which map downstream to the cyt *b*. The dynamics of nucleotide substitutions and indels are constrained to preserve the secondary structure in functional tRNAs; thus, these sequences must fold in energetically stable secondary structures and substitutions might be compensated in the more conserved stem regions (Mindell *et al.*, 1998). Free energies at 37°C were calculated using the efn server (M. Zuker, Washington University), which uses calculations based on Walter *et al.* (1994).

Sequence Alignment and Phylogenetic Analyses

The CR and cyt *b* sequences were aligned using CLUSTAL X with the default options (Thompson *et al.*, 1997). The cyt *b* sequences were uniform in length; so, alignment was straightforward. The CR alignment was manually edited using SE-AL (version 1.0; A. Rambaut; http://evolve.zoo.ox.ac.uk/Se-Al/Se-Al.html). The boundaries of the CR-I, CR-II, and CR-III domains and of the inner, outer, and transmembrane cyt *b* domains were mapped following Randi and Lucchini (1998) and Zhang *et al.* (1998), respectively. Substitution rates in the different partitions of the sequences were estimated by the maximum-likelihood (ML) procedure implemented in the computer program LRHET (Gaut and Weir, 1994). A likelihood test for regional heterogeneity of substitution rates was performed using PLATO (version 2.01; N. Grassly and A. Rambaut; http://evolve.zoo.ox.ac.uk/Plato/Plato2.html). Compositional stationarity and reliability of substitution models to compute genetic distances were tested with MODELS (Rzhetsky and Nei, 1995).

Length differences were limited to short parts of CR-I and CR-III, which required the insertion of indels usually shorter than three to four nucleotides. Regions with indels were difficult to align and thus we excluded 44 gapped sites from the phylogenetic analyses. Intraspecific divergence was lower than 0.5% (range

0.005–0.001), and the addition of multiple individuals did not affect the topologies nor the bootstrap values of the trees (not shown); so, we used one sequence per species for all the phylogenetic analyses.

Phylogenetic congruence of the combined cyt *b*+CR alignment was assessed by the partition-homogeneity test (Farris *et al.*, 1995), with 1500 replicates, as implemented in PAUP* (version 4.0b2a; Swofford, 1998). Phylogenetic analyses were performed by (1) the exhaustive maximum-parsimony procedure (MP; Swofford, 1998), excluding all uninformative nucleotide positions, with unordered and equally weighted characters; (2) the neighbor-joining algorithm (NJ; Saitou and Nei, 1987), with Tamura and Nei's (1993) DNA distances (TN93) and HKY model (Hasegawa *et al.*, 1985); and (3) the heuristic maximum-likelihood procedure (Felsenstein, 1981), as implemented in PAUP*.

Additional heuristic MP searches, with 10 random addition replicates, TBR and MulTree options in use, were computed using the following weighting schemes: (1) transversions (Tv) and transitions (Ti) were weighted differently, accordingly to the substitution trends observed in the two genes (see Results), to confer more weight to the more slowly evolving and putatively less homoplasious types of changes; (2) the parsimony-informative characters were individually reweighted by maximum values of the consistency index (CI), rescaled consistency index (RI), and retention index (RC), to maximize the phylogenetic contribution of the sites showing the lowest value of homoplasy; and (3) six-parameters inverse weights (Williams and Fitch, 1990), a procedure which may improve phylogenetic analyses of mtDNAs (Cunningham, 1997), were obtained as follows: first, the number of each type of nucleotide substitution in the MP tree was estimated using MACCLADE (version 3.05; Maddison and Maddison, 1992); second, raw weights proportional to the total number of mutations divided by the number of mutations in a particular class were computed; and, finally, the weight matrix implied by these raw weights was corrected for violation of the triangle inequality using MACCLADE.

The best-fit ML model of DNA substitution was selected by likelihood ratio tests among a suite of models of increasing complexity, performed according to the following procedure (Huelsenbeck and Crandall, 1997): we obtained the exhaustive unweighted MP tree using the combined cyt *b* + CR alignment; this topology was used to compute the likelihood scores for substitution models of increasing complexity; the best-fit models were assessed by the likelihood ratio test $\Delta = 2(\log L_1 - \log L_0)$, with $\log L_1$ = likelihood of the most complex model and $\log L_0$ = likelihood of the simpler model. The Δ values, which are distributed accordingly to a χ^2 distribution (Huelsenbeck and Crandall, 1997), were used to determine whether simpler models should be rejected in favor of the more complex models.

Among-site substitution rate heterogeneity was modeled by the discrete-rate γ-distribution (Yang, 1994). The values of shape parameter α of the γ-distribution and the Ti over Tv ratios, for cyt *b*, CR, or the combined sequences, were estimated by PAUP* via heuristic ML, using the best-fit substitution model and four discrete-rate categories.

Robustness of the phylogenies was assessed by bootstrap percentages (BP; Felsenstein, 1985), with 1000 random resamplings with replacement. We have considered the clades with BP >70% to be well supported (Hillis and Bull, 1993; Kimball *et al.*, 1999). Moreover, we have computed the decay index (DI; Bremer, 1988), which corresponds to the number of extra parsimony steps required to disrupt the monophyly of each clade, using the computer program AUTODECAY (version 4.0; T. Eriksson, 1998; http://www.botan.su.se./Systematik/Folk/Torsten.html). Statistical differences among alternative phylogenetic trees were tested by Kishino and Hasegawa's (1989; KH) likelihood test as implemented in PAUP*.

RESULTS

Authenticity of the Mitochondrial Sequences

When numt sequences are present, the nuclear and mitochondrial products are often coamplified by PCR, and sequences from these products may exhibit double peaks and ambiguities at some positions (Sorenson and Fleischer, 1996). In our study all the cyt *b* and CR PCR products appeared as clean single bands of the expected molecular weight when controlled using 1% agarose gel electrophoresis. The electropherograms from all sequencing reactions did not exhibit multiple peaks, suggesting that only a single PCR product was sequenced. Thus, it did not appear that we were coamplifying both nuclear and mitochondrial copies of the cyt *b* and CR genes in any sample.

The CR sequences of *Lophura* were easily aligned with the homologous CR sequences from *G. gallus* and *Coturnix japonica*, which were obtained from clones of purified mtDNA which were not PCR-amplified (Desjardins and Morais, 1990, 1991). The CR sequences that we obtained from alkaline lysis mtDNA extractions were identical to the sequences amplified from total DNAs in *P. colchicus* and in two species of *Lophura* (see also Randi and Lucchini (1998), which obtained similar results in a study of mtDNA CR from the *Alectoris* partridges). Although the alkaline lysis procedure does not exclude possible nuclear DNA contaminations, these results suggest that the PCR products are authentic mtDNA CR rather than numt sequences.

All the cyt *b* sequences from *Lophura* and the outgroups had an open reading frame without indels or internal stop codons. However, in the cyt *b* sequenced

from three unrelated individuals of *L. diardi*, we observed CTG, a leucine codon, in the position where the stop codon normally would be located (Fig. 2a). Moreover, three unrelated samples of *L. ignita* had TTG, another leucine codon, at that position (Fig. 2a). Leucine codons were present in all individuals of *L. diardi* and *L. ignita* examined. Therefore, these unusual mutations are likely to be representative of these species. All the other *Lophura* species, *Phasianus*, and *Crossoptilon* had a typical mitochondrial TAG stop codon (Fig. 2b; Kornegay *et al.*, 1993; Randi, 1996; Kimball *et al.*, 1999).

Several lines of evidence indicated that the sequences from *L. diardi* and *L. ignita* represented functional cyt *b* genes and not numts. First, *L. diardi*, *L. ignita*, and all the other sequenced *Lophura* and outgroups contained a full-length open reading frame that encoded a protein with high similarity to other cyt *b* proteins, including the cyt *b* from the complete *G. gallus* mitochondrion (Desjardins and Morais, 1990). The heme-ligating histidines and other conserved residues (Howell, 1989; Zhang *et al.*, 1998) could be identified, suggesting that our sequences were functional genes (e.g., Kornegay *et al.*, 1993; Arctander, 1995). Comparisons of functional genes and their numts (Sorenson and Quinn, 1998) indicated that numts often accumulate mutations that would result in amino acid changes in highly conserved regions even in the absence of insertions and deletions (Arctander, 1995), making an examination of such regions a suitable method to detect numts.

Second, a comparison of the tRNAs downstream from cyt *b* indicated that those sequences were likely to represent mitochondrial sequences. In the highly conserved $tRNA^{Thr}$, we observed a single insertion in *P. colchicus*, which was in a loop and not in a stem (Fig. 2a). The free energy of $tRNA^{Thr}$ from *G. gallus* (Desjardins and Morais, 1990) is the same as that in *L. diardi* and *L. ignita* (i.e., −10.6 kcal/mol), providing no evidence of deleterious mutations likely to have accumulated in a numt. The more variable $tRNA^{Pro}$ has several mutations in the stem regions. However, an examination of those mutations indicates some compensatory mutations within the stem (Fig. 2a; see asterisks). Using the complement of the sequences shown in Fig. 2a, the estimated free energy of $tRNA^{Pro}$ was lower in *L. diardi* and *L. ignita* (−11.2 kcal/mol) than in *G. gallus* (−15.2 kcal/mol). An examination of the free energies from mitochondrial $tRNA^{Pro}$ sequences (Mindell *et al.*, 1998) indicates that the free energy of $tRNA^{Pro}$ is quite variable and can be as low as −8.1 kcal/mol (*Smithornis sharpei;* Mindell *et al.*, 1998). The presence of compensatory mutations in the stem regions and an absence of mutations that reduce the overall stability of the tRNAs provides additional evidence that the sequences were mitochondrial and not nuclear.

An examination of the autoradiographs indicated that a single band was present in *L. diardi* and *L. ignita* for all four enzymes examined (not shown). Therefore, there is no evidence for a cyt *b* numt in *L. diardi* or *L. ignita*.

We suggest that RNA editing (Börner *et al.*, 1997; Smith *et al.*, 1997), which can generate a functional stop codon by cleavage after the downstream T (Fig. 2b, region B) and polyadenylation of the resulting transcript to form a UAA stop codon, is the most likely explanation for the observed CTG and TTG codons (see Discussion).

Structure and Sequence Variability of the cyt b and CR Genes of Lophura

All the *Lophura* cyt *b*, except those from *L. diardi* and *L. ignita*, were 1143 nt long. Nucleotide substitutions were homogeneously distributed across the sequences, and PLATO did not detect any regions evolving at significantly different rates. The proportions of variable and parsimony-informative sites were similar at inner, outer, and transmembrane domains (Table 2). Substitution rates among sites were heterogeneous, with $\alpha = 0.18$, and, as expected, third codon positions showed the greatest proportion of variable sites (Table 2).

The base composition of the cyt *b* sequences was biased (25.8% A, 26.5% C, 14.0% G, and 33.7% T, on average), with a bias against G, which is usual for the mtDNA sense strand of vertebrates (Wolstenholme, 1992). Second codon positions had the most biased G + C frequency (Table 2), and third positions had the highest C values (46%) and the lowest G value (2.4%). However, nucleotide frequencies were not significantly different among species and stationarity was not rejected by MODELS ($I = 32.29$, $P = 0.50$). The TN93

FIG. 2. (a) Alignment of the C-terminal region of cytochrome *b*, $tRNA^{Thr}$, $tRNA^{Pro}$, and the C-terminal region of ND6. Mitochondrial light-strand sequences are aligned to the sequence of the complete mitochondrial genome of the domestic chicken, *Gallus gallus* (Desjardins and Morais, 1990). Boundaries for the open reading frame of coding genes, tRNAs, and stem regions are those used by Desjardins and Morais (1990). A, Stop codon of cytochrome *b* in most avian taxa; B, intergenic region separating cytochrome *b* and $tRNA^{Thr}$; C, intergenic region separating $tRNA^{Thr}$ and $tRNA^{Pro}$; D, intergenic region separating $tRNA^{Pro}$ and ND6. Brackets around tRNA sequences indicate the base-paired stem regions. Cytochrome *b* and $tRNA^{Thr}$ are encoded on the light strand (indicated with a solid line); $tRNA^{Pro}$ and ND6 are encoded on the heavy strand (indicated with a dashed line). (b) The C-terminal end of cytochrome *b* and downstream sequence from a range of galliform species. A, Stop codon of cytochrome *b* in most avian taxa; B, intergenic region separating cytochrome *b* and $tRNA^{Thr}$. Sequences are from this study, Kimball *et al.* (1997, 1999), and T. M. Crowe (pers. comm.). GenBank Accession Nos. of the new tRNA sequences are: AF314632 (*L. diardi*), AF314633 (*L. ignita*), AF314634 (*L. nycthemera*), and AF314635 (*P. colchicus*).

a

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                 cyt b
G. gallus        ACCATCCTACTTATCCTCTTCCCCACAATCGGAACACTAGAAAACAAAATACTCAACTAC
L. diardi        ..T..T.....A............G........G.T..G................T...
L. ignita        ..T........A............G........G.C............T.......T...
L. nycthemera    ..T..T.....A............G.......G..T....................T...
P. colchicus     .....T.....C........T..TG..........C................T....T.

                 A   B   tRNAThr
G. gallus        TAA AAT ACTCTAATAGTTTA-TGAAAAACATTGGTCTTGTAAACCAAAAACTGAAGACTC
L. diardi        CTG --. ..............-.......................................A
L. ignita        .TG --. ..............-.......................................A
L. nycthemera    ..G --. ..............-.......................................G
P. colchicus     ..G --. .............A.A.....................................A

                                 C
G. gallus        CACCCTTCTTAGAGTA -- TCAGAAAAGGAGGGCTCAAACCTCCATCTCCAGCTCCCAAAG
L. diardi        ................ AC ........AA...A.........TTC................
L. ignita        ................ AC ........AA...A..T......TTC................
L. nycthemera    ................ AC ........AA...A..T.......TC................
P. colchicus     ................ AC ........AA...A..T.....TTC................
                                        **                  **

                 tRNAPro      D                ND6
G. gallus        CTGGTATTTTCAAATAAACTACTCTCTG AAA-----CCC TTAAACCGCCCGAATTGCCCC
L. diardi        ...A......-..........T.T.... ..GAGGCC... C..............N.....
L. ignita        ...A......-..........T.T.... ..G---CC... C..............C.....
L. nycthemera    ..........-..........T.T.... ...-----... C..............N.....
P. colchicus     ...A.....-.....T.T..... ...-----T.. C..............C.....
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b

	cyt b	A	B	tRNAThr
Lophura diardi	CTCAATTAC	CTG	T	ACTCTAATAGTTTATGA
Lophura ignita	CTCAATTAC	TTG	T	ACTCTAATAGTTTATGA
Lophura nycthemera	CTCAATTAC	TAG	T	ACTCTAATAGTTTATGA
Phasianus colchicus	CTTAACTAC	TAG	T	ACTCTAATAGTTTAATA
Crossoptilon crossoptilon	CTCAACTAC	TAG	T	ACTCTAACAGTTTATGA
Catreus wallichi	CTCAACTAC	TAG	T	ACTCTAATAGTTTATGA
Chrysolophus pictus	CTCAACTAC	TAG	T	ACTCTAATAGTTTATGA
Syrmaticus reevesi	CTCAACTAC	TAA	GT	ACTCTAATAGTTTATGA
Gallus gallus	CTCAACTAC	TAA	AAT	ACTCTAATAGTTTATGA
Bambusicola thoracica	CTCAACTAC	TAA	AAT	ACTCTAATANTTNATGA
Francolinus francolinus	CTCAACTAC	TAA	AT	ACTCTAATAGTTTATGA
Afropavo congensis	CTAAATTAC	TAA	AAT	ACTCTAATAGTTTATGA
Pavo muticus	CTAAACCAC	TAA	AAT	ACTCTAATAGTTTATGA
Argus argusianus	CTCAACCAC	TAA	AAT	ACTCTAATAGTTTATGA
Polyplectron bicalcaratum	CTCAACTAC	TAA	AAT	ACTCTAATAGTTTATGA
Pucrasia macrolopha	CTTAATCAC	TAA	AT	ACTCTAATAGTTTATGA
Lophophorus impeyanus	CTAAACCTC	TAA	AT	ACTCTAATAGTTTATGA
Perdix perdix	CTTGACTAC	TAG	AT	ACTCTAATAGTTTATGA
Tympanchus phasianellus	CTCAGCCAC	TAA	AT	ACTCTAATAGTTTATGA
Cyrtonyx montezumae	CTTAAACTC	TAA	T	ACTCTAATAGTTTATAA
Crax pauxi	CTCTATCAC	TAA	AAT	ACTCTAATAGTTTACAA
Alectura lathami	CTGAACTAC	TA		ACTCTAATAGTTTATAA
Leipoa ocellata	CTGAACTAC	TA		ACTCTAATAGTTTATAA

TABLE 2

Nucleotide Composition and Sequence Variability at First, Second, and Third Codon Positions, at Inner, Transmembrane, and Outer Cytochrome *b* Domains (Defined after Zhang, 1998), and at Control Region IA, IB, II, and III Domains (Defined after Randi and Lucchini, 1998) of *Lophura*

	cyt *b* Codon positions			cyt *b* Membrane domains			CR domains			
Domains	First	Second	Third	Inner	Trans	Outer	IA	IB	II	III
Nucleotides	381	381	381	294	519	330	188	128	468	365
GC proportion	0.50	0.39	0.49	0.44	0.48	0.47	0.42	0.43	0.51	0.28
Variable sites[a]	0.06	0.01	0.40	0.20	0.23	0.24	0.11	0.41	0.07	0.10
Parsimony sites[b]	0.75	0.80	0.48	0.51	0.45	0.48	0.60	0.65	0.39	0.46
Observed Ti/Tv	3.1	<1	12.15	8.87	14.71	8.89	1.62	3.78	2.92	3.53
Substitution rates[c]	0.023	0.004	0.195	0.067	0.074	0.071	0.050	0.212	0.087	0.048

[a] Observed proportion of total nucleotides that are variable.
[b] Observed proportion of variable nucleotides that are parsimony informative.
[c] Average proportion of nucleotide substitutions, as estimated by maximum-likelihood procedure, using LRHET.

model was an appropriate estimator of genetic distances ($T = 6.94$, $P = 0.22$).

The length of the CR of *Lophura* was relatively conserved (average 1148 nt, min. 1145, max. 1152 nt), and addition of indels was limited to the hypervariable parts of CR-I and CR-III. Nucleotide substitutions occurred more frequently in the peripheral domains and particularly in the second part of CR-I (CR-IB), where a region spanning 128 nucleotides, which corresponds to 11% of CR length, showed 41% of the total variable sites. On the contrary, the first part of CR-I (CR-IA) was conserved (Table 2). The average proportions of nucleotide substitutions at CR-IA, CR-IB, CR-II, and CR-III among *Lophura* were significantly different ($P < 0.01$; Table 2). The faster substitution rate of CR-IB was detected by PLATO, using either a uniform or a γ-distributed substitution model ($Z = 32.9$, $P < 0.052$ and $Z = 22.5$, $P < 0.05$, respectively). Site-to-site substitution rates were also heterogeneous, with $\alpha = 0.09$.

The CR of *Lophura* exhibited a biased base composition (26.9% A, 32.0% T, 26.6% C, and 14.5% G). The average G + C content (41.1%) was similar to that of other birds (e.g., Baker and Marshall, 1997; Randi and Lucchini, 1998) and not so extreme as that reported for other vertebrates (e.g., G + C was only 30% in the CR of rainbow fishes; Zhu *et al.*, 1994). The G content was particularly low at CR-III (G = 7.8%), whereas the nucleotide composition was less biased at CR-II (Table 2). However, nucleotide frequencies were not significantly different among species and stationarity was not rejected by MODELS ($I = 30.25$; $P = 0.30$), which indicated that the TN93 model is an appropriate estimator of genetic distances ($T = 5.97$; $P = 0.31$).

Comparative Rates of Sequence Evolution in the cyt b and CR Genes

A plot of TN93 + γ genetic distances showed that the CRs evolved faster than the cyt *b* in comparisons involving the less divergent species (those with genetic distances <0.10), but evolved more slowly than most cyt *b* sequences in comparisons among the most divergent species and among *Lophura* and their outgroups (Fig. 3a). With the exception of the hypervariable CR-IB, the proportions of nucleotide substitutions were comparatively higher in cyt *b* than in CR (Table 2). To compare rates of CR and cyt *b* divergence, TN93 + γ distances for the CR were divided by the distances for cyt *b*, and these values were then averaged across all comparisons. These ratios showed that the CR evolved, on average, about 1% more slowly than cyt *b*, suggesting that, except for CR-IB, the mtDNA control regions do not evolve faster than cyt *b* (see also Randi and Lucchini, 1998).

The average transition to transversion ratios (estimated by TN93 + γ substitution model) were Ti/Tv = 6.7 in *Lophura* and 3.0 among *Lophura* and the outgroups for the CR, and Ti/Tv = 18.7 in *Lophura* and 11.4 among *Lophura* and the outgroups for the cyt *b*. Thus, the cyt *b* showed about three times more Ti, but fewer Tv than the CR (Figs. 3b and 3c), in agreement with the well-known functional constraints acting on protein-coding genes (Irwin *et al.*, 1991; Kumar, 1996). As expected, most nucleotide substitutions, mainly transitions, accumulated at third codon positions of cyt *b* (Table 2). Therefore, the main contribution to sequence divergence among the species in *Lophura* was due to third codon Ti in the cyt *b* and to Tv substitutions in the CR.

Phylogenetic Relationships among the Lophura

The partition-homogeneity test indicated that cyt *b* and CR display congruent phylogenetic signals ($P = 0.407$, using 230 parsimony-informative characters). Thus, we analyzed the concatenated sequence alignment, which was 2291 nt long, on average. Analyses of the individual cyt *b* and CR partitions produced results similar to those obtained using the catenated data.

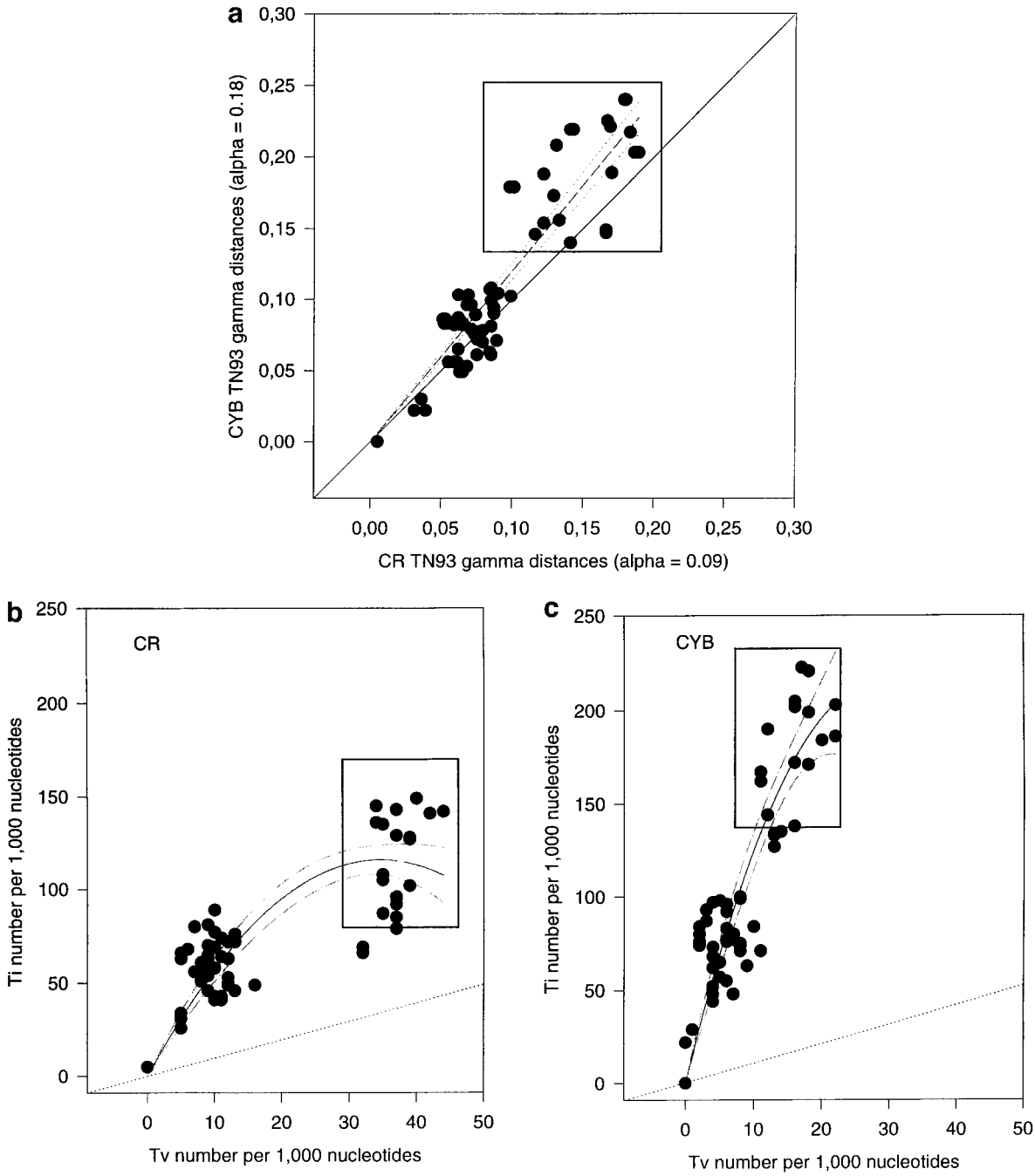

FIG. 3. (a) Cytochrome *b* (cyt *b*) vs control region (CR) TN93-γ distances among *Lophura* (lower left) and between *Lophura* and the outgroups (upper right, boxed). (b) Transition (Ti) vs transversion (Tv) number per 1000 nucleotides in the CR sequences of *Lophura* and the outgroups (boxed). (c) Transition (Ti) vs transversion (Tv) number per 1000 nucleotides in the cyt *b* sequences of *Lophura* and the outgroups (boxed). The diagonal dotted lines indicate the expected values in case of identical rates of Ti and Tv substitutions in cyt *b* and CR genes. The fitted functions are second order polynomials (±95% confidence intervals). DNA distances and substitution numbers may be phylogenetically correlated; thus, the fitted polynomials are intended to show the trends of the plottings and not to analyze their statistical properties.

A single MP tree (length L = 521; CI = 0.495; RI = 0.472; $g_1 = -1.1030$; $P < 0.01$; Hillis and Huelsenbeck, 1992) was obtained by exhaustive search using 230 parsimony-informative and equally weighted characters (Fig. 4a). In this tree, *Lophura* was split into four distinct clades: *L. bulweri* (clade 1), basal to all the other species; *L. diardi–L. ignita* (clade 2); *L. erythrophthalma–L. inornata* (clade 3); *L. leucomelanos–L. nycthemera* (clade 4a); and *L. swinhoii–L. edwardsi–L. hatinhensis* (clade 4b). The relationships among sister

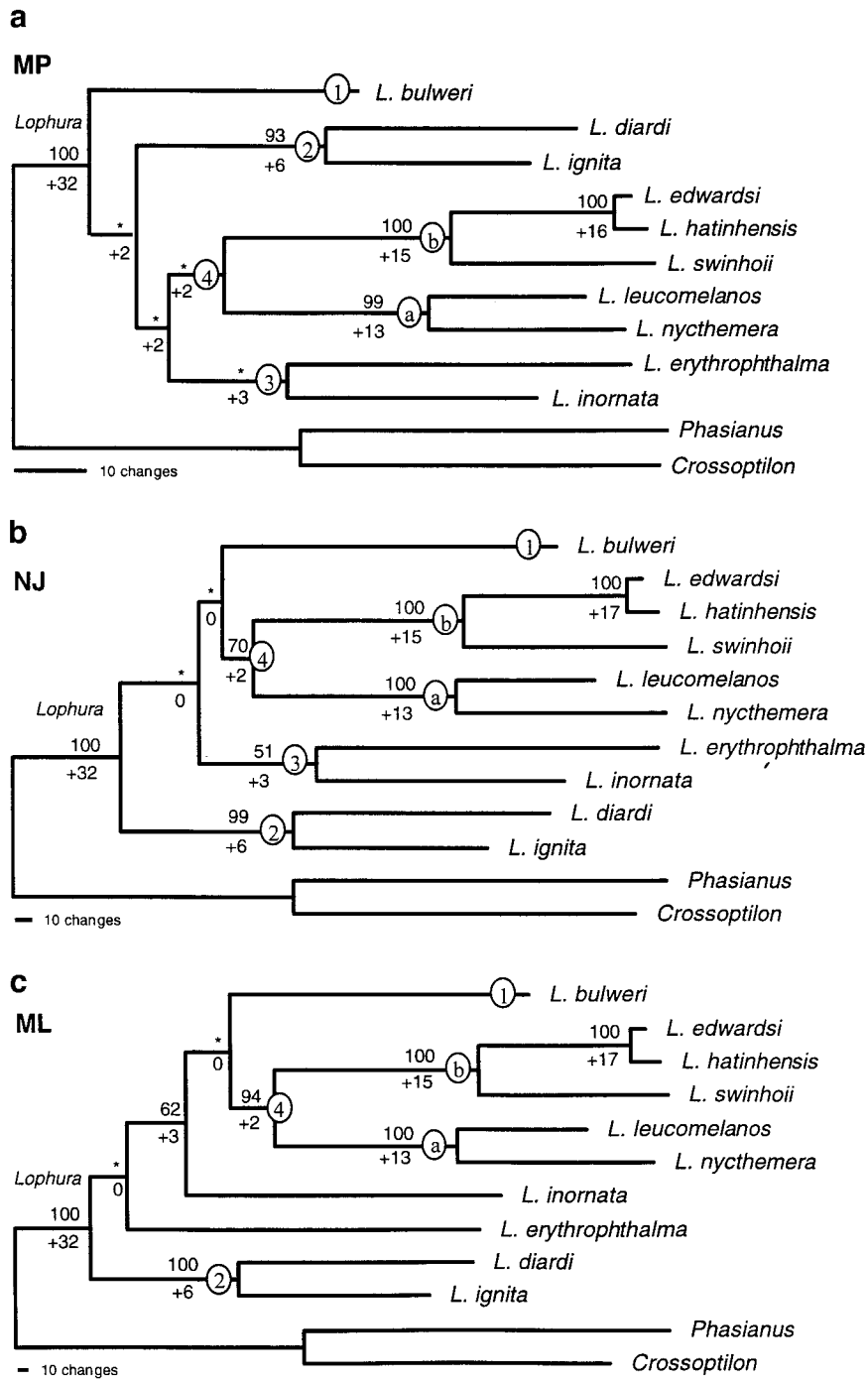

FIG. 4. (a) Unweighted maximum-parsimony tree of *Lophura* obtained by exhaustive search with the concatenated CR + cyt *b* alignment; (b) neighbor-joining tree obtained using TN93 genetic distances; (c) heuristic maximum-likelihood tree computed using HKY + γ substitution model. The main clades are identified by the numbers at internodes; BP and DI values are reported above and below the internodes, respectively (asterisks indicate BP values <50%). The trees are rooted using *Phasianus colchicus* and *Crossoptilon crossoptilon* as outgroups.

species joining in clades 2, 4a, and 4b were supported by high BP (>90%) and DI (> +6) values, whereas the pairing of *L. erythrophthalma* and *L. inornata* (clade 3) and the basal relationships among the five clades were weakly supported (Fig. 4a).

Heuristic searches conducted with individual characters reweighted by the maximum values of CI, RI, and RC produced a single MP tree, with the same topology in each case, identical to the equally weighted MP tree (see Fig. 4a). Heuristic searches conducted

with either Tv = 6Ti (the average Ti to Tv ratio, estimated by ML heuristic procedure, with HKY + γ, four categories, and $\alpha = 0.63$, was 6.17) or six-parameters inverse weighting produced a single MP tree, showing clade 2 (*L. diardi–L. ignita*) in basal position to *L. bulweri* and to all the other species in *Lophura* (not shown). Bootstrap analyses supported clades 2, 4a, and 4b, but did not support clade 3 (*L. inornata–L. erythrophthalma*) nor the inferred basal relationships among the main clades. Thus, the results of MP analyses were sensitive to the different weighting schemes, but the support to the weaker internodes and the resolution of basal relationships among the four main clades did not improve.

The NJ trees, computed with TN93, with or without γ-distribution ($\alpha = 0.6$), differed from the MP trees by placing clades 2 (*L. diardi–L. ignita*) and 3 (*L. erythrophthalma–L. inornata*) in basal position and *L. bulweri* in closer relation to clade 4 (Fig. 4b). Basal relationships among clades 1, 2, and 3 were not supported. BP and DI values supported strongly clades 2, 4a, and 4b (BP > 99%; DI > +6), marginally clade 4 (BP = 70%; DI = +2), and very weakly clade 3 (BP = 51%; DI = +3). The NJ trees were identical to the minimum-evolution trees, computed using heuristic searches with negative branches not allowed, TBR and MulTree options, and the TN93 or HKY substitution models.

Maximum-likelihood model fitting indicated that the most complex (parameter-rich) models are the most appropriate to describe the evolution of the concatenated sequences. Using the exhaustive MP tree as reference, the log L scores varied from −7382.18, for the simplest Jukes–Cantor (1969) model, to −6825.82, for the most complex general-time-reversible model (GTR; Lanave *et al.*, 1984). However, the GTR score was not significantly different from the less complex HKY model (log L = −6827.67, $\Delta = 3.70$, $df = 4$, $P > 0.05$), which was therefore used for subsequent analyses. The addition of a γ-distribution (HKY+γ with four categories) significantly improved the score (log L = −6811.81, $\Delta = 31.72$, $df = 1$, $P > 0.05$), whereas the constraint of a molecular clock worsened the fitting (HKY + γ + *c;* log L = −6819.13, $\Delta = 14.64$, $df = 3$, $P < 0.01$). We have, therefore, computed a ML tree with the HKY + γ model (four categories, $\alpha = 0.63$, Ti/Tv = 6.17 as estimated from the data, log L = −6803.44; Fig. 4c), which placed clade 2 in basal position to all the other species in *Lophura,* as in NJ, and disrupted clade 3, whereas *L. bulweri* was close to clade 4. BP values for the ML tree (estimated using the quartet-puzzling approximation) gave support to clades 2 and 4 (joining the strongly supported clades 4a and 4b), but not to the basal relationships. Thus, ML analysis did not improve the resolution of the basal relationships among clades in *Lophura,* but significantly increased the support to clade 4 (4a + 4b) and definitely disrupted the weakly supported clade 3 (*L. erythrophthalma–L. inornata*). However, KH likelihood tests showed that the MP, NJ, and ML topologies are not significantly different from each other ($P > 0.28$).

We do not feel that the basal relationships among the *Lophura* species are likely to be obscured due to saturation of the rapidly evolving sites of either cyt *b* or CR. Independent analyses of the cyt *b* and CR partitions also were unable to resolve these relationships (data not shown), suggesting that one partition did not obscure results obtained from the other. In addition, weighting schemes and analyses that should accommodate homoplasy were also unable to resolve basal relationships. Previous analyses of mtDNA CR and cyt *b* sequences from *Tragopan,* a galliform genus of similar age, indicated that inclusion of rapidly evolving and presumably saturated sites improved rather than obscured phylogenetic resolution (Randi *et al.,* 2000), suggesting that saturation may not be problematic.

DISCUSSION

RNA Editing of Anomalous cyt b "Stop" Codons in L. diardi and L. ignita

Sequences from *L. diardi* and *L. ignita* revealed the presence of an anomalous cyt *b* leucine codon instead of the expected stop codon at the 3′ end of the gene. However, experimental and analytical results indicate that these sequences represent authentic mitochondrial genes and not nuclear transpositions. RNA editing (Börner *et al.,* 1997; Smith *et al.,* 1997) might generate functional stop codons in these cyt *b* by cleavage of the observed CTG and TTG codons after the downstream T (Fig. 2a, region B). The resulting transcripts could then be polyadenylated to form a UAA stop codon, as has been observed in other vertebrate mitochondria (e.g., Ojala *et al.,* 1981). The stop codon would cleave directly between cyt *b* and $tRNA^{Thr}$ and would result in a protein with 381, instead of the usual 380, amino acids in *L. diardi* and *L. ignita.* An examination of downstream sequence data from a variety of galliform species suggests a high degree of variability in the C-terminal and putative intergenic region between cyt *b* and $tRNA^{Thr}$ (Fig. 2b). Cleavage directly upstream of the $tRNA^{Thr}$ is consistent with all species examined (Fig. 2b). In two species, *Alectura lathami* and *Leipoa ocellata* (both in the family Megapodiidae), the stop codon is cleaved after UA. In other species, cleavage occurs after the intergenic nucleotides, though the number and type of nucleotides varies. The observed variation in the region downstream of cyt *b* may allow variation and mutation in the stop codon region to be tolerated, consistent with the observation in *L. diardi* and *L. ignita.* Thus, we argue that, before concluding that anomalous sequences represent numts, careful analysis of the sequence data, including

additional information such as that from tRNAs or Southern hybridization, should be performed. If no other anomalies are present, alternative hypotheses, such as RNA editing, should be considered.

Comparative Molecular Evolution of the mtDNA CR and cyt b in Lophura

The evolution of CR and cyt *b* is controlled by different selective constraints acting on the two genes, in particular by the presence of conserved functional motifs in the CR (Sbisà *et al.*, 1997; Randi and Lucchini, 1998) and by the protein-coding nature of the cyt *b* (Moore and DeFilippis, 1997). Variable site-to-site substitution rates might generate rapid saturation of Ti at hypervariable positions, thus reducing the Ti/Tv value of the CR compared to protein-coding genes (Yang, 1998). However, the CR seems free to accumulate more Tv than cyt *b*, thus preserving significant phylogenetic signal at relatively deep sequence divergence, as shown in *Lophura* and other species (Lockard *et al.*, 1995; Randi and Lucchini 1988; Kimball *et al.*, 1999).

CR and cyt *b* evolve at comparable rates of change, on average (Fig. 3), and exhibit congruent evolutionary signals. However, the basal relationships among the main clades of *Lophura* were not resolved, computed either with the concatenated alignment or with cyt *b* and CR sequences independently (not shown). The deeper internodes of the phylogenetic trees of *Lophura* were unsupported, and the internal edges were short relative to the terminal branches, suggesting that the main clades might have originated almost contemporaneously. In particular, the slowly evolving Tv have accumulated mainly in the external branches leading to the terminal sequences and not along the internal edges of the trees. In fact, the average internodal lengths (estimated using TN93 model, Tv only) were <0.4 Tv per 1000 nt and the terminal branch lengths were >1.0 Tv per 1000 nt in the concatenated CR + cyt *b* sequences. Thus, sequence divergence among *Lophura* has been contributed mostly by substitutions within and not between clades. These findings suggest that the early evolutionary history of *Lophura* was marked by rapid speciation events, which makes cladogenic reconstruction of the phylogenetic trees difficult (Lanyon, 1988). Rapid evolutionary radiations and difficulties in the resolution of higher level relationships have been reported also in other groups of galliforms (Kimball *et al.*, 1999).

Phylogenetic Relationships within Lophura

Mitochondrial DNA sequences support the monophyly of *Lophura* (see also Randi *et al.* 1997; Hennache *et al.*, 1998). *Crossoptilon* and *Phasianus* are among their most closely related extant sister genera (Kimball *et al.*, 1999). However, resolution of the position of *Lophura* relative to the other phasianids, and the identification of their sister lineages, will require the addition of more taxa and sequence data.

Phylogenetic relationships in *Lophura* reveal the existance of four main clades, connected by weakly supported nodes, independent of the methods used to reconstruct the trees. The MP trees confer a basal position to *L. bulweri*, whereas the NJ and ML trees place clades 2 and 3 in basal positions and suggest that *L. bulweri* could belong to a more derived lineage. However, these topologies were not significantly different from each other, and the concatenated CR + cyt *b* sequences could not resolve the basal relationships among the main clades in *Lophura*. Several clades that are resolved in the molecular phylogeny, particularly clades 2, 4, 4a, and 4b, have also been suggested in previous phylogenies (Delacour, 1977; Johnsgard, 1986). These phylogenetic reconstructions, which are based on mtDNA sequences alone, should be considered gene trees and not species trees until additional sequences from nuclear genes become available.

L. inornata has been considered a generalized member of the genus and it was placed in basal position by Johnsgard (1986). However, Delacour (1977) suggested that *L. inornata* may be related to *L. edwardsi* and *L. erythrophthalma*. The number of retrices in *L. inornata*, 14 instead of 16, and the short, round, and flattened tail support the relationship between *L. inornata* and *L. erythrophthalma* that was weakly suggested by clade 3 (Fig. 4).

Previous studies and morphological data provide little insight into the position of *L. bulweri*, which is very distinctive and specialized. Delacour (1977) considered *L. bulweri* intermediate between *L. ignita*, *L. edwardsi*, *L. imperialis*, and *L. swinhoii*, similar to the suggestion of Johnsgard (1986), who considered *L. bulweri* most similar to *L. ignita*. Several traits suggest specific relationships to other species: the color of the wattles is similar to *L. ignita*, the retrices are curved outward as in *L. diardi*, the downy young are like those of *L. ignita*, the female plumage is similar to that of *L. edwardsi*, and the elaborate display suggests a relationship to *L. swinhoii*.

Divergence Time, Biogeography, and Speciation

The four main clades of *Lophura*, which join into the basal polytomy, diverged at values of 6.8 to 5.2% cyt *b* + CR sequence divergence. These divergence values cannot be calibrated specifically in *Lophura*, because there is no available independent fossil or biogeographical information that can be used. Using the "conventional" mtDNA clock of 2% sequence divergence per million years (Avise and Walker, 1998), we might infer that these clades diversified about 3.4 to 2.1 million years ago (mya), that is during the mid to late Pliocene and well before the major marine eustatic fluctuations that deeply affected the landscapes and the distribution of vegetation types in South East Asia and Sun-

daland during the last part of the Pleistocene (Heaney, 1986).

The structure of the mtDNA phylogenetic trees and current species' distributions suggest that speciation in *Lophura* could have been fostered mainly by isolation in allopatry or by ecological segregation among sympatric species in lowland and highland rainforest ecosystems. Allopatric sister species *L. diardi* (distributed in Indochina) and *L. ignita* (distributed in peninsular and insular Malaysia) share similar lowland to hill rainforest habitats (McGowan, 1994), but are geographically separated on the north and south sides of the isthmus of Kra (Fig. 1). They showed 4.4% sequence divergence, corresponding to a relatively ancient splitting (2.2 mya). *L. erythrophthalma* (distributed in peninsular Malaysia, Sumatra, and north Borneo) and *L. inornata* (endemic to the island of Sumatra) are sympatric in Sumatra, where they live separately in lowland (from sea level to 300 m) and highland rainforests, respectively. Current geographical distributions and extent of genetic divergence (6.0% sequence divergence, corresponding to 3.0 myr) suggest that these species belong to clades that diverged anciently in continental South East Asia, dispersed across the Sundaland region, and probably acquired ecological isolation following secondary contact in Sumatra. *L. erythrophthalma* and *L. bulweri* are sympatric in Borneo, but probably not syntopic, as they are spatially separated into lowland (up to 300 m) and highland (between 300 and 1500 m) rainforests.

Interspecific divergence among species joining into clade 4 was lower than 2.8%, suggesting that they might have originated early in the Pleistocene. In particular, (1) sister species *L. leucomelanos* and *L. nycthemera* (clade 4b), which are distributed allopatrically in the Himalayan region and in southeastern China, respectively, showed 2.8% sequence divergence and would have split about 1.4 mya, perhaps in consequence of the regional climate changes fostered by the final uplifting of the Himalayas and by the major glaciations in the northern hemisphere (Harrison *et al.*, 1992). Also, (2) *L. swinhoii* (whose current range is restricted to Taiwan) and the lowland Annamese species (*L. edwardsi* and *L. hatinhensis*, distributed in isolated rainforest patches in Vietnam) have an average genetic distance of 2.5% (divergence time of 1.2 mya) and could represent the surviving fragments of populations formerly widespread in continental South East Asia. *L. edwardsi* and *L. hatinhensis* are ecological specialists restricted to evergreen rainforest. The decrease of suitable habitats may have led to their very limited current populations. In contrast, *L. nycthemera* and *L. leucomelanos* are generalist species which may tolerate much wider habitat ranges.

In conclusion, speciation in *Lophura* was likely fostered by geographic isolation in continental South East Asia, with Sundaland being colonized more recently and independently by the different preexisting clades. However, the cyclical Pleistocene climate changes leading to the recurrent emergence of the Sunda shelf and land-bridge corridors could have facilitated the colonization of isolated rainforest areas in the Indonesian archipelago and the rates of population turnover in the islands. Thus, it is expected that extinctions and adaptation dynamics of local pheasant populations during the late Pleistocene may have determined current distributions and levels of intraspecific genetic divergence. Sea regressions during the Pleistocene may explain the reason that *L. bulweri* is present also in Bangka Island (near Sumatra) and may have determined the patterns of subspeciation in peninsular Malaysia and Sumatra. Estimating the extent of genetic diversity within and among natural populations and subspecies of *Lophura* is now the major task to be accomplished to complete the reconstruction of their evolutionary history and biogeography and to cast the basis for their conservation.

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