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# Mitochondrial DNA diversification among the subspecies of the Silver and Kalij Pheasants, *Lophura nycthemera* and *L. leucomelanos*, Phasianidae

SIBYLE MOULIN,[1]* ETTORE RANDI,[2] CRISTIANO TABARRONI[2] & ALAIN HENNACHE[1]
[1]*Laboratoire de Conservation des Espèces Animales, Muséum National d'Histoire Naturelle, 57 rue Cuvier, 75290 Paris cedex 05, France*
[2]*Istituto Nazionale per la Fauna Selvatica (INFS), Via Cà Fornacetta 9, 40064 Ozzano dell'Emilia (BO), Italy*

The taxonomic status of the pheasant superspecies *Lophura leucomelanos* and *Lophura nycthemera* has been unclear since 1948. Molecular techniques provided the opportunity to clarify the situation. Using sequences of mitochondrial DNA (800 nucleotides from the D-loop, plus 400 from the cyt *b*) from 49 specimens belonging to 10 subspecies (plus two outgroups), we constructed a phylogeny of the subspecies of *L. nycthemera* and *L. leucomelanos*. Our data support the monophyly of both species. *L. l. lineata* and *L. l. crawfurdi* belong to *L. leucomelanos* and not to *L. nycthemera* (suggested by other authors). Our data also confirm a northern locality of origin (Central Buthan) for *L. l. moffitti*, and have clarified the relationships between subspecies within each species: there are three groups within *L. leucomelanos* and two within *L. nycthemera*.

The evolutionary relationships and taxonomy of the subspecies of the Silver and Kalij Pheasants (*Lophura nycthemera* and *L. leucomelanos*; Phasianidae) are unclear. Delacour (1949) included them in a single superspecies, and pointed out the following systematic problems: (1) their description in a large number of genera before his revision, 49 taxa of Silver and Kalij Pheasants being split into 28 species belonging to four genera (Del Hoyo *et al*. 1994); (2) their large geographical range with a limited number of specimens available to study; (3) the occurrence of natural hybridization in contact zones where populations of the two species of his new classification overlapped. Therefore, Delacour suggested that some subspecies could have derived from past episodes of hybridization.

About 15 subspecies of *L. nycthemera* and nine subspecies of *L. leucomelanos* (Table 1) are currently recognized (Del Hoyo *et al*. 1994, Johnsgard 1999). However, McGowan and Panchen (1994) suggested that three subspecies of *L. leucomelanos* (*L. l. crawfurdi*, *L. l. lineata* and *L. l. oatesi*) should be attributed to *L. nycthemera* and seven subspecies of *L. nycthemera* (underlined in Table 1) do not deserve the status of subspecies, belonging to a morphocline.

*Corresponding author.
Email: smoulin@mnhn.fr

In the wild these two species inhabit forests from bushy hills to medium sized mountains (700–2000 m), from Pakistan to south-western China. In summary (Johnsgard 1999): east of the Irrawaddy River there is only *L. leucomelanos*, and west of this natural limit *L. nycthemera* plus the three subspecies of *L. leucomelanos* (*L. l. lineata*, *L. l. crawfurdi* and *L. l. oatesi*) that are claimed to belong to *L. nycthemera* by McGowan and Panchen (1994) (see Fig. 1).

McGowan and Garson (1995) recommend the assessment and clarification of the relationships among 15 subspecies of *L. nycthemera* and nine subspecies of *L. leucomelanos*, in order to improve the conservation strategies for this group. The previous description of trifling differences between specimens resulted in the proposal of many subspecies that are obviously very similar and which may have resulted from introgressive hybridization. Although the majority of the Silver and Kalij Pheasant taxa are not considered endangered, some subspecies with limited ranges have potential conservation problems owing to habitat loss or degradation, clearance for agriculture and hunting for food (McGowan & Garson 1995), particularly *L. n. annamensis*, *L. n. whiteheadi* and *L. n. engelbachi*, which are listed 'endangered', whereas *L. n. lewisi*, *L. l. lineata* and *L. l. crawfurdi* are considered 'vulnerable' and *L. l. moffitti* 'insufficiently known' (Mace & Lande

**Table 1.** Subspecies of *Lophura leucomelanos* and *L. nycthemera*.

| Code | Scientific name | English name | Descriptor | Type locality |
|---|---|---|---|---|
| cra | *L. l. crawfurdi* | Crawfurd's Kalij Pheasant | (Gray, 1829) | Ava, Hat Sanuk, Ko Lak, Western Siam |
| ham | *L. l. hamiltoni* | White-crested Kalij Pheasant | (Gray, 1829) | Simla Almora, India |
| lat | *L. l. lathami* | Black-brested Kalij Pheasant | (Gray, 1829) | Sylet |
| leu | *L. l. leucomelanos* | Nepal Kalij Pheasant | (Latham, 1790) | Nepal |
| lin | *L. l. lineata* | Lineated Kalij Pheasant | (Vigor, 1831) | Straits of Malacca, East Pegu Hills |
| mel | *L. l. melanota* | Black-backed Kalij Pheasant | (Hutton, 1848) | Darjeeling |
| mof | *L. l. moffitti* | Black Kalij Pheasant | (Hachisuka, 1938) | unknown (ship from Calcutta, India) |
| oat | *L. l. oatesi* | Oates's Kalij Pheasant | (Ogilvie-Grant, 1898) | Prome, Prome division, Arakan Hills |
| wil | *L. l. williamsi* | Williams Kalij Pheasant | (Oates, 1896) | Kalewa, Chin Hills, Burma |
| ann | *L. n. annamensis* | Annamese Silver Pheasant | (Ogilvie-Grant, 1906) | Nhatrang, Southern Annam |
| bea | *L. n. beaulieui* | Lao Silver Pheasant | (Delacour, 1948) | Xieng-Kouang, Laos |
| bel | *L. n. beli* | Bel's Silver Pheasant | (Oustalet, 1898) | Huê Annam |
| ber | *L. n. berliozi* | Berlioz's Silver Pheasant | (Delacour & Jabouille, 1928) | Laobao Quangtri, Annam |
| eng | *L. n. engelbachi* | Bolovens Silver Pheasant | (Delacour, 1948) | Boloven Plateau, Laos |
| fok | *L. n. fokiensis* | Fokien Silver Pheasant | (Delacour, 1948) | NW Fokien |
| jon | *L. n. jonesi* | Jones's Silver Pheasant | (Oates, 1903) | 20 miles West of Kentung, S. Shan States |
| lew | *L. n. lewisi* | Lewis's Silver Pheasant | (Delacour & Jabouille, 1928) | Bokor, Cambodia |
| nyc | *L. n. nycthemera* | True Silver Pheasant | (Linnaeus, 1758) | China, probably Chinese Tonkinese border |
| occ | *L. n. occidentalis* | Western Silver Pheasant | (Delacour, 1948) | Hills north-west of Tengyueh, Yunnan |
| ome | *L. n. omeiensis* | Szechwan Silver Pheasant | Cheng, Chang & Tang, 1954 | Mont Omei Setchouan |
| rip | *L. n. ripponi* | Rippon's Silver Pheasant | (Sharpe, 1902) | Southern Shan States |
| ron | *L. n. rongjiangensis* | Rang Jiang Silver Pheasant | (Tan & Wu, 1981) | Rang Jiang |
| ruf | *L. n. rufipes* | Ruby mines Silver Pheasant | (Oates, 1898) | Ruby Mines, Burma |
| whi | *L. n. whiteheadi* | Hainan Silver Pheasant | (Ogilvie-Grant, 1899) | Five Fingers Mountains, Hainan |

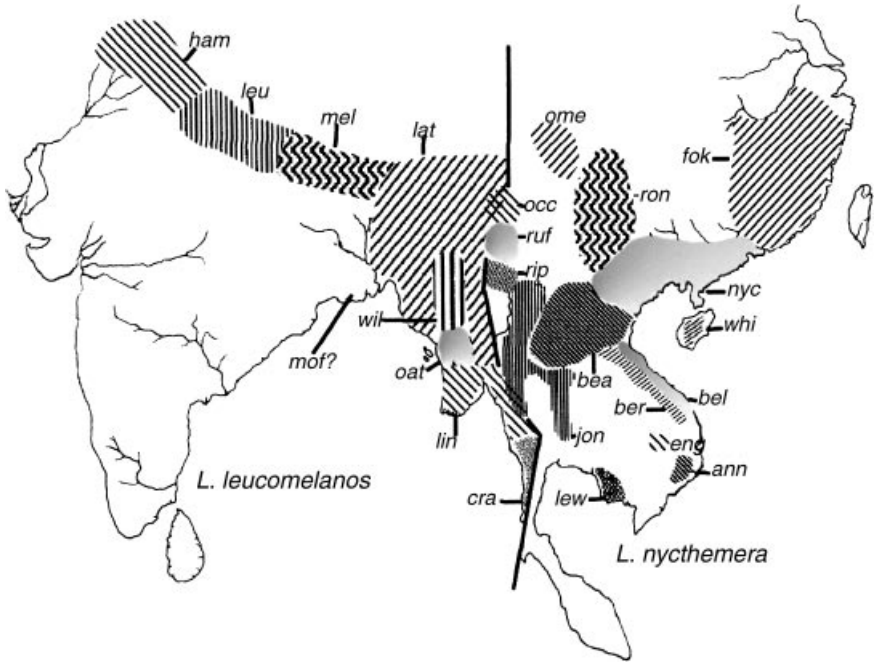


**Figure 1.** Repartition map of the different subspecies; *L. leucomelanos* is on the left of the black line and *L. nycthemera* on the right. Arrows show intergradation between subspecies, modified from Johnsgard (1999) and Delacour (1949).

1991). It is clearly essential for the purpose of considering their conservation that the systematics of this group are clarified.

This study aimed to obtain reference mitochondrial DNA (mtDNA) sequences for some of the subspecies of the Silver and Kalij Pheasants to assess the validity of the subspecies. This is a preliminary work performed on birds belonging to a limited number of subspecies owing to the difficulty of collecting samples. Indeed it is virtually impossible to get material from the wild for most of the subspecies. Worldwide, however, there are many captive populations of these birds. Therefore to assess the phylogenetic relationships of subspecies, we have sampled captive birds of different origin. It is well known that the *Lophura* species hybridize easily in captivity as well as in the wild (Hennache 1997, Johnsgard 1999). It is likely that captive populations of *L. l. leucomelanos* and *L. n. nycthemera* contain many hybrids because the relatively inexperienced breeders of these birds rarely pay regard to the subspecies. Fortunately, however, these subspecies are not threatened in the wild. In contrast, limited captive stocks of *L. l. moffiti*, *L. l. crawfurdi*, *L. l. lineata*, *L. n. annamensis* and *L. n. lewisi* are kept by experienced breeders who even know the ancestries of their birds and their wild origins. It is very unlikely that these include hybrids.

The mitochondrial DNA genome is maternally inherited and does not recombine, thus allowing the identification of the different maternal lineages that are present in wild and captive populations. The identification of the mtDNA haplotypes (unlike with nuclear DNA) allows recognition of hybrids if another source of information is added, e.g. where the phenotype of the specimen disagrees with the genotype. The mtDNA control region and third position codon of the cytochrome *b* gene are highly variable and can be used reliably to estimate the extent of divergence between subspecies (Mundy *et al.* 1997, Kidd & Friesen 1998). The non-coding control region contains the replication and transcription promoters, and can be divided into three domains which vary differently: the central domain evolves about 10–20 times more slowly than the two peripheral domains (Baker & Marshall 1997, Randi & Lucchini 1998). The control region can evolve accumulating both point mutations and insertions/deletions (indels). In contrast, cytochrome *b* is a coding gene and most mutations are synonymous substitutions at the third codon position.

## MATERIALS AND METHODS

### Samples

Forty-six feather and tissue samples were collected from captive stock in Belgium, Czech Republic, France, Germany, Latvia, Poland, Singapore, the UK, the USA and Vietnam (Table 2), often from institutions that knew the histories of the Silver or Kalij Pheasant phenotypes in their possession. Some specimens were from a second captive generation, with known ancestries. Five feather samples were collected from wild birds. We obtained DNA sequences from 51 samples (Table 2). Two other *Lophura* species, *L. edwardsi* and *L. swinhoei* (from Parc de Clères [MNHN]), were also sequenced and used as outgroups because they are close enough to the ingroup to avoid long branch attraction (Felsenstein 1978). More than one outgroup reduces errors in character polarization; the choice and the order of outgroups can influence the topology of trees (Barriel & Tassy 1998).

### Molecular analyses

Total DNA was extracted from feathers preserved in 99% ethanol, and dried cell cultures from skin biopsies using the GuSCN method (Gerloff *et al.* 1995). The oligonucleotide primers used for polymerase chaine reaction (PCR) are summarized in Table 3. PCRs were performed in a Perkin-Elmer 9600 thermocycler using 2 mM of $MgCl_2$ with 0.25 μM primer concentration in the reaction buffer, and the following thermal cycle: 94 °C × 2 min; 30 cycles at (94 °C × 15 s + 55 °C × 15 s + 72 °C × 1 min); 72 °C × 10 min; 4 °C × 10 min; 15 °C forever. The amplified mtDNA control region (CR) and cytochrome *b* (cyt *b*) were sequenced using the Perkin-Elmer AmpliTaq FS Dye Terminator kit in an ABI373 automatic sequencer. Some 'touch Down' PCR procedures were used for the second part of the D-loop: 94 °C × 2 min; nine cycles with a 1 °C decrease at each cycle (94 °C × 15 s + 59–50 °C × 15 s + 72 °C × 1 min) then 30 cycles at (94 °C × 15 s + 50 °C × 15 s + 72 °C × 1 min); 72 °C × 10 min; 4 °C × 10 min; 15 °C thereafter.

An enzymatic purification was then performed on the amplified DNA, using EXO-SAP (37 °C × 30 min + 80 °C × 15 min), and a sequence reaction as follows: 25 cycles at (94 °C × 10 s + 50 °C × 5 s + 60 °C × 4 min); 72 °C × 10 min; 4 °C × 10 min; 15 °C thereafter. Using these protocols we sequenced the entire mtDNA control region (CR), and the 3′ terminal part of the cytochrome *b* gene (cyt *b*) in all 51 DNA samples.

### Alignment and phylogenetic analyses

Alignment of the nucleotide sequences was obtained using Se-Al (version 1.0; A. Rambaut; http://evolve.zoo.ox.ac.uk/software/Se-Al/main.html). The alignment of the cyt *b* was straightforward, and the alignment of the CR was performed manually.

Phylogenetic reconstructions were computed using the concatenated cyt *b* + CR sequences by the Neighbour-Joining method (NJ) (Saitou & Nei 1987) on percentage divergence estimated by Kimura's two-parameter formula and maximum parsimony (MP) trees using PAUP (Swofford 1999). We also performed likelihood analyses (ML) using PUZZLE 4.0 (Strimmer & Von Haeseler 1996) with the HKY model (Hasegawa *et al.* 1985) and substitution rate heterogeneity with two rates (invariable vs. variable sites) or with the gamma distribution (four and eight discrete categories).

Support for the internodes was assessed by Bootstrap (Felsenstein 1985) with 100 resamplings. We also computed the Bremer decay index (Bremer 1988) with AUTODECAY (Eriksson 1998).

## RESULTS

Between 1003 and 1131 nucleotides (nt) for the CR, and between 377 and 391 nt for the cyt *b* gene were sequenced. The first base of the aligned CRs sequenced in this study corresponds to nt number 37 in the alignment of *Lophura* in Randi *et al.* (2001). The first base of the aligned cyt *b* corresponds to nt 753 in the alignment of the homologous *Alectoris* sequences (Randi 1996).

It appears that we have no nuclear copy of the cyt *b* because all sequences were aligned without problem (Kidd & Friesen 1998), and no stop codon appears inside. The structure of the CR appears to be conserved (Randi & Lucchini 1998, Freeland & Boag 1999).

The pairwise percentage sequence divergence between *Lophura nycthemera* and *L. leucomelanos* and the outgroups was about 4–5%. The consensus tree obtain by MP was 476 steps long (CI = 0.538, RI = 0.829 and g1 = –0.39655). The NJ and MP consensus trees differ only for minor topological details. The ML tree differs from NJ and MP consensus trees for a trifurcation joining two clades of *L. leucomelanos* and *L. nycthemera* (Fig. 2).

**Table 2.** Origin of the samples.

| Name | Code | Origin | Sex | Captive/wild |
|---|---|---|---|---|
| *Lophura swinhoei* | Swi 34 | Clères zoo | m | captive |
| *Lophura edwardsi* | Edw 36 | Clères zoo | m | captive |
| *L. l. crawfurdi* | Cra 50 | Private breeder | f | captive |
| | Cra 96 | Private breeder | m | captive |
| *L. l. hamiltoni* | Ham 95 | Private breeder | f | captive |
| | Leu 35 | Clères zoo | m | captive |
| *L. l. leucomelanos* | Leu 2 | Villars zoo | f | captive |
| | Leu 6 | Villars zoo | m | captive |
| | Leu 10 | Ostrava zoo | unk | captive |
| | Leu 11 | Ostrava zoo | unk | captive |
| | Leu 12 | Riga zoo | unk | captive |
| | Leu 13 | Riga zoo | unk | captive |
| *L. l. lineata* | Lin 86 | Private breeder | f | captive |
| | Lin 103 | Private breeder | m | captive |
| | Lin 122 | Private breeder | f | captive |
| | Lin 123 | Private breeder | m | captive |
| | Lin 124 | Private breeder | m | captive |
| | Lin 126 | Private breeder | | captive |
| *L. l. moffitti* | Mof 15 | Private breeder | m | captive |
| | Mof 16 | Private breeder | | captive |
| | Mof 17 | Private breeder | f | captive |
| | Mof 19 | Private breeder | m | captive |
| *L. n. annamensis* | Ann 31 | Hanoi zoo | m | wild |
| | Ann 91 | Saigon zoo | | wild |
| *L. n. berliozi* | Ber 4 | Villars zoo | m | captive |
| | Ber 7 | Villars zoo | f | captive |
| | Ber 37 | Clères zoo | m | captive |
| | Ber 54 | Private breeder | m | captive |
| *L. n. jonesi* | Jon 38 | Private breeder | m | captive |
| | Jon 108 | Private breeder | f | captive |
| | Jon 117 | Private breeder | f | captive |
| | Jon 118 | Private breeder | m | captive |
| *L. n. lewisi* | Lew 47 | Private breeder | m | captive |
| | Lew 48 | Private breeder | f | captive |
| | Lew 94 | Private breeder | m | captive |
| | Lew 113 | Private breeder | f | captive |
| | Lew 114 | Private breeder | m | captive |
| | Lew 115 | Private breeder | f | captive |
| *L. n. nycthemera* | Nyc 30 | Hanoi zoo | m | wild |
| | Nyc 33 | Clères zoo | m | captive |
| | Nyc 99 | Private breeder | | captive |
| | Nyc100 | Private breeder | m | captive |
| | Nyc 101 | Private breeder | f | captive |
| | Nyc 102 | Private breeder | m | captive |
| | Nyc 111 | Private breeder | m | wild |
| | Nyc 112 | Private breeder | | wild |
| *L.* ssp. | Ssp 9 | Ostrava zoo | unk | captive |
| | Ssp 25 | Warsaw zoo | unk | captive |
| | Ssp 28 | Racine zoo | unk | captive |
| | Ssp 29 | Racine zoo | unk | captive |
| | Ssp 32 | Upie zoo | unk | captive |

**Table 3.** Primers used for PCR with references.

| Name | Primer sequence 5′–3′ | Reference |
|---|---|---|
| PHDL | AGG ACT ACG GCT TGA AAA GC | modified from Fumihito *et al.* (1995) |
| PH-L818 | GGA ATG ATC TTG ACA CTG ATG CAC T | E.A. Scott pers. comm., in Randi and Lucchini (1998) |
| PHDH | CAT CTT GGC ATC TTC AGT GCC | modified from Fumihito *et al.* (1995) |
| L400 | ATT TAT TGA TCG TCC ACC TCA CG | E.A. Scott pers. comm., in Randi and Lucchini (1998) |
| Ph1 h | TTA TGT GCT TGA CCG AGG AAC CAG | E.A. Scott pers. comm., in Randi and Lucchini (1998) |
| HB6 | GTC TTC AGT TTT TGG TTT ACA AGA C | |
| L14990 | CCA TCC AAC ATC TCA GCA TGA TGA AA | Kocher *et al.* (1989) |

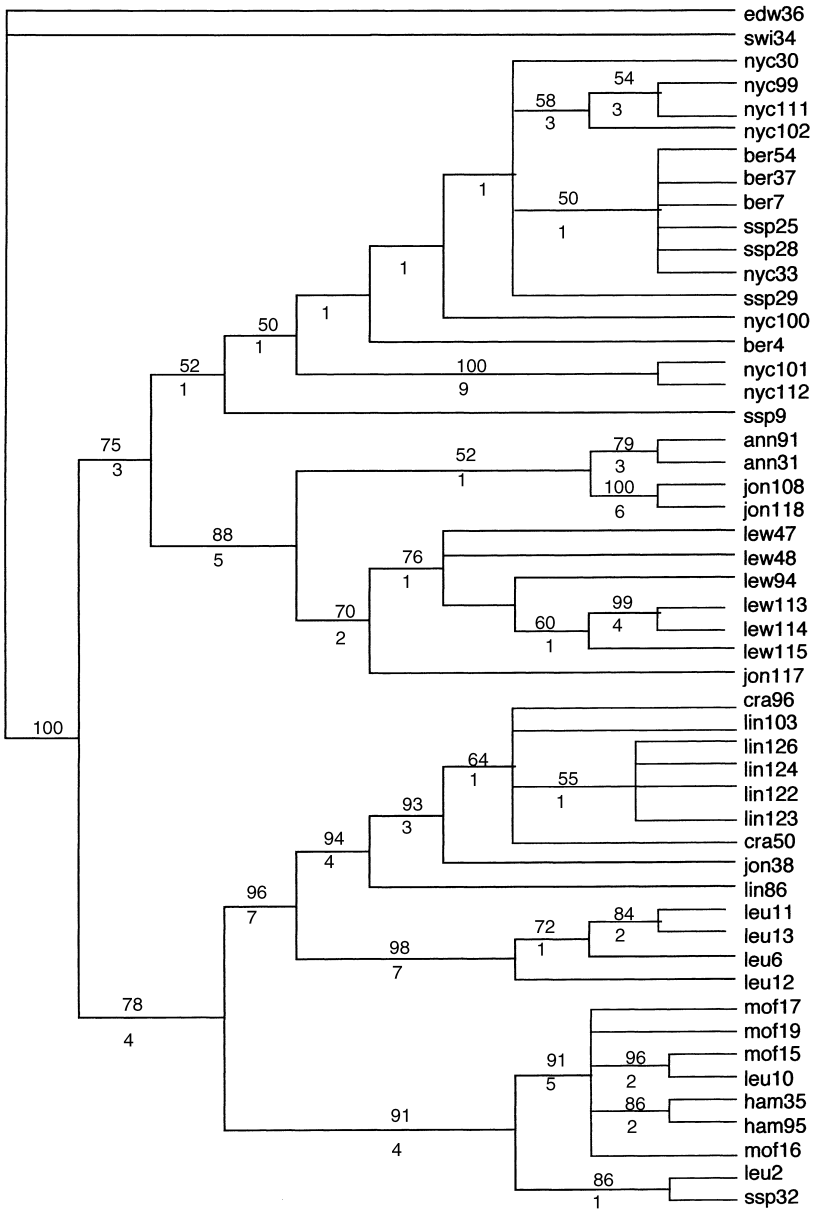


**Figure 2.** MP consensus tree with bootstrap values above the line (not shown under 50%) and Bremer index under the line.

**Table 4.** Summary of likelihood model results.

| | 1 invariable + 1 variable | gamma 4 categories | gamma 8 categories |
|---|---|---|---|
| expected ratio Ti/Tv | 3.60 | 2.56 | 2.53 |
| estimated ratio Ti/Tv | 3.66 | 2.60 | 2.58 |
| shape parameter α | | 0.02 | 0.08 |
| Likelihood value | Log L = –5576.35 | Log L = –5231.98 | Log L = –5202.39 |

The average base composition is for cyt *b* and CR: (A = 26.4%; 26.8%, C = 11.3%; 26.2%, G = 33.5%; 14.7%, T = 28.6%; 31.1%) and other parameters of the ML trees are shown in Table 4.

Both outgroups (*L. edwardsi* and *L. swinhoei*) remain sister groups of the superspecies (bootstrap value from 95% to 100%, Bremer index 23 for the MP tree), whichever phylogenetic reconstruction is used, leading to the conclusion that the ingroup is monophyletic. The use of an outgroup belonging to another genus such as *Gallus gallus* leads to the same result. Nucleotide sequences from the Silver and Kalij Pheasants split into two main branches, supported by bootstrap values of 70–93% for *L. nycthemera* and 78–73% for *L. leucomelanos*. Furthermore, each species splits into two well-supported main groups resulting in four clusters: (1) a first cluster includes all *L. nycthemera nycthemera* (nyc) and *L. nycthemera berliozi* (ber) (bootstrap = 52–91%); (2) a second one joins all the other *Lophura nycthemera* (*L. nycthemera lewisi* [lew], *L. nycthemera jonesi* [jon] and *L. nycthemera annamensis* [ann]) with bootstrap = 77–98% and Bremer index = 5 for the MP tree; (3) a third group includes *L. leucomelanos lineata* (lin) and *L. leucomelanos crawfurdi* (cra) (from 57 to 95%, Bremer index 7 for the MP tree) and a branch of *L. leucomelanos leucomelanos* (leu) (from 85 to 99%, Bremer index 7 for the MP tree); (4) a last cluster gathers *L. leucomelanos leucomelanos* (leu), *L. leucomelanos hamiltoni* (ham) and *L. leucomelanos moffitti* (mof) (from 57 to 100%, Bremer index 4 for the MP tree).

## DISCUSSION

As the majority of samples came from captive stock, it is necessary to check that there is no major bias among samples: biogeographical details provide a good argument for no bias (cf. Fig. 3). Sequences from the Silver and Kalij Pheasants form a monophyletic group (clade A) with two main branches (B and C) leading to the two different species. Phylogenetic structure among subspecies of *L. nycthemera* is relatively well supported: there are two clusters (D and E). Cluster (D) includes *L. n. nycthemera* (nyc), *L. n. berliozi* (ber) and some samples for which the subspecies is unknown. Some of the *L. n. nycthemera* (nyc 30, nyc 111, nyc 112) were caught in the wild. *L. n. berliozi* and *L. n. nycthemera* cannot be separated. This might have resulted from a past hybridization between the two captive stocks as there is no contact between these two forms in the wild. Alternatively, it could mean that there is no need to distinguish two subspecies as they belong to the same evolutionary unit. The latter case raises a new difficulty as there are other subspecies whose distribution range lies between them: *L. n. beli* and *L. n. beaulieui*, from which we did not obtain samples. Subspecies of *L. nycthemera* from the east form a clade.

The other clade (E) of *L. nycthemera* separates three subspecies: *L. n. lewisi*, *L. n. annamensis* (both specimens ann 31 and ann 91 were caught in the wild) and *L. n. jonesi*. *L. n. annamensis* and *L. n. lewisi* have an isolated geographical distribution (Delacour 1948, Johnsgard 1999). This clade regroups the darker subspecies of *L. nycthemera* from the south-east. McGowan and Panchen's (1994) conclusion was to combine all subspecies of *L. nycthemera*, but they used subspecies of *L. nycthemera* that do not include ours (except *L. n. nycthemera*), and from a geographical perspective they had no subspecies belonging to the clade (E).

For the clade (F) containing *L. l. crawfurdi* and *L. l. lineata*, we are not able to separate the subspecies from each other. Neither were McGowan and Panchen (1994): they combine these subspecies with *L. l. oatesi* in only one category. We can make the same assumptions as for *L. n. berliozi* and *L. n. nycthemera*. Therefore, they would form only one evolutionary unit. Linked to this clade there is another (G) containing *L. l. leucomelanos* (leu 6 from

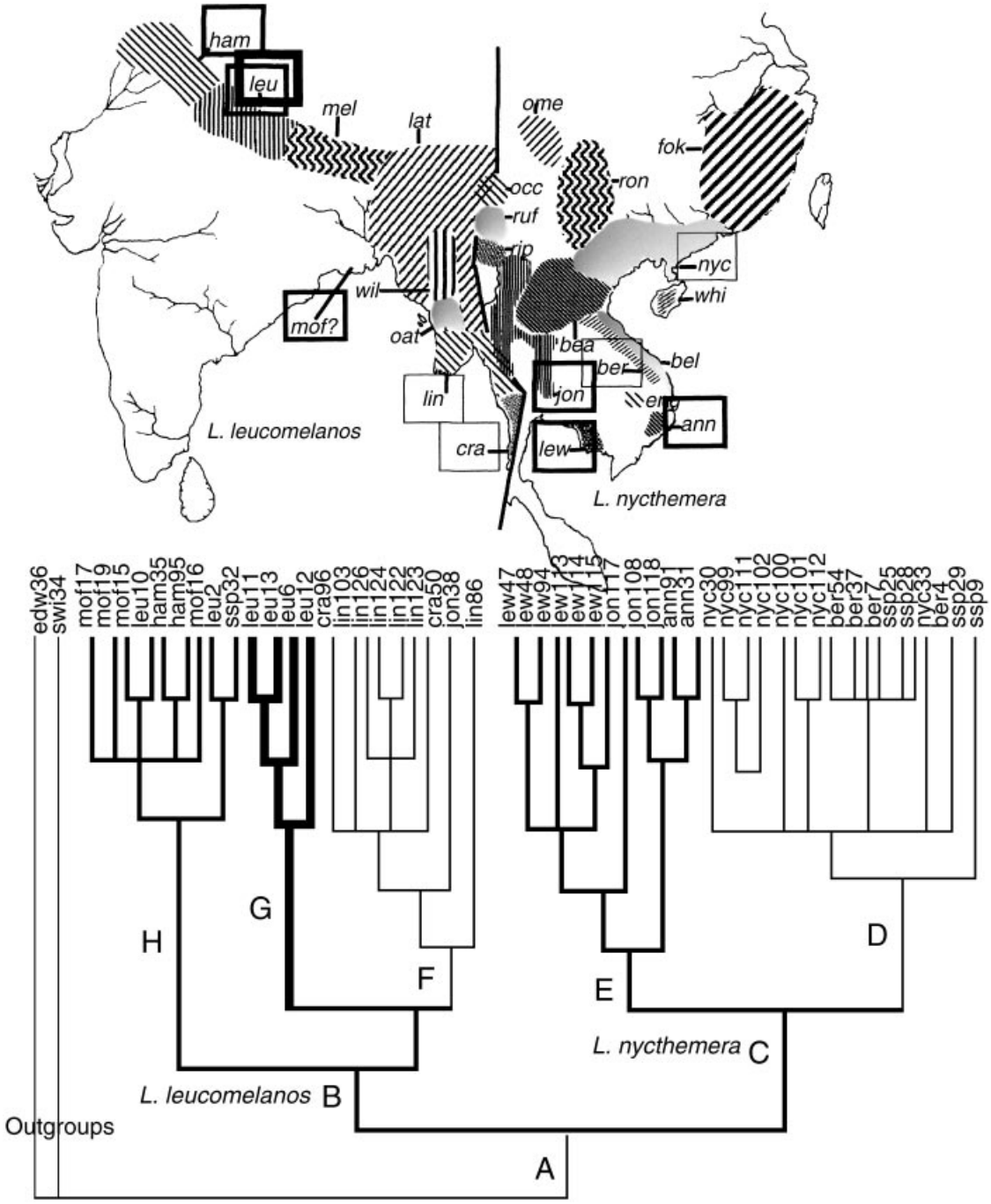


**Figure 3.** Phylogenetic relationships between subspecies in relation to their distribution range.

France, leu 11 from Ostrava Zoo, leu 12 and leu 13 from Riga Zoo). It is rather surprising to find these specimens close to the south-east subspecies of *L. leucomelanos*. Either hybridization in captivity has to be considered or the subspecific identity has been mistaken. Indeed, McGowan and Panchen (1994) have grouped in the same plumage category the following subspecies: *L. l. leucomelanos*, *L. l. hamiltoni* and *L. l. lathami* and indicated possible confusion between them. Furthermore, the ranges of *L. l.*

*lathami* and *L. l. lineata* could abut. The hypothesis of two different evolutionary units with the same phenotypic pattern must also be considered. These specimens could have been attributed to the wrong subspecies. This is the clade of the south-west (west of the range of *L. nycthemera*).

The last clade of *L. leucomelanos* (H) contains species from the north-west, *L. l. moffitti*, *L. l. hamiltoni* and some other samples from *L. l. leucomelanos*. McGowan and Panchen (1994) had specimens from *L. l. leucomelanos*, *L. l. hamiltoni* and *L. l. lathami*, but none from *L. l. moffitti*. The range of *L. l. moffitti* specimens was unknown for a long time because a few pairs were shipped from Calcutta in 1934 (Fig. 3) (Delacour 1949). Without knowing their origins accurately, Delacour (1949) stated that they inhabit 'Central Bhutan, around the Mo Chu Valley, between the known ranges of *melanota* and *lathami*. Or it might inhabit somewhere to the south in the western Kashia Hills'. Ali and Ripley (1987) wrote that K. S. Ranjitsinhji of Wankaner saw a male of *L. l. moffitti* in 1965 in central Bhutan. The geographical analysis of our molecular data is fully in agreement with the northern origin of this subspecies.

The position of some specimens is more enigmatic, e.g. jon 38. We have been told that it is a bird caught in the wild (*L. n. jonesi*): morphologically this bird definitely belongs to *L. nycthemera* (we had the opportunity to examine this specimen). But it clusters with southern *L. leucomelanos*. Either it is a natural hybrid resulting from a secondary contact (Davison 1996) with *L. l. lineata* or this bird is not wild born but a descendant from a past hybridization in captivity. It could be an example of secondary contact (Davison 1996). Ssp 32 (we have seen this one too) also shows an *L. nycthemera* morphotype and clusters with northern *L. leucomelanos*, but we have no data on the whereabouts of this bird. More than 95% of the specimens have a phenotype that corresponds to the mitochondrial genotype and clusters in accordance with the biogeographical pattern (out of 42 specimens only two hybrids have been detected). It seems, however, that potential hybridization problems do not interfere much at the systematic level because of the well-known properties of mtDNA.

The divergence percentages found between species appear to be of the same order of magnitude as those found by other authors. Hennache *et al.* (1999) found a divergence rate of 2.8% between *L. leucomelanos* and *L. nycthemera* and about 0.5% inside the *L. edwardsi* group, which is close to the figures that we found within the different subspecies.

In the Long-billed Lark *Certhilauda curvirostris* complex, Ryan and Bloomer (1999) found 2–9% divergence between species and 0.2–0.4% between subspecies with cyt *b* sequences of 483 bp. They found no amino acid differences either between or within groups. Grapputo *et al.* (1998) failed to discriminate two subspecies of Reed Bunting *Emberiza schoeniclus schoeniclus* and *E.* s. *intermedia* which have different bill sizes and very different behaviour patterns (one is migratory and the other sedentary), with cytochrome *b* haplotypes, but succeeded with four microsatellite loci, and estimated divergence time at about 500 000 years. Mundy *et al.* (1997) analysed 200 bp of the Dloop plus 200 bp of cytochrome *b* of four subspecies of the Loggerhead Shrike *Lanius ludovicianus*, and found low variability and different frequencies of the four haplotypes within each subspecies.

Such analyses help us to purify the captive stock in order to use it for future captive breeding plans (Hennache 1997). *Lophura nycthemera* and *L. leucomelanos* are among the three groups of pheasants known to hybridize in the wild, and species belonging to the genus *Lophura* are able to produce hybrids with a large number of genera in the family (from *Tragopan* to *Chrysolophus*, Johnsgard 1999).

Some of our results suggest a few cases of past hybridization in the captive stocks. These mixed captive subspecies populations are no longer of much interest for conservation. The present aim of *ex situ* conservation is to save taxa from extinction in the wild: thus captive stock management must aim to keep the stock conforming to the type. Regardless of their phenotypic variation and the taxonomic status they receive, these two species of *Lophura* show a great deal of genetic variability, which is important to consider.

Whatever their ultimate status (subspecies, morphocline, etc.), the results show that the distribution of the *Lophura* seems to be consistent with their phylogenetic relationship, which justifies, *a posteriori*, our captive sampling. Their range in the wild is rather threatened, for example by deforestation in Vietnam (Nguyen Cu & Eames 1992) and Pakistan (Chaudhry 1992), and where population status in the wild is known, other human activities are also found to threaten pheasants in the wild. Furthermore there are no recent data about the protected areas within Burma and Cambodia (McGowan *et al.* 1999).

## CONCLUSIONS

Phylogenetic trees constructed from mitochondrial DNA from 49 specimens of 10 subspecies clearly show two monophyletic species, with a clear geographical structure showing a strong east/west separation and a less marked north/south one, as suggested by Darlington (1963). These results were obtained mostly from captive birds, showing the value of these collections, even though confirmation by wild caught specimens and/or museum samples would be most welcome. The captive stock seems to be of major importance for the conservation of genetic diversity *ex situ*. Although some subspecies seem to be valid, it has not been possible to conclude definitely on the case of the *L. n. berliozi*/*L. n. nycthemera* ambiguity and the origin of the different *L. l. leucomelanos* samples, as more subspecies need to be added. Further research should aim to sample more from the wild from untested subspecies and to investigate the nuclear DNA field to detect any hybrid from the paternal line by using microsatellites. Accurate information on the genetic diversity of these species and subspecies is also needed in case they become extinct in the wild, in order to understand the intraspecific evolutionary process, and to improve the present captive stocks (Haig & Avise 1996).

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