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Genetic diversity, phylogenetic relationships and conservation of Edwards's Pheasant *Lophura edwardsi*

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Summary

An historical study shows that the present day *ex situ* population of Edwards's Pheasant *Lophura edwardsi*, now numbering over 1,000 living specimens, originated in the 1920s from at least six different sources and *c.* 30 potential founders, including more males than females. Partial sequences, 820 nucleotides long, of the mitochondrial DNA (mtDNA) control-region were obtained in representatives of about half of 21 captive-reared bloodlines, identified from data in the revised International Studbook. All captive-reared birds had identical sequences. Sequences of mtDNA obtained from museum skins and samples of birds collected in the wild were slightly different from that representing the captive-reared birds. The lack of mtDNA variability in the captive stock is probably due to the small number of founding females and genetic drift during *c.* 30 generations of captive breeding. Estimation of true extent of mtDNA sequence variability in historical and living wild Edwards's Pheasants awaits the procurement and analysis of more specimens. At least one bloodline of captive Edwards's Pheasant is polluted with exogenous genes resulting from past hybridization with Swinhoe's Pheasant *L. swinhoii*. Edwards's Pheasant is more closely related to Vietnamese Pheasant *L. hatinhensis* and Imperial Pheasant *L. imperialis* than to several other taxa regarded as full species in the genus *Lophura*. However it is not yet possible to determine the extent of their evolutionary divergence and of their proper taxonomic rank. The initial results of this genetic research suggest that there should be efforts to (1) expand field sampling and genetic analyses of wild populations of *Lophura* species, (2) purge the captive stock of Edwards' Pheasant of all hybrids, (3) apply microsatellite analyses to estimate the level of genetic diversity in nuclear DNA.

Introduction

Edwards's Pheasant *Lophura edwardsi* is one of only four pheasant taxa currently classified as critically endangered according to the IUCN (1994) Red List Criteria (McGowan and Garson 1995, P. Garson, unpubl.). This is mainly a consequence of extensive habitat loss throughout its former range in the Annamese lowlands of central Vietnam. Several field surveys in Quang Tri and Thua Thien Provinces after the War failed to locate the species (Eames *et al.* 1992), which was consid-

ered extinct in the wild until its rediscovery in late 1996 (Eve 1997). The conservation of Edwards's Pheasant, as well as two other critically endangered forms also endemic to this small area of Vietnam, the Imperial *Lophura imperialis* and Vietnamese *Lophura hatinhensis* Pheasants, is a high priority (McGowan and Garson 1995). Edwards's and Vietnamese Pheasants are currently breeding both in Vietnam and elsewhere. However, if these captive stocks are to constitute a valuable conservation resource for the future, they need extremely careful management.

Although generally considered to be a valid species (Collar *et al.* 1994), only slight plumage differences distinguish the Imperial and Vietnamese from Edwards's Pheasant. Thus it is important to determine their evolutionary relationships in order to decide the extent to which they deserve separate conservation action (McGowan and Garson 1995). The aims of this paper are to report on the extent of genetic variability in the Edwards's Pheasant captive stock, and to explore the molecular phylogenetics of these three *Lophura* taxa, with the object of contributing vital information to the process of defining a global conservation programme for them. However, our first task is to review all known historical information on the captive population of Edwards's Pheasant.

The Edwards's Pheasant European endangered species programme

In 1994, under the aegis of the World Pheasant Association (WPA), Han Assink and Alain Hennache accepted responsibility for running an International Studbook for the Edwards's Pheasant, and initiated a European Endangered Species Programme (EEP) under the European Association of Zoological Parks and Aquaria (EAZA). This EEP now has 51 participants (18 different countries) managing about 300 birds.

The declared aim of the Edwards's Pheasant EEP is to establish and maintain an *ex situ* population in order to preserve and maintain 90% of its original genetic diversity for a period of at least 100 years. The determination of the size of population required to achieve this result depends on the available historical information, including knowledge of the number of original founders, their breeding history and transfers, as well as information on the current population size and its growth rate. Moreover, reliable information on historical and current populations size and structure is critical for estimating the expected amount of genetic diversity that has been retained so far in the captive stock.

The maintenance of genetic diversity is important for the viability of a captive population. Loss of genetic diversity, through random drift and the occurrence of inbreeding depression can threaten the survival of any small population, as a result of lowered reproductive performance and raised sensitivity to infectious diseases (Ralls and Ballou 1986). It is also likely that some interspecific hybridization has polluted the captive stocks (Carpentier *et al.* 1975), making them unsuitable for a conservation breeding programme.

In response to these problems with the *ex situ* population of Edwards's Pheasant, a research agreement was signed in 1996 between the Museum National d'Histoire Naturelle (MNHN), Paris, France, and the Istituto Nazionale per la Fauna Selvatica (INFS), Bologna, Italy, with the aim of obtaining DNA sequence data on samples from wild and captive-reared Edwards's Pheasant with which to investigate these problems objectively.

Origin of the captive stock

The first priority for the Edwards's Pheasant EEP was to review the historical information in an effort to determine the number of founders and their breeding and transfer history. The history of Jean Delacour's expeditions to Indochina, during which the first Edwards's Pheasants were collected, has been researched in detail by Ciarpaglini and Hennache (1995). Seven expeditions were organized between 1922 and 1939. At least 58–59 Edwards' Pheasants were collected during the first five expeditions in Quang Tri and Thua Thien Huê provinces between 1922 and 1929 (Table 1), although only 28–29 of those, including just 9 females, were exported with certainty (Ciarpaglini and Hennache 1995). Three countries benefited from these imports: France (Clères) in 1924, 1928 and 1930, Japan in 1926 and Great Britain in 1928.

These *c.* 30 Edwards's Pheasants probably included the founders of the present captive population. However, since 1995, new information has become available indicating that Jean Delacour was probably not the only person to import this species from Vietnam. Three other possible sources are of importance (Ciarpaglini and Hennache 1997): (1) Walter Goodfellow imported Edwards's Pheasants and other Indochinese birds to England in 1936 (Anonymous 1936), but nothing more is known than this; (2) American soldiers, returning after the war in Vietnam, may have brought living Edwards's Pheasants to the U.S.A. (T. Lovel *in litt.*, R. Sumner *in litt.*), but this is not properly documented anywhere to our knowledge; and (3) in the early 1980s, eggs of Edwards's Pheasants were received from Vietnam by a well-known private breeder in the former East Germany. From these eggs he reared two females one of which bred later with one of his captive males. According to Dang Gia Tung (*pers. comm.* 1996) the export of anything from central Annam was very difficult in the 1980s because of the widespread destruction of roads. It is therefore possible that these eggs came from northern Annam, within what is now known to be the range of the Vietnamese Pheasant. Thus these females may have been Vietnamese Pheasants, and the captive stock of Edwards's Pheasant may therefore contain some hybrid lines.

Some of the Edwards's Pheasants imported by J. Delacour bred soon after their arrival: in France in 1925, in Japan in 1927, and in England in 1929. In France, reproduction by captive Edwards's Pheasants was very irregular and Delacour did not produce as many Edwards's Pheasants at Clères as he reported in the literature. Only 18–25 Edwards's Pheasants were certainly reared at Clères between 1925 and 1939 (Ciarpaglini and Hennache 1997) and some of these were transferred to other breeders in France, Belgium, Germany and England (Ciarpaglini and Hennache 1997). No data are available for Japan. In England, John Spedan Lewis gave his first Edwards's Pheasant offspring to other English breeders (Ciarpaglini and Hennache 1997). Unfortunately the origin of the American population is completely unknown.

Documented transfers

While the ornithological literature reveals the names of the breeders who obtained the first offspring bred from wild Edwards's Pheasants at Clères, no records exist that allow us to pinpoint accurately the first transfers between continents or countries. World War II destroyed most of the pheasant collections in

Table 1. Edwards's Pheasant captures and importations between 1922 and 1929

Collectors	Years	Number captures known	Females known	Males known	Registered in Jabouille notes	Localities
Jabouill	1922	3	1	2	0	Hailang, Vinhlinh
Jabouille	1922/23	4	0	4	0	Hailang, Vinhlinh
Delacour/Jabouille	1923/24	22	3	19	(2,2) Câmlo (No 15, 5, 19, 7) ; (3,0) Huong Hoa (No 16, 17, 18) ; (1,0) Ba Long (No 8) ; (1,1) Mailanh (No 20,10)	
Jabouille	1925	1	1	0	0	Unknown
Delacour/Lowe	1925/26	7	0	7	(2,0) Mailanh (No 19,282) ; (3,0) Langkhoai (No 945,946,947) ; (2,0) Hai Van Pass (No 1788,1789)	
Jabouille	1926	6/7 (including 1 juvenile)	4/5	1	(1,0) Langkhoai (No 180) ; 1 juv. Langkhoai (No 21)	
Delacour/Jabouille/Lowe	1926/27	5 ?	1	1	(1,1)Thua Luu, Hai Van Pass (No 4130, 4129)	
Delacour/Webb	1927/28	6	2	3	(3,0) Hai Van Pass (No 2882, 2729, 2873)	
Jabouille	1928/29	4	2	2	(1,0) Quang Tri (No 147)	
Total collect known	**58/59**	**14/15**	**39**	**25**		
Total imported birds	**28/29**	**9**	**19/20**			

continental Europe, but were reconstituted just afterwards, using birds presumably obtained from American and English aviaries. Some Edwards's Pheasants were transferred from Clères to John Spedan Lewis in 1937, others were exported from England to the U.S.A. in 1945 (Powell 1997) or from the U.S.A. to France just after the War (J. Delacour *pers. comm.*). The International Studbook documents only the most recent international transfers, from 1950 to 1996, i.e. 130 Edwards's Pheasants exchanged between 19 countries, including Europe (mainly The Netherlands, France, England, Belgium, Czech Republic, Germany, Estonia, Latvia and Russia), Canada, U.S.A., Japan, India and Vietnam. Past exchanges between Japan and The Netherlands suggest that a great part of the Japanese population probably originated from Europe.

In conclusion, these historical studies suggest that: (1) there were relatively few founders in the original captive stock and the number of females did not exceed nine individuals; (2) the *ex situ* population passed through at least two bottlenecks. The first occurred at the very start because many of the imported birds did not reproduce. The second took place during World War II, when there was widespread destruction of pheasant collections throughout continental Europe. After 30–35 generations the present captive population has grown to over 1500 birds (only 300 of which are included in the EEP population), most probably without any further wild founders being added for a period of at least the past 60 years. Although the present captive population originated from three different stocks (France, Japan, England) founded by wild birds captured in different localities and years, the destruction of pheasant collections in continental Europe during World War II and the international transfers before and after this period led to some mixing of all the blood lines in Europe and probably in Japan.

Identification of the bloodlines

The reconstruction of the genealogical relationships among all the Edwards's Pheasants registered in the International Studbook performed using the software packages ZRBOOK (Princee 1991) and SPARKS (Scobie 1996) suggested the existence of several different bloodlines. To assist in the following discussion we define three terms here: (1) a *founder* is an animal from the wild population that produced offspring and has had descendants in the captive population; (2) a *pseudofounder* is a dead or living captive animal of unknown origin that contributed descendants to the captive population; and (3) a *bloodline* is the subpopulation of dead or living individuals derived from a founder or pseudofounder.

We have identified all the putative pseudofounders, of which there are more than 200 in the studbook, as well as finding all their offspring in the records. These groups of pseudofounders and all their offspring have been referred to as bloodlines. Most of the pseudofounders did not have any known offspring, or very few offspring, or could be linked with a high degree of certainty to other known pseudofounders and their blood lines. As a result of this analysis we have identified 14 individuals as potential pseudofounders in the Studbook. Many Edwards's Pheasants distributed all over Europe originated from these birds and their offspring are recorded over six generations.

To these 14 we have added another seven bloodlines on the basis of more anecdotal information. Five of them are linked to small regional populations

resulting from the efforts of private breeders entirely within one country. One is connected with putative wild females that a private breeder apparently imported at the beginning of the 1980s (see above), and the last to one female imported in 1975 by the World Pheasant Association from the U.S.A. to the U.K., although the fate of her offspring is unknown. Thus we have defined 21 blood lines, recorded as A to T and X.

Aims of the DNA study

In the rest of this paper we report the first results of nucleotide sequencing of the mitochondrial DNA (mtDNA) control region in samples from captive Edwards's Pheasants representative of 10 of the 21 recognized bloodlines. We have also analysed samples from other captive birds actually not included in the Studbook, as well as some historical and recent samples of wild birds. Samples from other *Lophura* taxa were analysed in order to make a first attempt to resolve the phylogeny of the three Vietnamese lowlands forms (*L. edwardsi, L. hatinhensis* and *L. imperialis*). Preliminary molecular analyses suggested that they have only diverged very slightly so far, and their taxonomic status remains unclear (Scott 1997, Randi *et al.* 1997).

The mtDNA is a maternally inherited part of the genome about 16,800 nucleotides long in most vertebrates. It includes protein-coding genes, tRNA and rRNA genes, and the control region, a non-coding region containing the promoters for mtDNA replication and transcription (Clayton 1992). The mtDNA has two interesting properties: (1) its rate of molecular evolution is about five times faster than any nuclear gene (Baker and Marshall 1997), and (2) it is a haploid molecule, which does not recombine. As a result, forms of the mtDNA with different nucleotide sequences (haplotypes) can be used as markers for different maternal lineages in captive and wild population (Haig and Avise 1996). On average, control region is the fastest evolving region of the mtDNA, although it accumulates point mutations and insertions/deletions at very different rates in its different domains. The very conservative central domain evolves about 10–20 times more slowly than hypervariable blocks within the two peripheral domains (Baker and Marshall 1997, Randi and Lucchini in press.). Nucleotide sequencing of the mtDNA control region is therefore a powerful tool for estimating genetic diversity within and among closely related populations, as well as for reconstructing phylogenetic relationships among more divergent taxa like congeneric species (Randi and Lucchini *in press.*). A knowledge of mtDNA control region sequences also allows one to identify the maternal species of any offspring, and therefore detect any maternal hybridization.

Methods

Sampling

Feather and blood samples were collected from captive Edwards's Pheasants, representing the main existing bloodlines and reared in the U.S.A., England, The Netherlands, Belgium, Germany, Japan and France. For this study we have sequenced 820 nucleotides of the mtDNA control region in 27 captive-reared

Table 2. Edwards's and Vietnamese Pheasants sampled for the mtDNA gene analysis

Sex	Std#	Source	Blood lines (%)[a]	Maternal lineage	Sample ID
?	—	R. Sumner	Q (Dallas)	Q	LED10
M	0287	Jersey	A(25) B(25) C(50)	C	LED17
M	0258	Jersey	A(25) B(25) C(50)	C	LED18
M	0239	Jersey	A(12,5) B(12,5) D(25) F(50)	B	LED22
F	0502	Jersey	R	R	LED29
F	0226	Jersey	A(25) B(25) C(50)	C	LED31
M	0311	Private	J(50) K(50)	K	LED32
F	0607	Private	S	S	LED34
M	0172	Clères	H(100)	H	LED35
F	0298	Clères	E(50) I(50)	E	LED36
M	0386	Clères	J(50) K(50)	K	LED37
F	0608	Antwerp	OTR	O	LED41
M	0781	Clères	R	R	LED44
F	0825	Clères	E(25) H(50) I(25)	E	LED46
F	0827	Clères	E(25) H(25) I(25) J(12,5) K(12,5)	E	LED48
M	0828	Clères	E(25) H(50) I(25)	E	LED49
F	0860	Paris	E(25) H(50) I(25)	E	LED50
M	0619	Private	P	P	LED51
M	0713	Clères	P	P	LED52
M	0881	Private	OT	O	LED53
F	0882	Private	OT	O	LED54
F	0759	Clères	E(25) H(25) I(25) J(12,5) K(12,5)	E	LED61
F	0058	Powell	QR	Q	LED62
F	0235	Powell	R	R	LED63
M	0983	Private	OT	O	LED64
F	0985	Private	OT	O	LED65
F	0984	Private	OT	O	LED66
M	Museum Paris no 878	Delacour 1931 (F1 Clères ?)		W?	LED55
F	Museum Paris no 922	Delacour 1924 (Câm Lo)		W	LED56
M	Museum Paris no 2882	Delacour 1927 (Hué)		W	LED57
?		Hanoi	Vietnam (August 1996)	W	LED58
?		Hanoi	Vietnam (August 1996)	W	LED59
?		Hanoi	*Lophura hatinhensis*		LHA1
?		Hanoi	*Lophura hatinhensis*		LHA4

[a]The bloodlines are referenced as follows : A, Netherlands 1 ; B, USA 1 ; C, USA 2 ; D, England 1 ; E, Belgium 1 ; F, Belgium 2 ; G, Belgium 3 ; H, Belgium 4 ; I, England 2 ; J, Netherlands 2 ; K, Netherlands 3 ; L, Netherlands 4 ; M, Netherlands 5 ; N, England 3 ; O, Germany 1 ; P, Japan ; Q, USA 3 ; R, England 4 ; S, Netherlands 6 ; T, Germany 2 ; W, Wild ; X, USA 4.

individuals representative of the following maternal bloodlines: B (1 pheasant studied), C (3), E (6), H (1), K (2), O (6), P (2), Q (2), R (3), and S (1) (Table 2). Additionally, we have obtained skin samples from wild Edwards's Pheasant specimens collected by J. Delacour in 1924 (sample labelled LED56 in this paper), 1927 (LED57) and 1931 (LED55), and preserved at Museum National d'Histoire Naturelle, Paris, and from two wild Edwards's Pheasants collected in central Vietnam in August 1996 (LED58 and LED59; Table 2).

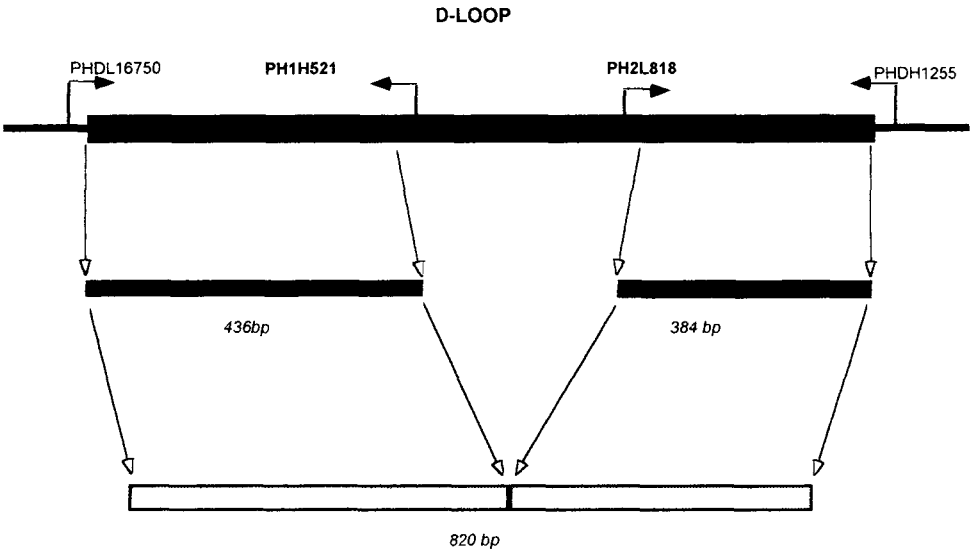

Figure 1. Diagram of the mtDNA control region of Edwards's Pheasants. PHDL 16750 and PHDH 1255 are the primers used for DNA amplification and sequencing. PH1 H521 and PH2 L818 are the internal sequencing primers (see Methods).

Phylogenetic relationships among *Lophura* taxa were reconstructed using nucleotide sequences of the entire (about 1,100 nucleotides) mtDNA control region from the following samples: two Vietnamese Pheasants reared in captivity at Hanoi Zoo; two specimens each of Swinhoe's *L. swinhoii*, Kalij *L. leucomelana*, Silver *L. nycthemera*, and Salvadori's *L. inornata* Pheasants, and Elliot's Pheasant *Syrmaticus ellioti* as the outgroup. All these samples were from captive birds reared in Zoological Park of Clères, France, and sequenced in double to check for the quality of the results.

Laboratory methods

For these genetic analyses we have used methods based on the polymerase chain reaction (PCR), which permits the copying and amplification of selected genes *in vitro*, with a high level of fidelity, from a disparate array of biological samples (Baker and Palumbi 1994). The PCR makes it possible to use samples containing a very few molecules of DNA, even if they are partially degraded, from sources such as feathers, animals found dead in the field, small portions of tissue, skin and bones, or material from old museum specimens. All these samples can be preserved safely in ethanol at room temperature without any further degradation of DNA.

Total DNA was extracted from feather and blood samples using a silica procedure (Hoss and Paabo 1993), or simply by boiling the sample (E. Valsecchi *pers. comm.*). The PCR protocol was optimized to amplify the entire control region of the mtDNA of Edwards's Pheasants (Figure 1), using primers PHDL16750 and PHDH1255 (Fumihito *et al.* 1994), 2 mM $MgCl_2$ with 0.25 μM primer concentration in the PCR buffer, and the following thermal cycle: 94 °C × 120 s; 30 cycles

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at 94 °C × 15 s, 50 °C × 15 s, 72 °C × 60 s; 72 °C × 300 s, performed in a PerkinElmer 9600 machine. The copied mtDNA control regions have been sequenced using the PerkinElmer AmpliTaq FS Dye terminator kit in an ABI 373 automatic sequencer, with the two PCR primers and the following internal sequencing primers (kindly provided by E. A. Scott, Manchester Metropolitan University, U.K.):

PH1H521 (5′ – TTATGTGCTTGACCGAGG AACCAGA – 3′) and PH2L818 (5′ – GGAATG ATCTTGACACTGATGCACT – 3′) (Figure 1).

Analysis of the sequences

Nucleotide sequences were aligned using CLUSTAL W (Thompson *et al.* 1994). Phylogenetic reconstructions were conducted by the neighbour-joining (NJ) method (Saitou and Nei 1987) on percentage divergence estimated by Kimura's (1980) two-parameters formula using the computer program MEGA (Kumar *et al.* 1993). We obtained maximum parsimony (MP) trees using PAUP (Swofford 1993). Robustness of the phylogenies was assessed by the bootstrap method (Felsenstein 1985), with 1,000 resamplings followed by a distance (NJ) or a maximum parsimony reconstruction (bootstrap option in PAUP).

Results

We have obtained partial sequences, spanning 436 nucleotides of the peripheral left and 384 nucleotides of the peripheral right domains (Figure 1) of the mtDNA control region in 27 captive-reared Edwards's Pheasants representatives of 10 maternal bloodlines (Table 2). All these sequences were identical, with the exception of the sample labelled LED64 (*L. edwardsi* 64), which differed by about 1.5% from all the others. After comparisons with other species of *Lophura*, sequence LED64 proved to be identical to the control region sequence of *L. swinhoii* (Figure 3). Thus LED64 is presumed to be a hybrid that has retained the maternal *L. swinhoii* mtDNA.

All the contemporary captive-reared Edwards's Pheasant samples studied (*L. edwardsi* 29, Figure 2) had an identical nucleotide sequence, which was slightly divergent from both the historical and recent samples from wild birds. These wild samples showed two different mtDNA control region sequences: the historical *L. edwardsi* 55 had only one nucleotide difference from the captive-reared (one G–A transition at position no. 142), whilst the other two (nos. 56 and 57) and the recent samples (nos. 58 and 59) had five differences (all transitions) and clustered separately in the phylogeny, at about 0.5% sequence divergence from the captive reared birds (Figure 2).

Sequences for *L. hatinhensis* were very similar to those of *L. edwardsi*: they had fixed nucleotide substitutions at position nos. 201, 297 (C–T transitions) and 463 (deletion of a C in Edwards's Pheasant). Interestingly, the two *L. hatinhensis* samples had two nucleotides shared with the captive Edwards's Pheasant and the wild LED55 (at position nos. 72 and 150), while three nucleotides were shared with all the other historical and recent wild Edwards's Pheasants (at position nos. 142, 205 and 310). Consequently, *L. hatinhensis* is nested within the two

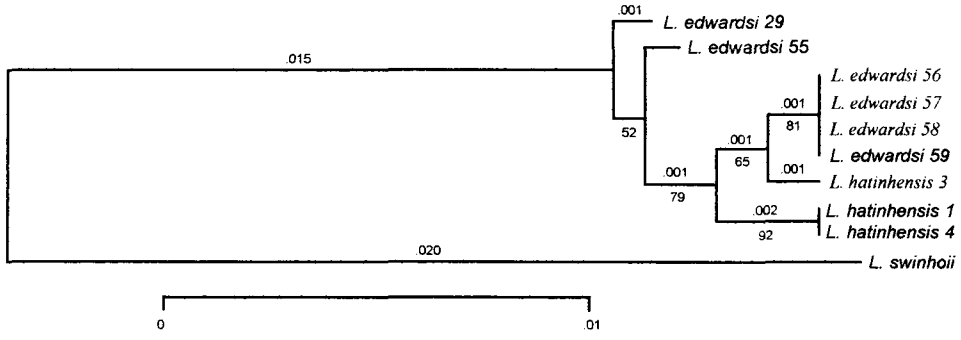

Figure 2. NJ tree showing relationships among the sequenced mtDNA control regions of *in situ* and *ex situ* populations of Edwards's and Vietnamese Pheasant. Above and below the internal branches are the estimated percentage sequence divergence and bootstrap support values. *L. swinhoii* is used as an outgroup to root the tree.

groups of Edwards's Pheasants, at about 0.4% and 0.5% sequence divergence, respectively (Figure 2). Details of the alignment of 820 mtDNA control region nucleotides of *Lophura* pheasants can be obtained from the authors.

Notwithstanding the very low level of divergence amongst all sequences (the maximum genetic distance was <0.5%), the statistical support for the phylogenetic tree showed in Figure 2 was generally high. The bootstrap values, which measure the percentage of recurrence of sequences joined at each internode after 1,000 random sampling of nucleotides in the alignment, were larger than 87%, except for the separation between LED29 and LED55, which received a lower value of 67%. The same topology and very similar bootstrap values were obtained by maximum parsimony procedures.

A provisional phylogeny for the whole series of *Lophura* taxa samples was obtained in the same way, using sequences for the entire control region in *L. swinhoii*, *L. leucomelana*, *L. nycthemera*, *L. inornata*, and in the outgroup *Syrmaticus ellioti*. The peripheral left domain of the control region of Galliformes contains a hypervariable region, spanning about 100 nucleotides, which evolves very quickly and generates phylogenetic noise (Baker and Marshall 1997, Randi and Lucchini, in press). We have therefore excluded this region from the phylogenetic analyses.

Salvadori's Pheasant *L. inornata* has a basal position in the phylogenetic tree (Figure 3). Then there were two strongly supported branches, one leading to the sister taxa *L. leucomelana* and *L. nycthemera* (bootstrap support value = 95%), and the other to *L. swinhoii*, *L edwardsi* and *L. hatinhensis* (bootstrap support value = 82%). NJ and MP phylogenetic trees and bootstrap values were concordant.

Discussion

Edwards's Pheasant ex situ

Nucleotide sequencing of the mtDNA control region suggests that the present day captive-reared Edwards's Pheasant stock has retained a very low level of

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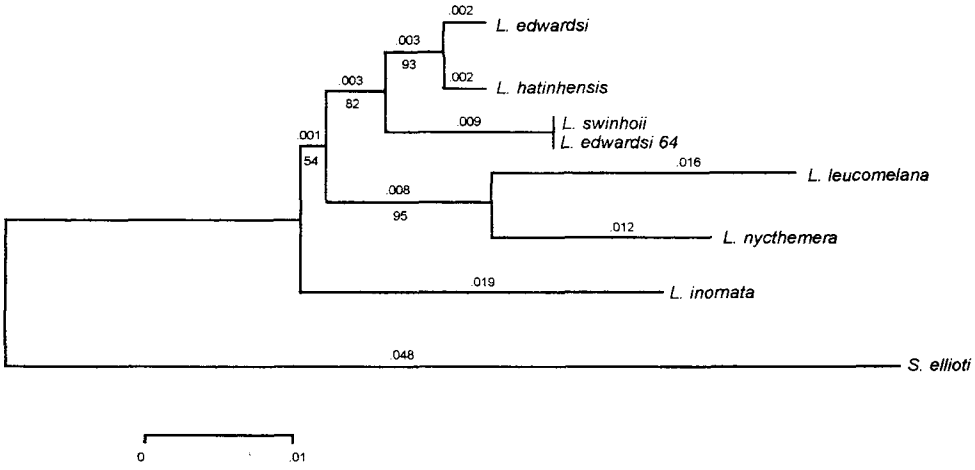

Figure 3. NJ tree showing phylogenetic relationships among the sequenced mtDNA control regions of several *Lophura* taxa. Above and below the internal branches are the estimated percentage sequence divergence and bootstrap support values. *Syrmaticus ellioti* is used as an outgroup to root the tree.

genetic diversity, probably as a consequence of the low number of founder females and at least two bottlenecks during more than 30 generations of captive breeding. There are no apparent mtDNA differences amongst or within the studied bloodlines, even though our sequences include the hypervariable part of peripheral left domain of the control region. This is known to be highly polymorphic in many birds (Baker and Marshall 1997, Randi and Lucchini in press) and, especially so in wild populations of Galliformes, including Alpine species of partridges and tetraonids (E. Randi, V. Lucchini and P. DeMarta unpubl. data). Although we have studied samples belonging to only 10 of the 21 supposed Edwards's Pheasant bloodlines, it seems unlikely that an enlarged sample would reveal additional variability. The number of founding females was low whilst transfer of pheasants among breeders (i.e. gene flow) was high until very recently (Ciarpaglini and Hennache 1997).

Population genetics theory predicts that small isolated populations will lose genetic diversity at a rate which is inversely proportional to their size (Lande and Barrowclough 1987). The founder effect and random genetic drift are seen as powerful forces that can quickly reduce the genetic variability held in small and isolated populations. Computer simulations based on this body of theory (Crow and Kimura 1970) predict that: (1) populations with an effective population size (N_e) of less than 10–20 have high probability of losing all their genetic diversity at nuclear diploid loci within 10–20 generations; (2) as the effective size of mtDNA (as a maternally inherited haploid molecule) is 1/4 N_e of nuclear genes (Wilson *et al.* 1985), it is expected that mtDNA will become monomorphic within $4N_{ef}$ (effective population size of females) generations. This means that populations with $N_{ef} < 5$–10 have a high probability of losing all their mtDNA genetic variability within 20–40 generations. Thus, it is entirely expected that the captive Edwards's Pheasant population will have retained very low or no genetic

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Table 3. Morphological characters of the Vietnamese Pheasant *Lophura hatinhensis*

Features	Vietnamese pheasant	Edwards's pheasant	Remarks
General aspect			Like Edwards's (except white tails feathers)
Tarsus length	87 mm	75 mm	Like Imperial Pheasant (87 mm)
General colour of females	Chestnut reddish	Chestnut brown	More like Imperial Pheasant
Crest	White	White	Like Edwards's Pheasant
Chick			More like Imperial Pheasant
General colour	Chestnut brown	Chestnut brown to yellowish	Darker than Edwards's Pheasant
Crown	Blackish	Brownish	
Line on the eye	Black	Blackish	Larger than Edwards's
Line on the head	Black	Blackish	Larger than Edwards's
Throats, cheeks and sides	Fulvous	Yellowish	
Legs	Reddish	Pinkish	
Base of the bill	Blackish	Yellowish	

diversity within bloodlines, in both the nuclear and mitochondrial genomes (respectively), although the process of independent founding of the first stocks in France, England and Japan and the later independent management of populations in America, could have resulted in random fixation of different alleles in these different bloodlines.

In contrast, comparisons between historical and recent wild Edwards's Pheasant mitDNA sequences suggest that genetic variability is present in the wild population. However the low number of samples from the wild currently makes it impossible to estimate the extent of genetic diversity in this species. Fieldwork aimed at collecting moulted feather and blood or tissue samples from trapped birds would be useful in allowing further work to be done.

Relationships amongst Edwards's, Vietnamese and Imperial Pheasants

Vietnamese and Imperial Pheasants have been found in the same area at Cat Bin in 1990 (Robson *et al.* 1993) whilst Edwards's Pheasant has been rediscovered further south, although still within its historical range in central Annam. If this represents the ancestral distribution pattern, one may expect Imperial and Vietnamese Pheasants to be more similar to each other than either is to Edwards's Pheasant.

Observations made in Clères using living chicks of both Vietnamese and Edwards's Pheasants, and museum skins of Imperial, Vietnamese and Edwards's Pheasants (Table 3) support previous findings (Scott 1997 and pers. comm.) in suggesting that the Imperial Pheasant could be closely related to the other two species of lowland Vietnamese pheasants. The very low degree of genetic divergence shown by the fast-evolving mtDNA control region, makes it difficult to reconstruct a reliable phylogeny for the three lowland Vietnamese *Lophura* taxa and their rearest relatives. The nucleotide sequences presented in this study do

not separate *L. edwardsi* and *L. hatinhensis* into two distinct clades. Instead, *L. hatinhensis* is nested within *L. edwardsi* in paraphyletic position. It is possible that a very recent evolutionary separation by these two taxa has so far resulted only in incomplete segregation of mitochondrial DNA haplotypes, so that the two populations still retain shared polymorphisms.

The information available to date does not allow any conclusive statement to be made about evolutionary relationships between the lowland Vietnamese *Lophura* taxa. However it seems safe to suggest that Edwards's, Vietnamese and Imperial Pheasants diverged very recently (Scott 1997).

More samples are required in order to evaluate if morphological and DNA diagnostic traits are concordant amongst different populations, or if they represent polymorphisms shared across populations. Particularly in birds, genetic divergence alone cannot be used as a yardstick to assign taxonomic rank, because reproductive isolation can be achieved through the evolution of a novel few plumage traits that act as courtships signals in a species recognition system (Zink 1996).

Relationships within the genus Lophura

Sequencing the control region of mtDNA has allowed us to discriminate between several other different *Lophura* taxa that are already accepted as species on morphological biogeographical grounds (Delacour 1977, Johnsgard 1986). The molecular phylogenetic trees we show in this paper are preliminary, however, and need to be integrated with data from more species and longer DNA sequences. Nevertheless our trees suggest that: (1) *L. inornata* is the most primitive amongst *Lophura* species sequenced so far, as suggested also by its morphology; (2) *L. leucomelana* and *nycthemera* may be sister species, as is consistent with parapatric distributions; and (3) *L. swinhoii* and the three lowland Vietnamese *Lophura* forms appear to be closely related, as has been suggested already on morphological grounds. A detailed discussion of these conclusions is premature at this time. A larger-scale and wider analysis of DNA sequences is required before a definitive phylogeny can be derived.

Conservation implications

The data available so far suggest that the captive stock of the Edwards's Pheasant has the very low genetic variablity to be expected of a population bred for many generations from few founders. Our results also suggest that there has also been some genetic pollution of the captive gene pool due to hybridization with Swinhoe's Pheasant. Thus all the Edwards's Pheasants currently registered in the International Studbook should now be sampled so that any hybrid can be removed from the population. This goal can be achieved rapidly and at reasonable cost if all breeders of registered birds agree to collaborate.

Our analysis also suggests that the wild Edwards's Pheasant populations has some genetic variability and the particular mtDNA control region sequence, which has become fixed in the captive stock has not been found in a wild specimen. A careful estimate of the extent of genetic diversity in the rediscovered surviving wild Edwards's Pheasant population is critical to an assessment of the

value of the captive stock (when purged of hybrids) as a genuine insurance against species extinction, and as a source of birds for any introduction or restocking programmes in future.

An improved phylogenetic framework for the genus *Lophura*, the largest among Pheasants, is needed to evaluate the pattern and extent of divergence between the populations representing established species and accepted subspecies. Only this kind of analysis, for the genus as a whole, will allow an objective assessment of the status of problematical taxa such as the three Annamese lowland pheasants. For the moment we hesitate to draw definite conclusions regarding the relative taxonomic status of these three pheasants, pending the results of further molecular, morphological and field research. Thus the provisional position on their conservation management must be that individuals belonging to the three forms should not be cross-bred in captivity, and their wild populations should be managed independently, as if they belong to three different species. Speciation is not (excepting polyploidy) an all-or-nothing process, and many species originate from the accumulation of slight morphological and molecular changes. The careful conservation of the existing pheasant taxa in central Vietnam therefore calls for preserving and not merging *L. edwardsi*, *hatinhensis* and *imperialis* either *ex situ* or *in situ*.

Conclusion

The collaboration between INFS and MNHN is set to continue for several more years, and aims to (1) identify and sequence nuclear genes in order to complete the genetic analyses of the captive stocks of *Lophura*; (2) suggest a protocol for molecular identification of hybrids and enforce its application in order to purge all captive stocks; (3) suggest a captive breeding plan aimed to maximize the retention of genetic diversity and minimize inbreeding in all the separate captive stocks; (4) obtain a complete phylogenetic tree of *Lophura* sequence by using data from mitochondrial and nuclear genes. This phylogenetic tree should ideally include taxa now regarded as full species and as subspecies.

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References

Anonymous (1936) Correspondence. *Avicult. Mag.* 5^{e}s., I, 10 :280.
Baker, A. J. and Marshall H. D. (1997) Mitochondrial control region sequences as tools for

understanding evolution. Pp. 51–82 in D. P. Mindell, ed. *Avian molecular evolution and systematics*. San Diego, California: Academic Press.

Baker, C. S. and Palumbi S. R. (1994) Which whales are hunted? A molecular genetic approach to monitoring whaling. *Science* 265: 1538–1539.

Carpentier, J., Yealland, J. J., Van Bocxstaele, R. and Van Den Bergh, W. (1975) Imperial Pheasant hybridization at Antwerp Zoo. *Int. Zoo Yb.* 15: 100–105.

Ciarpaglini, P. and Hennache, A. (1995) J. Delacour's expeditions to Vietnam from which the captive stock of Edwards' Pheasant originated. *Tragopan* 3: 6–8.

Ciarpaglini, P. and Hennache, A. (1997) Les origines de la population captive du Faisan d'Edwards (*Lophura edwardsi*). Pp. 57–60 in A. Hennache, coord. *The International Studbook for the Edwards's Pheasant and its conservation*. Paris, Muséum National d'Histoire Naturelle, Service des publications scientifiques.

Clayton, D. A. (1992) Transcription and replication of animal mitochondrial DNAs. Pp. 217–323 in D. R. Wolstenholme and K. W. Jeon, eds. *Mitochondrial genomes*. San Diego, California:Academic Press.

Collar, N. J., Crosby, M. J. and Stattersfield, A. J. (1994) *Birds to watch 2: the world list of threatened birds*. Cambridge, U.K.: BirdLife International (BirdLife Conservation Series 4).

Crow, J. F. and Kimura, M. (1970) *An introduction to population genetics theory*. New York: Harper and Row.

Delacour, J. (1977) *Pheasants of the world*. Second edition. Hindhead, U.K.: Spur Publications and World Pheasant Association.

Eames, J. C., Robson, C. R., Nguyen Cu and Truong Van La (1992) *Forest bird surveys in Vietnam 1991*. Cambridge, U.K.: International Council for Bird Preservation (Study Report 51).

Eve, R. (1997) Edwards's Pheasant, naturalists and the history of its rediscovery. Pp. 65–67 in A. Hennache, coord. *The International Studbook for the Edwards's Pheasant and its conservation*. Paris: Muséum National d'Histoire Naturelle, Service des publications scientifiques.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 83–791.

Fumihito, A., Miyake, T., Sumi, S-I., Takada, M., Ohono, S. and Kondo, N. (1994) One subspecies of the red jungle fowl (*Gallus gallus gallus*) suffices as the matriarchic ancestor of all domestic breeds. *Proc. Natl. Acad. Sci., USA* 91: 12505–12509.

Haig, S. M. and Avise, J. C. (1996) Avian conservation genetics. Pp. 160–189 in J. C. Avise and J. L. Hamrick, eds. *Conservation genetics*. New York: Chapman & Hall.

Hoss, M. and Paabo, S. (1993) DNA extraction from Pleistocene bones by a silica-based purification method. *Nucl. Acid Res.* 21: 3913–3914.

Johnsgard P. A. (1986) *The pheasants of the world*. Oxford: Oxford University Press.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111–120.

Kumar, S., Tamura, K. and Nei, M. (1993) MEGA: Molecular evolutionary genetic analysis, version 1.01. University Park, Pennsylvania: Pennsylvania State University.

Lande, R. and Barrowclough, G. F. (1987) Effective population size, genetic variation, and their use in population management. Pp. 87–123 in M.E. Soulè, ed. *Viable populations for conservation*, Cambridge, U.K.: Cambridge University Press.

McDonald, D. M. and Potts, W. K. (1997) DNA microsatellites as genetic markers at several scales. Pp. 30–49 in D. P. Mindell, ed. *Avian molecular evolution and systematics*. San Diego, California: Academic Press.

McGowan, P. J. K. and Garson, P. J. (1995) *Pheasants: status survey and conservation action plan 1995–1999*. Gland, Switzerland: IUCN.

Powell, E. (1997) The history of the Edwards's Pheasant in United Kingdom. Pp. 94–95 in

A. Hennache, coord. *The International Studbook for the Edwards's Pheasant and its conservation*. Paris: Muséum National d'Histoire Naturelle, Service des publications scientifiques.

Princee, P. G. (1991) ZRBOOK (ZooResearch Studbook Management). Amsterdam, National Foundation for Research in Zoological Gardens.

Ralls, K. and Ballou, J. (1986) Captive breeding programs for populations with a small number of founders. *TREE* 1: 19–22.

Randi, E. and Lucchini, V. (in press) Organization and evolution of the mitochondrial DNA control region in the avian genus *Alectoris*. *Mol. Biol. Evol.*

Randi, E., Lucchini, V. and Hennache, A. (1997) Searching for mtDNA genetic diversity in the captive Edwards's Pheasants. Pp. 117–123 in A. Hennache, coord. *The International Studbook for the Edwards's Pheasant and its conservation*. Paris: Muséum National d'Histoire Naturelle, Service des publications scientifiques.

Robson, C. R., Eames, J. C., Ngyuen Cu and Truong Van La (1993) Further records of birds from Viet Nam. *Forktail* 8: 25–52.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406–425.

Scobie, P.N. (1996) SPARKS (Single population analysis and records keeping system). Apple Valley, Minneapolis:International Species Information System.

Scott, E. A. (1997) Systematic relationships of endemic Vietnamese Pheasants. Pp. 26–30 in A. Hennache, coord. *The International Studbook for the Edwards's Pheasant and its conservation*. Paris: Muséum National d'Histoire Naturelle, Service des publications scientifiques.

Swofford, D. L. (1993) PAUP (Phylogenetic analysis using parsimony) version 3.1. Champaign, Illinois: Natural History Survey.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalities and weight matrix choice. *Nucl. Acid. Res.* 22: 4673–4680.

Vo Quy (1975) Chim Viet Nam (Birds of Vietnam). Hanoi: Nha Xuat ban, KHKT (in Vietnamese).

Wilson, A. C. *et al.* (1985) Mitochondrial DNA and two perspectives on evolutionary genetics. *Biol. J. Linn. Soc.* 26: 375–400.

Zink, R. M. (1996) Species concepts, speciation, and sexual selection. *J. Avian Biol.* 27: 1–5.

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