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The *Lanius excubitor* (Aves, Passeriformes) conundrum—Taxonomic dilemma when molecular and non-molecular data tell different stories

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ABSTRACT

The phylogeny of 18 taxa in the *Lanius excubitor* complex, and the related species *L. sphenocercus*, *L. ludovicianus* and *L. somalicus*, was estimated based on the mitochondrial cytochrome *b* gene and the non-coding D-loop (in total ~1.3 kb). According to the mitochondrial gene tree, *Lanius excubitor* s.l. is non-monophyletic, with some of its subspecies being more closely related to *L. sphenocercus*, *L. ludovicianus*, and *L. somalicus*. Also the division of the *L. excubitor* complex into a northern (*L. excubitor*) and a southern (*L. meridionalis*) species, as has been proposed based on morphological and ecological similarity and geographical distributions, is not compatible with the mitochondrial tree. Overall, genetic divergences among the ingroup taxa are small, indicating a recent radiation. A tree based on the nuclear ornithine decarboxylase (ODC) introns 6–7 is unresolved with respect to the ingroup, but provides strong support for a clade containing the *Lanius excubitor* complex, *L. sphenocercus*, *L. ludovicianus* and *L. somalicus*. We discuss the incongruence between the current taxonomy and the mitochondrial gene tree, and conclude that based on the latter the *Lanius excubitor* complex may be treated as at least six species, *L. borealis*, *L. elegans*, *L. excubitor*, *L. lahtora*, *L. meridionalis*, and *L. uncinatus*, but that other taxonomic treatments are also possible. However, uncertainty regarding to which extent the mitochondrial gene tree reflects the species phylogeny prevents us from recommending taxonomic change without further investigation. This study highlights the possible danger of relying on a single molecular marker, such as mitochondrial DNA, in taxonomic revisions and phylogenetic inference.

1. Introduction

Despite that birds are generally well known with respect to the geographical variation in morphology, vocalisations, other behaviours, ecology, distribution, and degree of reproductive isolation between different populations, species limits are frequently the subject of continual debate (e.g. Baker et al., 2003; Collinson et al., 2006; Isler et al., 1999; Parkin et al., 2004; Yésou, 2002). This is an inescapable problem, caused by the gradual nature of evolutionary change, which produces populations that are difficult to classify, irrespective of species concept. The advent of molecular tools has frequently cast doubt upon traditional classifications, hence leading to increased rather than decreased disputes. However, one problem with molecular markers is that gene trees may differ from the organismal phylogeny they are contained within (reviews in e.g. Avise, 1994, 2000; Edwards et al., 2005; Funk and Omland, 2003; Maddison, 1997; Page and Charleston, 1998). This underscores the importance of using independent data when inferring phylogenies and basing taxonomic recommendations on molecular data.

The Great Grey Shrike *Lanius excubitor* complex in the avian family Laniidae is an example of a taxonomically contentious group. Traditionally, it has been treated as a single species with approximately 20 subspecies (Rand, 1960; Vaurie, 1959; Fig. 1 and Table 1) distributed over much of the Palearctic, northern North America, and in northern Africa south to the Sahel region (Fig. 1). Some of the subspecies are highly distinctive in plumage, while others are very similar (del Hoyo et al. 2008; Harris, 2000; Lefranc and Worfolk, 1997). Based on morphological and ecological characteristics and geographical distributions, several authors have divided this species into two subspecies groups, a northern and a southern one (Cramp and Perrins, 1993; Eck, 1994; Glutz

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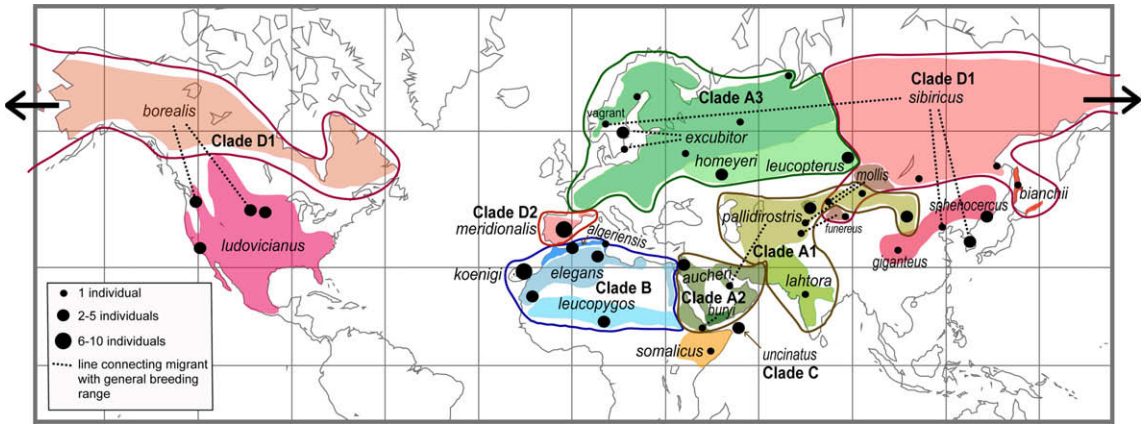

Fig. 1. Breeding distribution of the taxa in the *Lanius excubitor* complex (based on e.g. Cheng, 1987; Cramp and Perrins 1993; Harris, 2000; Lefranc and Worfolk, 1997). The sites of the DNA samples used in the present study are marked by filled circles (sizes representing samples sizes, as explained in figure). Lines encircling ranges conform with the clades in Fig. 2. Filled circles denoting individuals of migratory species collected away from the breeding grounds are connected to the appropriate taxon by a dashed line.

von Blotzheim and Bauer, 1993; Panov, 1983; Schön, 1998; Vaurie, 1959; Fig. 2 and Table 1). Most recent authors have gone one step further and treated these subspecies groups as two polytypic species, Great (or Northern) Grey Shrike *L. excubitor* and Southern Grey Shrike *L. meridionalis* (Clements, 2000; del Hoyo et al., 2008; Dickinson 2003; Harris, 2000; Isenmann and Bouchet, 1993; Lefranc and Worfolk, 1997; Fig. 2 and Table 1). This is based on alleged sympatry between the northern and southern groups in two areas, without any evidence of interbreeding, in combination with differences in morphology and ecology. Lack of interbreeding has been reported from Mongolia, where *mollis* and *pallidirostris* are stated to occur in sympatry (Panov, 1995), and from SW France, where *excubitor* and *meridionalis* have been suggested to breed sympatrically (Isenmann and Bouchet, 1993; Lefranc, 1999). Previously, Grant and Mackworth-Praed (1952) split the complex in two species based solely on morphology, but they used the name *L. elegans* for the southern species (*meridionalis* s.s. was not dealt with). Isenmann and Bouchet (1993) treated all taxa but erroneously also used *elegans*, although *meridionalis* has priority (see Table 1).

Table 1
Examples of previous classifications of the *Lanius excubitor* complex.

Rand (1960)	Panov (1983)	Lefranc and Worfolk (1997), Harris (2000), Dickinson (2003), del Hoyo et al. (2008)
L. excubitor	*L. excubitor* (excubitor group)	*L. excubitor*
borealis	*borealis*	*borealis*
invictus	*invictus*	*invictus*[1]
sibiricus	*sibiricus*	*sibiricus*
excubitor	*excubitor*	*excubitor*
bianchii	—	*bianchii*
mollis	*mollis*	*mollis*
funereus	*funereus*	*funereus*
homeyeri	*homeyeri*	*homeyeri*
leucopterus	—	*leucopterus*[2]
—	*melanopterus*	—
	L. excubitor (*meridionalis* group)	*L. meridionalis*
meridionalis	*meridionalis*	*meridionalis*
koenigi	—	*koenigi*
algeriensis	*algeriensis*	*algeriensis*
elegans	*elegans*	*elegans*
leucopygos	*leucopygos*	*leucopygos*
—	—	*jebelmarrae*[3]
aucheri	*aucheri*	*aucheri*
buryi	*buryi*	*buryi*
uncinatus	*uncinatus*	*uncinatus*
lahtora	*lahtora*	*lahtora*
pallidirostris	*pallidirostris*	*pallidirostris*
—	—	*theresae*[4]

[1] Not recognised by Dickinson (2003).
[2] Not recognised by Dickinson (2003) and del Hoyo et al. (2008).
[3] Only recognised by Dickinson (2003).
[4] Not recognised by Harris (2000).

The Chinese Grey Shrike *L. sphenocercus* and North American Loggerhead Shrike *L. ludovicianus* are generally considered to be closely related to the *L. excubitor* complex based on morphological and ecological similarity (Cramp and Perrins, 1993; Harris, 2000; Lefranc and Worfolk, 1997). A study of mitochondrial control region sequence data by Mundy and Helbig (2004) found *L. ludovicianus* to be nested within *L. excubitor* s.l. These authors also found a tandem repeat in the mitochondrial control region of Loggerhead Shrike *L. ludovicianus* and *L. excubitor* s.l., but not in Fiscal Shrike *L. collaris*, Red-backed Shrike *L. collurio*, Isabelline Shirke *L. isabellinus*, Long-tailed Shrike *L. schach*, or Woodchat Shrike *L. senator*. Mundy and Helbig (2004) also suggested that the Iberian *meridionalis* s.s. was sister to the North American *invictus*, with the mainly NW Palearctic *excubitor* s.s. in a basal position. The fragment length polymorphism resulting from different numbers of repeats was further studied in the *L. excubitor* complex by Hernández et al. (2004), who found that the proportion of number of repeats per individual differed between taxa. For example, in *excubitor* s.s., 11% of the individuals had two repeats and 80% had three repeats, while in *pallidirostris* 93% had two repeats and 7% had three repeats. Based on these results, Hernández et al. (2004) proposed that *L. excubitor* s.l. be divided into three species: *L. excubitor*, *L. meridionalis* and *L. pallidirostris*. Evidence of further divergence was presented by Klassert et al. (2008) who, based on mitochondrial cytochrome *b*, showed that only the Iberian *meridionalis* s.s. is closely related to the North American *invictus* (junior synonym of *borealis*, Dickinson, 2003), while the Canary Islands *koenigi* and North African *algeriensis* are sisters to *excubitor* s.s., *aucheri* (Indian subcontinent) and *pallidirostris*. They also confirmed that *L. sphenocercus* is part of this complex. A similar hypothesis was presented by Gonzalez et al. (2008) based on sequence data from the mitochondrial cytochrome *b* and the nuclear introns ornithine decarboxylase and myoglobin, although they lacked data on *aucheri*, *pallidirostris* and *L. sphenocercus*. Taken together, the studies of Mundy and Helbig (2004), Hernández et al. (2004), Klassert et al. (2008) and Gonzalez et al. (2008) point in the same direction: there seems to be a basal dichotomy separating one branch

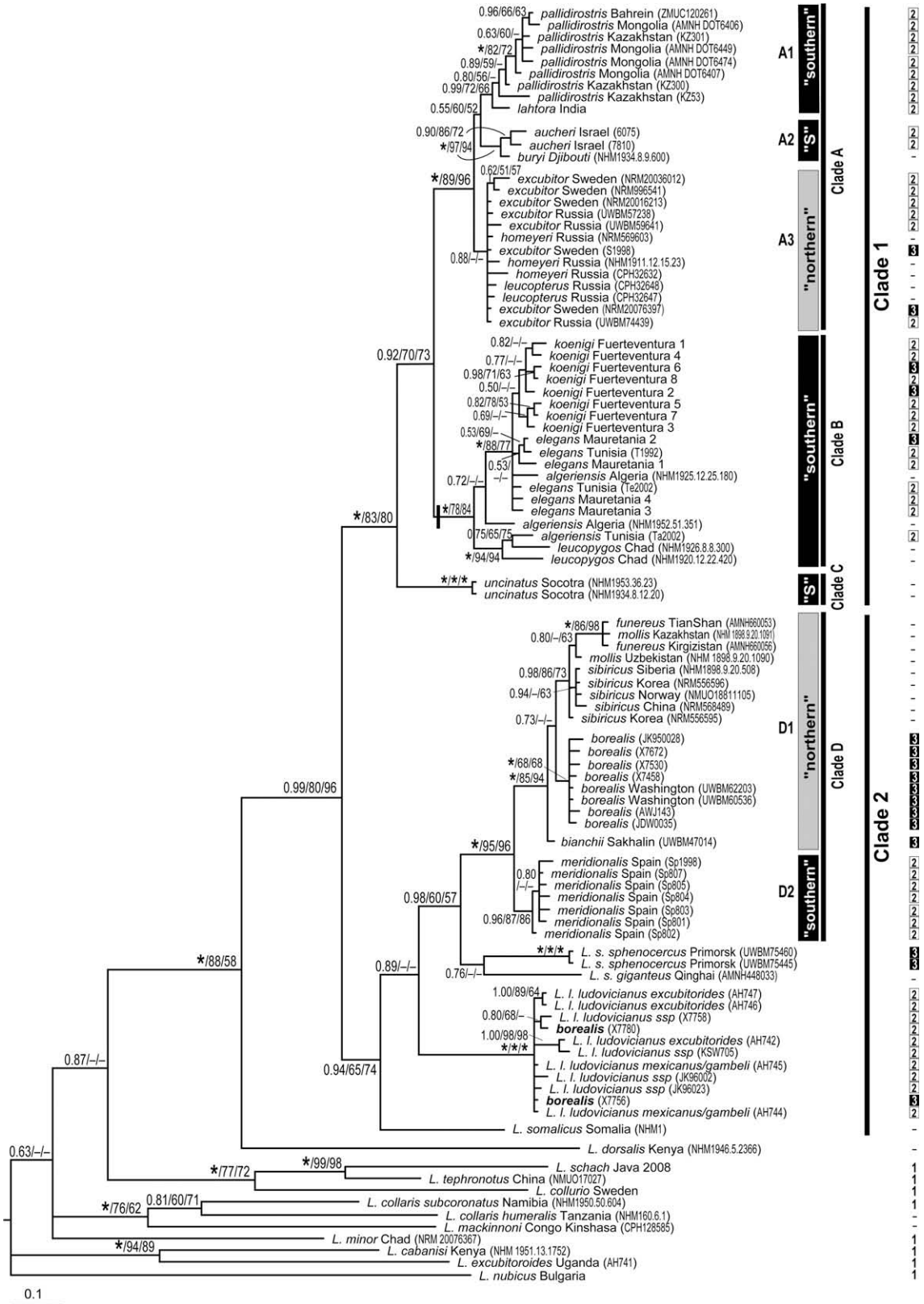

Fig. 2. Relationships of the *Lanius excubitor* complex and its nearest relatives, estimated by Bayesian analysis of the mitochondrial cytochrome *b* gene and part of the mitochondrial control region D-loop (in total 1.3 kbp). The broad grey and black vertical bars denote the common division into a northern and southern group, respectively. The former is now usually treated *L. excubitor* s.s. and the latter as *L. meridionalis*. The vertical bar at the base of clade B indicates a unique synapomorphic single-base pair deletion in the D-loop alignment. The column on the far right gives the number of tandem repeats in the mitochondrial control region found in each individual in this study; "–" denotes missing data. Values at branches indicate, in sequence, posterior probability, maximum likelihood bootstrap and parsimony bootstrap (all ≥0.50/50%); ∗ denote posterior probability or bootstrap value of 1.00/100%.

Table 2
Pairwise cytochrome *b* distances within and between major groups or clades.

Group or clade	% Distance uncorrected *p*	% Distance HKY + Γ + *I* corrected
Clade A1	0–1.0	0–1.1
Clade A2	0.3–0.4	0.3–0.4
Clade A3	0–0.6	0–0.6
A1–A2	0.6–1.3	0.6–1.4
A1–A3	0.3–1.2	0.3–1.2
A2–A3	0.4–1.0	0.4–1.1
Clade B (all)	0.1–2.1	0.1–2.3
Clade B *(elegans/koenigi)*	0.1–0.6	0.1–0.6
Clade B *(algeriensis–elegans/koenigi)*	0.5–1.6	0.5–1.7
Clade A–clade B	1.3–3.6	1.4–4.3
Clade A–clade C	2.3–3.5	2.6–4.2
Clade B–clade C	2.3–3.3	2.6–3.8
Clade 2	0–4.9	0–6.3
Clade D1 *(borealis)*	0–0.5	0–0.5
Clade D1 *(borealis–sibiricus/mollis)*	0.3–1.3	0.3–1.3
Clade D2	0–0.4	0–0.4
Clade D1–clade D2	0.8–1.8	0.8–1.9
Clade 1–clade D	2.5–6.9	2.8–9.1
Clade 1–clade 2	2.5–6.9	2.8–9.1
L. ludovicianus–clade 1	4.4–6.0	5.7–7.8
L. sphenocercus–clade 1	3.6–6.2	4.3–8.6
L. somalicus–clade 1	3.7–5.9	4.7–7.8
L. ludovicianus–clade D	2.5–3.7	2.9–4.3
L. sphenocercus–clade D	2.6–4.1	3.0–4.9
L. somalicus–clade D	3.9–5.1	4.8–6.5
L. sphenocercus–L. ludovicianus–L. somalicus	3.9–4.7	4.9–6.0
L. ludovicianus clade	0–0.6	0–0.6
L. s. sphenocercus–L. s. giganteus	3.2	3.8

containing *excubitor*, *aucheri, pallidirostris, koenigi* and *algeriensis* from a branch containing *meridionalis, invictus, L. sphenocercus* and *L. ludovicianus*. These results challenge both the traditional classification of the *L. excubitor* complex and the more recent separation into a northern and a southern species.

We here present a phylogenetic hypothesis for the *Lanius excubitor* complex based on two mitochondrial loci, the cytochrome *b* gene and the non-coding D-loop region, and the nuclear ornithine decarboxylase (ODC) introns 6–7. We include 18 taxa that are traditionally placed in the *L. excubitor* complex, as well as the closely related *L. sphenocercus* and *L. ludovicianus* (two subspecies of each). The taxa *bianchii, buryi, funereus, homeyeri, lahtora, leucopterus, leucopygos, mollis, sibiricus* and *uncinatus* in the *L. excubitor* complex are here included in a molecular phylogenetic study for the first time. Twelve additional taxa in the genus *Lanius* are included, most of which show morphological similarity to the *L. excubitor* complex. We discuss the results in comparison with the traditional classification, and discuss discordances between these data.

2. Material and methods

2.1. Study group

For taxon names and taxonomy, we follow Dickinson (2003) (Table 1), although we are aware of some instances where a revision is called for based on examination of the collections in several museums (unpubl.). We obtained samples of 97 individuals of 34 taxa in the genus *Lanius* (Appendix). In the *Lanius excubitor* complex we obtained samples from all of the taxa recognised by Dickinson (2003), except *jebelmarrae* and *theresae* (Appendix). All of these, except one sample of *excubitor*, one *pallidirostris*, three *sibiricus* and all *borealis*, *mollis* and *funereus*, were collected on or near the breeding grounds. The ones that were collected on migration or in their winter quarters were identified based on their distribution (*excubitor* and *borealis*) or by morphological characters (in the case of *mollis, funereus* and *sibiricus* in comparison with large series of museum specimens; L.S.); the Norwegian record of *sibiricus* (a 19th century specimen) was identified by L.S. and later confirmed by mitochondrial DNA. Fifty-one of the samples were from museum specimens (Appendix).

2.2. DNA extraction and sequencing

DNA was extracted from blood, feathers, muscle or museum specimen toepads, using QIA Quick DNEasy Kit (Qiagen, Inc) according to the manufacturers instructions, but with 30 µl DTT added to the initial incubation step of the extraction of feathers. Samples obtained from old museum specimens were initially incubated for 2–4 days.

We sequenced the mitochondrial cytochrome *b* gene and the non-coding D-loop from 97 individuals of 34 *Lanius* taxa, and the nuclear ornithine decarboxylase (ODC) introns 6–7 for at least one individual per taxon for 29 taxa (Appendix). The tandem repeat region of the mitochondrial control region was amplified and characterized for all samples for which fresh material was available.

Amplification and sequencing followed the protocols described in Olsson et al. (2005) for the cytochrome *b* gene; Allen and Omland (2003), Friesen et al. (1999), and Irestedt et al. (2006) for intron 6–7 of the nuclear ODC gene; and Mundy et al. (1996), and Mundy and Helbig (2004) for the D-loop and tandem repeat region of the mitochondrial control region. Amplification of DNA from museum specimens was done with specifically designed primers, following a different protocol, available upon request. Except for the material from old museum specimens, the cytochrome *b* gene was amplified as one fragment to decrease the risk of amplifying nuclear pseudocopies (cf. e.g. Arctander, 1995; Quinn, 1997; Quinn and White, 1987; Sorensen and Quinn, 1998).

2.3. Phylogenetic analyses

Sequences were aligned using MegAlign 4.03 in the DNAstar package (DNAstar Inc.). Phylogenies were estimated by Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The choice of model for the BI was determined based on the Akaike Information Criterion (Akaike, 1973) and a hierarchical likelihood ratio test (Posada and Crandall, 1998), both calculated in MrModeltest (Nylander, 2004). For all loci, the preferred model was HKY (Hasegawa et al., 1985), assuming rate variation across sites according to a discrete gamma distribution with four rate categories (Γ_4; Yang, 1994) and an estimated proportion of invariant sites (*I*; Gu et al., 1995). As the same model was suggested for both mitochondrial loci, data were analysed without being partitioned in the BI. Four Metropolis-coupled MCMC chains with incremental heating temperature 0.1 were run for 40 million generations and sampled every 100 generations. Every analysis was repeated four times, starting from random trees, and the results compared to ascertain that the chains had reached the same target distributions (as suggested by Huelsenbeck et al., 2002). The samples from the stationary phases of the independent runs were pooled to obtain the final approximation of the posterior distribution of trees. The posterior distributions were summarized as a majority-rule consensus tree. The first 10 million generations of each run, well after the chain reached apparent stationarity, were discarded as burn-in.

Maximum likelihood (ML) bootstrapping (1000 replicates) was performed in Treefinder Jobb et al., 2004; Jobb, 2008) using default settings and the same models as in the BI. Parsimony bootstrapping was performed in PAUP*: heuristic search strategy, 1000 replicates, starting trees obtained by stepwise addition (ran-

dom addition sequence, 10 replicates), TBR branch swapping, MulTrees option not in effect (only one tree saved per replicate).

2.4. Genetic distances

Pairwise distances, both uncorrected *p* and HKY + Γ + *I* corrected were calculated in PAUP* (Swofford 2001), with proportion of invariable sites and gamma shape estimated in the Bayesian analysis, performed as described above.

3. Results

3.1. Sequence characteristics

We obtained nucleotide data from a 1076 base pair portion of the cytochrome *b* gene and flanking region of tRNA-Thr (hereafter referred to as just cytochrome *b*), and a 256–257 base pair stretch of the D-loop from 73 individuals in the *Lanius excubitor* complex and 24 individuals of other *Lanius* species. For most samples that were obtained from museum specimens, we failed to sequence the entire cytochrome *b* fragment. Details of fragment lengths, origin and GenBank accession numbers are given in the Appendix.

We also obtained 696 base pairs of the nuclear ODC introns 6–7 for all fresh samples, but for several samples from museum specimens amplification failed or only partial sequences were obtained. Amplification of ODC failed entirely for *buryi, funereus, homeyeri, leucopterus* and *mollis*, and for *L. s. giganteus*. Differences between individuals in the ingroup consisted mainly of autapomorphic base substitutions, but also some ambiguous base calling at certain apparently heterozygous positions.

The phylogenetic analyses of the concatenated sequences of the light strand of the cytochrome *b* gene and the D-loop fragments contains 1333 characters, of which 205 (15%) are parsimony informative, 986 (74%) are constant, and 142 (11%) are variable but parsimony-uninformative. No unexpected start or stop codons that could indicate the presence of nuclear copies were observed in the cytochrome *b* sequences.

3.2. Tree topology

The *L. excubitor* complex forms a well-supported clade together with *L. somalicus*, *L. sphenocercus* and *L. ludovicianus* (Fig. 2). The taxa fall into two main clades (1 and 2). Clade 1 is strongly supported and comprises 12 taxa in the *L. excubitor* complex. Clade 2 comprises six taxa in the *L. excubitor* complex, as well as *L. ludovicianus*, *L. sphenocercus* and Somali Fiscal *L. somalicus*, with high posterior probability but lower bootstrap values (Fig. 2).

The taxa in the *L. excubitor* complex are divided into four major, well supported, clades (A, B, C, D; Fig. 2). Clade A comprises *lahtora* and *pallidirostris* from the Indian subcontinent, Central Asia and Mongolia (clade A1), *aucheri* and *buryi* from the Middle East/Arabia (clade A2), and the mainly European *excubitor*, SW Siberian *homeyeri* and N Kazakhstan *leucopterus* (clade A1). Clades A1 and A2 are inferred to be sisters, with poor support, and clade A3 is poorly supported by the molecular data. There is no structure among the three taxa in clade A3, while A1 and A2 have more structure. Clade B, which is sister to clade A with modest support, contains *algeriensis*, *elegans*, *leucopygos* and *koenigi* from North Africa and the Canary Islands. This clade is further corroborated by a unique one-base pair deletion in the control region alignment (Fig. 2). None of the taxa in this clade is strongly supported to have reciprocally monophyletic haplotypes, and *algeriensis* is nested both among *elegans/koenigi* and *leucopygos*, as well as on its own branch. The Socotran endemic *uncinatus* is sister to clades A and B with fairly strong support.

Clade 2 is moderately strongly supported by the molecular data. It includes several subclades, three of which are universally treated as separate species from the *L. excubitor* complex. Clade D comprises two main subclades, whose sister relation is strongly supported: *bianchii*, *funereus*, *mollis* and *sibiricus* of the NE Palearctic, and the North American *borealis* (clade D1), and the Iberian *meridionalis* (clade D2). The taxa *funereus*, *mollis* and *sibiricus* form a well-supported clade, although none of the taxa has reciprocally monophyletic haplotypes; *bianchii* is in a sister position to the others in clade D, although that is only weakly supported. In contrast, the *borealis* haplotypes are reciprocally monophyletic, with good support (but see below). The NE Chinese, Mongolian and Russian Far East *L. sphenocercus sphenocercus* and the Tibetan *L. s. giganteus* are inferred to be sisters to clade D, although the sister relationship between these two taxa is only weakly supported. The *L. ludovicianus* clade, which is inferred to be sister to clade D and *L. sphenocercus* with insufficient support, is unresolved with respect to the included subspecies. It also includes two samples of *L. e. borealis*. *L. somalicus* is sister to the others in clade 2, with moderate support.

A tree based on the nuclear ODC (Fig. 3) is unresolved with regard to the ingroup, but provides strong support that *L. somalicus*,

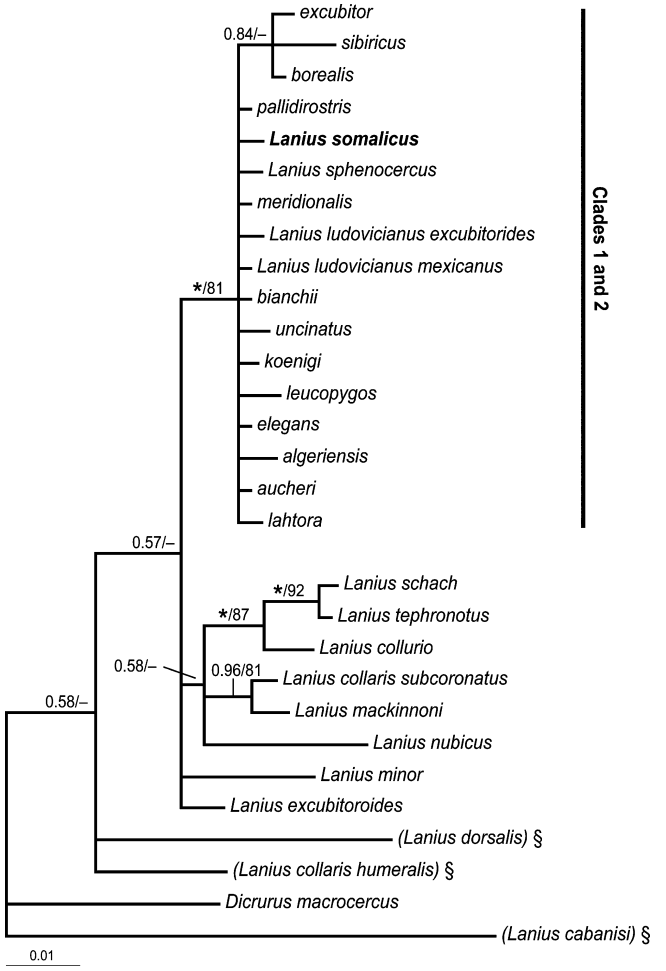

Fig. 3. Relationships of the *Lanius excubitor* complex and its nearest relatives, estimated by Bayesian analysis of the nuclear ornithine decarboxylate introns 6–7 and adjacent coding regions (ODC) (in total ~700 bp). The taxa that constitute clades 1 and 2 in Fig. 2 are here collapsed to a single, well-supported clade. Values at branches indicate, in sequence, posterior probability and parsimony bootstrap (≥0.50/50%); * denote posterior probability or bootstrap value of 1.00/100%. § The sequences of *L. cabanisi, L. collaris humeralis* and *L. dorsalis* are short, and the resulting low number of informative sites makes the placement of these taxa imprecise.

L. ludovicianus, L. sphenocercus and the *L. excubitor* complex belong to the same clade. For some taxa, particularly Taita Fiscal *L. dorsalis*, *L. collaris humeralis* and Long-tailed Fiscal *L. cabanisi*, the ODC sequences are short, making their placement imprecise.

3.3. Genetic divergences

The pairwise cytochrome *b* distances among the ingroup taxa (clades 1 and 2) are 0–6.9% (uncorrected) or 0–9.1% (HKY + Γ + *I* corrected) (Table 2). The differences between clades 1 and 2 are comparable to those between *L. sphenocercus*, *L. ludovicianus*, *L. somalicus* and clade 1. The divergences between clades A–B–C are minimum 1.3% (uncorrected) and maximum 4.3% (HKY + Γ + *I*); between *L. sphenocercus*–*L. ludovicianus*–*L. somalicus*–clade D minimum 2.5% (uncorrected) and maximum 6.5% (HKY + Γ + *I*); between A1–A2–A3 minimum 0.3% (uncorrected and HKY + Γ + *I*) and maximum 1.4% (HKY + Γ + *I*); and between D1–D2 minimum 0.8% (uncorrected and HKY + Γ + *I*) and maximum 1.9% (HKY + Γ + *I*). The divergences within clades A1, A2, A3, B, D1, D2 and *L. ludovicianus* are minimum 0% (uncorrected and HKY + Γ + *I*) and maximum 2.3% (HKY + Γ + *I*). The divergence between *L. s. sphenocercus* and *L. s. giganteus* is minimum 3.2% (uncorrected) and maximum 3.8% (HKY + Γ + *I*).

Within the *Lanius excubitor* complex, the ODC sequences show only marginal differentiation, with a maximum divergence of 0.7% (uncorrected p), between *borealis* and *excubitor*, but the same divergence exists between two individuals of *borealis*.

Among the outgroup taxa, a surprisingly large cytochrome *b* divergence is evident between *subcoronatus* and *humeralis*, which are presently treated as subspecies of *L. collaris* (Dickinson 2003; del Hoyo et al., 2008; Harris, 2000; Lefranc and Worfolk, 1997; Rand, 1960).

3.4. Tandem repeats

Amplification of the tandem repeat region of the mitochondrial control region yielded fragments of different lengths. Some were sequenced and used as calibration standards against which the remainder were compared. All individuals in the clade consisting of *Lanius excubitor, L. sphenocercus, L. ludovicianus* and *L. somalicus* has two or more repeats, while none of the samples outside this clade has more than one repeat (Fig. 2). A single case shows a trace of a band that appears to contain four repeats (Fig. 2). The commonest haplotype in this clade has two tandem repeats, with individuals containing three repeats scattered across clades 1 and 2. In clades A1, D2 and *L. ludovicianus* there are exclusively two tandem repeats, with the exception of one of the *borealis* individuals showing *L. ludovicianus* cytochrome *b* haplotype. In clade D1, all individuals that yielded a PCR product of the tandem repeat region contains three repeats, as do the two individuals in the *sphenocercus* clade.

4. Discussion

4.1. Tree topology

The mitochondrial tree is overall fairly well supported by our data. Each of the four main clades in the *L. excubitor* complex (clades A–D) are well supported. In agreement with previous phylogenetic hypotheses, based on mitochondrial genes (Klassert et al., 2008; Mundy and Helbig, 2004) and mitochondrial and nuclear (ODC, myoglobin) genes (Gonzalez et al., 2008), the data do not support monophyly of the *L. excubitor* complex (clades A–D). Instead, the taxa in clade D are inferred to be more closely related to *L. ludovicianus, L. sphenocercus* and *L. somalicus*. The two generally accepted groups/species within the *L. excubitor* complex, a "southern" and a "northern" one, are non-monophyletic according to our results: the taxa belonging to the *excubitor* ("northern") group/species are found in clades A3 and D1, while those belonging to the *meridionalis* ("southern") group/species constitute clades A1, A2, B, C and D2. These findings are consistent with previous molecular studies (Gonzalez et al., 2008; Klassert et al., 2008; Mundy and Helbig, 2004), and are remarkable both from a morphological and a biogeographical perspective (see below).

The ODC tree provides additional support for a close relationship between the *L. excubitor* complex, *L. sphenocercus*, *L. ludovicianus* and *L. somalicus*. We interpret the lack of differentiation in the comparatively rapidly evolving ODC and CHD1-Z introns (cf. Allen and Omland, 2003; Irestedt et al., 2006; Peters et al., 2005), in combination with the overall small cytochrome *b* distances among the taxa in the ingroup, as evidence of recent divergence of the entire group.

4.2. Incongruence between the gene tree and non-molecular traits

The mitochondrial gene tree is remarkably different from taxonomic arrangements based on morphological and ecological similarity and geographical distributions, which presume that the *L. excubitor* complex is monophyletic and separated into a northern and a southern group of subspecies or species (e.g. Cramp and Perrins, 1993; del Hoyo et al., 2008; Dickinson, 2003; Glutz von Blotzheim and Bauer, 1993; Harris, 2000; Lefranc and Worfolk, 1997; Panov, 1983; Vaurie, 1959). In particular, the strong morphological similarity between the allegedly geographically connected *excubitor* (clade A3) vs. *sibiricus* (D1), as well as the discontinuously distributed *excubitor* (A3) vs. *bianchii* (D1) and *pallidirostris* (A1) vs. *elegans* (B) (e.g. Cramp and Perrins, 1993; del Hoyo et al., 2008; Harris, 2000; Lefranc and Worfolk, 1997; Panov, 1983), are difficult to reconcile with the mitochondrial tree. The pronounced similarity between *excubitor* and *bianchii*, separated by a vast area of birds with more divergent plumage, was noted already by Hartert (1910). Moreover, the close relatedness and small cytochrome *b* divergence between the Iberian *meridionalis* and the NE Palearctic and North American taxa, as well as the non-monophyly of the *L. excubitor* complex, suggested by the mitochondrial data has never been suspected based on morphology. In addition, the Socotran endemic *uncinatus* was recently synonymized with *aucheri* due to their morphological similarity (Kirwan, 2007), which is contradicted by the present study.

The tree presented in Fig. 2 is based exclusively on mitochondrial DNA, which calls for a cautious interpretation, since gene trees can differ from the species phylogeny (reviews in Avise, 1994, 2000; Edwards et al., 2005; Funk and Omland, 2003; Maddison, 1997; Page and Charleston, 1998). The incongruence between the gene tree and non-molecular data could have any (or several) of the following molecular bases: (1) incorrectly inferred tree; (2) amplification of paralogous sequences; (3) incomplete lineage sorting or fixation of "mis-sorted" ancestral haplotypes; or (4) introgressive hybridization. Alternatively, it could have a morphological foundation, such as (1) retention of plesiomorphic morphological character states in unrelated taxa, and/or (2) parallel morphological evolution. The first of these possibilities seems unlikely, since the inferred tree is overall strongly supported, both in model-based and parsimony analyses. There is also complete agreement with three independent studies (Gonzalez et al., 2008; Klassert et al., 2008; Mundy and Helbig, 2004). The second point also seems improbable, since there is much variation among the haplotypes of all taxa for which multiple samples are available. Moreover, all of the coding sequences translate into amino acids.

Effects of lineage sorting cannot be eliminated with certainty, and larger sample sizes and independent data will be required to evalu-

ate whether these processes may have affected the gene tree. Moore (1995) argued that trees based on mitochondrial DNA are much less likely to be affected by lineage sorting problems than trees based on nuclear DNA, since the effective population size (N_e) of mitochondrial DNA is only a quarter of that of nuclear-autosomal DNA (since mtDNA is effectively haploid and transferred only through the matriline). Mitochondrial gene trees would thus conform with the species phylogeny more often than nuclear gene trees—unless the internodes between splitting events are short relative to the internodal effective population size, in which case also mitochondrial gene trees might suffer from lineage sorting problems. In the *Lanius* gene tree the internodes uniting the main clades are relatively short, increasing the chance that lineage sorting may be a problem. Fixation of "mis-sorted" haplotypes would lead to permanent discordance between the gene tree in question and the organismal phylogeny (Edwards, 1997). Unfortunately, our attempts to obtain an independent estimate of the phylogeny have been unsuccessful. Most of the nuclear ODC sequences are identical among the ingroup taxa, or differ by single autapomorphic base substitutions. However, they strongly support that *L. sphenocercus, L. ludovicianus* and *L. somalicus* are part of the same clade as the *L. excubitor* complex, although the precise positions of the three former are uncertain. Moreover, Z-chromosome-linked CHD1-Z intron sequences of *meridionalis*, *koenigi*, *pallidirostris*, *L. sphenocercus* and *L. collurio* are available on GenBank (accession numbers AY180171–75). However, all these are identical except *L. collurio* which differs from the others by 0.7% (uncorrected *p*). For this reason, we dismissed this marker, and did not produce any sequences of our own.

There are only two cases of probable recent introgression between main subclades: two haplotypes of *borealis* in the *L. ludovicianus* clade. However, more ancient introgression and subsequent fixation of introgressed haplotypes cannot be eliminated, and actually seems to be a possibility (see below). Cases of mitochondrial introgression have been suggested several times (e.g. Alström et al., 2008; Alström and Mild, 2003 and references therein; Andersson, 1999; Degnan, 1993; Irwin et al., 2009; Kulikova et al., 2005; Peters et al., 2007; Weckstein et al., 2001).

If the gene tree indeed represents the organismal phylogeny, parallel evolution of morphological traits and/or retained ancestral states are likely to explain the incongruence between the gene tree and the traditional classification. For example, the overall similarity in multiple traits between clades A1 and B could be due to retention of these traits, as a result of stabilising selection or only weak divergent selection in the relatively homogeneous habitat occupied by these taxa. The selection for divergence in morphology would probably have been stronger in clade A3, after it diverged from a common ancestor with A1/A2 and spread northward into a different biome. However, the strong morphological similarity between the taxa in clades A3 and D1, both in adult and juvenile plumage, is harder to explain, and suggests that other processes, notably lateral transfer and subsequent fixation of foreign haplotypes in either clade A or D, might have affected the gene tree. The surprisingly small cytochrome *b* divergence between the geographically and morphologically divergent clade D and *meridionalis* (0.8–1.9%) adds to the suspicion that horizontal transfer might have affected the mitochondrial tree.

Independent data, such as fast-evolving nuclear markers, are required to clarify whether or not the mitochondrial gene tree conforms with the organismal phylogeny.

4.3. Taxonomic implications

4.3.1. Inappropriateness of current classifications and taxonomic dilemma

The mitochondrial gene tree challenges the traditional classification of the taxa in clades A, B, C and D as belonging to a single polytypic species, *L. excubitor* (e.g. Cramp and Perrins, 1993; Glutz von Blotzheim and Bauer, 1993; Hartert, 1910; Rand, 1960; Vaurie, 1959; Voous, 1977). It also strongly contradicts the division into a northern and a southern polytypic species, *L. excubitor* and *L. meridionalis*, respectively, as none of these species, as presently circumscribed (Clements, 2000; del Hoyo et al., 2008; Dickinson, 2003; Harris, 2000; Lefranc and Worfolk, 1997), is monophyletic. All of the taxa in these clades have essentially disjunct or parapatric breeding distributions. The only evidence of overlapping breeding ranges is between *mollis* and *pallidirostris* (Panov, 1995), although Isenmann and Bouchet (1993) erroneously interpreted the parapatric breeding ranges of *excubitor* and *meridionalis* in S France as more or less equivalent to sympatry in their assessments of species limits. The proven or supposed non-interbreeding between two northern and two southern taxa has been important in the treatment of the northern and southern groups as separate species (Clements, 2000; del Hoyo et al., 2008; Dickinson, 2003; Harris, 2000; Lefranc and Worfolk, 1997; Panov, 1995). However, this was based on an underlying assumption that these groups are reciprocally monophyletic, which is here suggested not to be the case, in agreement with the mitochondrial trees presented by Mundy and Helbig (2004), Klassert et al. (2008) and Gonzalez et al. (2008). Moreover, the cytochrome *b* divergences between the members of each presumably non-interbreeding pair (3.5–5.5% uncorrected, 4.4–7.1 HKY + Γ + I corrected between *mollis* and *pallidirostris*; 3.9–5.5% uncorrected, 4.8–7.8% HKY + Γ + I corrected between *excubitor* and *meridionalis*) are among the largest of all pairwise comparisons (cf. Table 1). The alleged reproductive isolation between these taxa has little bearing on the taxonomy of the others in the *L. excubitor* complex. Moreover, there is a lack of information regarding reproductive interaction between several of the geographically adjacent pairs of taxa.

Since the mitochondrial gene tree deviates substantially from the (non-cladistic) interpretation of relationships based on morphological and ecological characteristics, and there are indications that the gene tree might not fully conform with the organismal phylogeny, any proposed taxonomy is uncertain. This does not only concern the taxa traditionally placed in the *L. excubitor* complex, but also the three universally accepted species *L. sphenocercus*, *L. ludovicianus*, and *L. somalicus*. Within and between clades 1 and 2, there are extremely few documented cases of sympatric breeding, making species delimitation under the "biological" species concept sensu Mayr (1942) subjective. Species delimitation under the "phylogenetic" species concept sensu Cracraft (1989) is equally tricky for several of the taxa, which grade into each other. This situation is rather similar to that in the Yellow Wagtail *Motacilla flava* complex, which is another recently evolved complex with intricate plumage variation and incongruence between mitochondrial gene trees and other data, and problems with species delimitation irrespective of species concept (Alström and Mild, 2003). The taxonomy of the taxa in clades 1 and 2 is discussed in more detail below.

The apparently large divergences between taxa currently placed in *L. sphenocercus* and *L. collaris*, respectively, merit further investigation (see below regarding the former).

4.3.2. Taxonomy of clade 1

Based on the mitochondrial tree, several taxonomic options are possible with respect to clade 1. One possibility would be to treat all of the taxa as one species, *L. excubitor*. This would correspond to a large, well-supported clade, which has a similarly large sister group, from which it has been demonstrated to be reproductively isolated in at least two cases (see below). However, this approach does not consider the isolation between clades A, B and C, which is implied by the gene tree.

Recognition of three species, representing clades A, B and C, would also be acceptable based on the gene tree. The first one

would then be *L. excubitor* s.s., the second one *L. elegans*, and the third one *L. uncinatus*, in agreement with the principle of priority (ICZN, 1999). These clades are well supported, and the taxa included are geographically largely separated. However, morphologically, clade A is heterogeneous, as the taxa in clade A1 and A2 are more similar to the ones in clades B and C than to those in clade A3 (del Hoyo et al., 2008; Harris, 2000; Lefranc and Worfolk, 1997). Moreover, intergrades between *aucheri* (clade A2) and *elegans* (clade B) have been reported from areas where they occur in close vicinity, such as SW Israel, E Egypt and NE Sudan (Fry and Keith, 2000; Shirihai, 1996; Vaurie, 1959), indicating the possibility of ongoing gene flow between them. Our sample sizes and geographical coverage does not allow examination of this, and further studies are required.

A third solution would be to recognise clade A1 as *L. lahtora*, clade A2 as *L. aucheri*, clade A3 as *L. excubitor* s.s., clade B as *L. elegans*, and clade C as *L. uncinatus*, in agreement with the principle of priority (ICZN, 1999). All of these clades except A3 are well supported by our data. Hernández et al. (2004) found evidence of reduced gene flow between *excubitor* and *pallidirostris*, which led them to propose that *excubitor* and *pallidirostris* be treated as specifically distinct. However, there is morphological evidence of possible gene flow between some of the populations in clade A (L.S., unpublished). In clade A3, no phylogenetic structure is present, and *excubitor*, *leucopterus* and *homeyeri* are intermixed. In contrast, there are pronounced plumage differences between the extremes in this clade (*excubitor–leucopterus*), although these are bridged by *homeyeri*.

In clade B, there is no support for reciprocal monophyly in any of the taxa. The three samples of *algeriensis* are scattered throughout the clade. One of these is nested among *elegans* and *koenigi*, another one is nested in the *leucopygos* clade, while the third individual is sister to the *elegans/koenigi* clade. The taxon *algeriensis* breeds in contact with *elegans*, and intermediates are well known (Vaurie, 1955, with further references), with at least two intermediate plumage types afforded subspecific names (*batesi* Grant and Mackworth-Praed, 1951 and *dodsoni* Whitaker, 1898). More research is needed on the variation in North Africa.

The cytochrome *b* divergences among clades A, B and C and, especially, among A1, A2 and A3, are relatively small—the latter are not much different from the variation within clade A1. Although genetic divergence as such can not be uncritically translated to a measure of "degree of speciation", the very low divergence in this case does not speak in favour of recognition of these clades as separate species.

The taxon *uncinatus* is restricted to the isolated island of Socotra, probably with little or no contact with any other populations in the *Lanius excubitor* complex. It is morphologically most similar to *aucheri* (Kirwan, 2007; Lefranc and Worfolk, 1997; synonymised with *aucheri* by former author), but differs from this by 2.3–2.7% (uncorrected) or 2.6–3.0% (HKY + Γ + I corrected), and is here placed as sister to the rest of clade 1 in the mitochondrial tree with strong support. The molecular evidence strongly suggests that this taxon represents an evolutionary lineage of long standing isolation.

4.3.3. Taxonomy of clade 2

As for clade 1, there are several taxonomic options for clade 2. One alternative is to treat the entire clade 2 as a single species. We are not in favour of this alternative, as this would create a species that is not only morphologically, but also ecologically and biogeographically highly heterogenous, and which would include several populations for which all evidence indicate that they are distinct evolutionary lineages. The species status of the three taxa *L. sphenocercus, L. ludovicianus* and *L. somalicus* have not been considered controversial by previous authors. However, the species rank of the two former is in fact poorly supported under the "biological" species concept (Mayr, 1942). The breeding range of *L. sphenocercus* is allopatric with that of the *L. excubitor* complex, except in the Ningxia Hui autonomous region in NC China, where both *sphenocercus* and *pallidirostris* are stated to occur (Cheng, 1987); however, this is presumably based exclusively on museum data and requires confirmation. We tentatively recommend continued treatment of *L. sphenocercus* as a separate species from the *L. excubitor* complex.

The Tibetan *L. sphenocercus giganteus* is 3.2% (uncorrected) or 3.8% (HKY + Γ + I corrected) divergent in cytochrome *b* from *L. s. sphenocercus*, and the sister relationship between them is weakly supported. Moreover, they differ markedly in external morphology and biometrics (Harris, 2000; del Hoyo et al., 2008; Lefranc and Worfolk, 1997). For these reasons, it seems reasonable to afford species rank also to *giganteus*. The distributions of *giganteus* and *sphenocercus* are non-overlapping (Cheng, 1987).

The North American *L. ludovicianus* and *L. excubitor* s.l. have non-overlapping breeding distributions, and their status under the "biological" species concept (Mayr, 1942) is therefore subjective. Indeed, our data suggest that there may be some geneflow between them, as two individuals identified as either *borealis* or *invictus* (here regarded as synonyms) on morphology (Andy Jones/BMNH, in litt.) have mitochondrial cytochrome *b* haplotypes matching *ludovicianus*. The most likely explanation for this would be hybridization between male *borealis* and female *ludovicianus*.

Our analysis includes representatives of two subspecies of *L. ludovicanus*, namely *excubitorius* and *mexicanus*, as well as a number of samples unidentified to subspecies (probably of these two taxa). Our data do not permit any conclusions as to whether the current classification of *L. ludovicianus* as comprising eight subspecies (Dickinson, 2003) is reasonable. However, there is no phylogenetic structure in the *L. ludovicianus* clade, in spite of the morphological differences and geographically widely separated ranges of the two taxa included. We provisionally prefer treatment of *L. ludovicianus* as specifically distinct, in agreement with previous classifications, based on the facts that it is phylogenetically and morphologically well separated from all other taxa in clade 2.

The range of *L. somalicus* is largely allopatric with the *L. excubitor* complex, but there is said to be an area of overlap with *leucopygos* in E Ethiopia, S Djibouti and NW Somalia (del Hoyo et al., 2008; Lefranc and Worfolk, 1997). *L. somalicus* is morphologically distinct, and there is no evidence of hybridization with *leucopygos*. The genetic divergence between them is 4.3–4.5% (uncorrected p), and 5.3–5.6% (HKY + Γ + I corrected). The genetic divergence between *L. somalicus* and the remainder of clade 2 is 3.9–5.1% (uncorrected), and 4.8–6.5% (HKY + Γ + I corrected). *L. somalicus* is considered to be monotypic (Dickinson, 2003), and we favour continued species rank for this taxon.

Clade D could be classified in different ways. Clades D1 and D2 could be treated as two separate species, *L. borealis* (with subspecies *borealis*, *bianchii, funereus, mollis* and *sibiricus*) and *L. meridionalis* (monotypic). These two clades are morphologically, geographically and ecologically well separated (Harris, 2000; del Hoyo et al., 2008; Lefranc and Worfolk, 1997). The genetic divergence between them is slight, with the lowest value (0.7%, HKY + Γ + I corrected), even lower than the greatest difference between samples of *pallidirostris* from Kazakhstan (0.8%, HKY + Γ + I corrected). However, the mitochondrial tree nevertheless indicates that they are separate reciprocally monophyletic lineages. The taxa in clade D1 appear to be more or less continuously distributed in the NE Palearctic and N Nearctic, except where interrupted by bodies of water. With the exception of *bianchii*, they are morphologically fairly similar (Harris, 2000; del Hoyo et al., 2008; Lefranc and Worfolk, 1997). The sister relationship between our single sample of *bianchii*, which is confined to Sakhalin and the Kurile Islands, and the other taxa in clade D1 is insufficiently supported.

In relation to *borealis*, the taxa *funereus, mollis* and *sibiricus* are reciprocally monophyletic with strong support in the mitochondrial tree. The cytochrome *b* divergence between the Palearctic and Nearctic clades is greater than the within-clade divergence (Table 1). These pieces of evidence suggest the possibility of lack of gene flow between the continents, but more detailed studies are needed to clarify this.

4.3.4. Taxonomic conclusions

We provisionally recommend continued treatment of *L. sphenocercus*, *L. ludovicianus* and *L. somalicus* as separate species from each other and from the *L. excubitor* complex, and suggest that *L. s. giganteus* probably merits species rank. We discuss alternative classifications of the *L. excubitor* complex, and note that all previous treatments are inconsistent with the mitochondrial gene tree. However, because of the disagreement between the mitochondrial and non-molecular data, we refrain from proposing any revised classification.

4.4. Tandem repeats

Our results differ in several respects from those presented by Hernández et al. (2004). For example the present study does not show the obvious difference in proportion of frequencies of two or three repeats between clades A1 and A3 (Fig. 1), which Hernández et al. (2004) took as evidence of limited geneflow between these populations. However, our sample size is small compared to that of Hernández et al. (2004) and vulnerable to stochastic effects. On the other hand, Hernández et al. (2004) stated that some of their data came from nestlings, without providing details as to whether all or only one of the nestlings per nest had been sampled. If samples from all nestlings in a nest were included in their study, their figures may be inflated as mitochondrial markers from nestlings are less likely to constitute independent samples. Instead, they would all be expected to show the same haplotype as their mother, thus representing a single haplotype per nest.

We did not obtain PCR products from either *L. somalicus* or *L. dorsalis*, as our samples came from museum specimens. Based on the fact that all other taxa in the ingroup that have been studied contain two or more repeats, we find it most parsimonious with a single origin of two repeats, and predict that *L. somalicus* will contain two or more repeats. Knowledge of the number of repeats in *L. dorsalis* would be crucial for establishing whether two tandem repeats arose in the ancestor of clades 1 and 2 or earlier.

4.5. Biogeography

Biogeographical interpretations hinge on the correct inference of the phylogeny, which in this case seems uncertain (see above). Clade 2 suggests a sister relationship between the Iberian *meridionalis* (D2) and five NE Asian/North American taxa (D1), and this clade includes two further taxa from E Asia (*sphenocercus*) and North America (*ludovicianus*), respectively, as well as the African *L. somalicus*. If the gene tree conforms with the organismal phylogeny, the most likely explanations for the geographically isolated position of *meridionalis* are that it is either the result of dispersal from North America or Asia, or that it was once part of a continuously distributed species, the range of which was later fragmented. If recent dispersal was the cause, we would expect *meridionalis* to be most closely related to either the Nearctic *borealis* or the E Palearctic *sibiricus/funereus/mollis/bianchii*. Instead, *meridionalis* is inferred to be sister to all of these, and this is the pattern to be expected as a result of fragmentation of a once continuous range. However, dispersal from an ancestral population, before the east Palearctic and Nearctic populations diverged, cannot be ruled out. A similar pattern of close relationship between an Iberian and an east Asian taxon is shown by the two *Cyanopica* taxa *cooki* and *cyanus*, which are generally considered conspecific, although recent studies have shown that they have been separated for considerable time (Fok et al., 2002; Kryukov et al., 2004). A similar example concerns the Corsican Nuthatch *Sitta whiteheadi*, which has been shown to be most related to the widely disjunt Chinese Nuthatch *S. villosa* (Pasquet, 1998).

5. Future challenges

The phylogenetic pattern is reasonably well supported in the present study, but the detailed topology is based on mitochondrial markers only. Future studies should strive to identify independent nuclear markers that are variable enough to provide a phylogenetic structure. An approach using the coalescent, perhaps combining evidence from sequences, microsatellites, AFLP or single nucleotide polymorphies (SNPs) is likely to shed further light on the evolution of this clade. Advances in sequencing technology is expected to make larger amounts of data available, which also holds a promise of new possibilities.

There are many specific details that deserve attention. In particular, the zones where the ranges of the taxa *excubitor–homeyeri*, *excubitor–sibiricus*, *homeyeri–pallidirostris*, *leucopterus–pallidirostris*, *aucheri–elegans*, *aucheri–buryi*, *sibiricus–borealis, pallidirostris–sphenocercus* and *giganteus–sphenocercus* meet or come in close proximity should be investigated. The previously studied contacts between *pallidirostris–mollis* and *leucopygos–somalicus*, as well as the close contact between *excubitor–meridionalis* deserve further attention, as well as the occurrence of *ludovicianus* mitochondrial haplotypes in *borealis*. A special case that would benefit from a detailed population genetic study is the apparently complex genetic population structure of *algeriensis* and its relation to *elegans*, *koenigi* and *leucopygos*. The presence or absence of interaction between the isolated taxa *uncinatus* and *bianchii* and taxa in their respective vicinity is worthy of further study.

Reciprocal illumination between phylogenetic and environmental or ecological evidence holds great promise of new insights. The *Lanius excubitor* complex includes populations at most conceivable stages of divergence, making the clade an excellent model for the study of the speciation process. Last but not least, it is also a perfect case study for the ever contentious question of how to delimit species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.11.010.

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