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Molecular Systematics of Owls (*Strigiformes*) based on DNA-sequences of the mitochondrial cytochrome b gene

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ABSTRACT

Sequence data of the mitochondrial cytochrome b gene provide a powerful tool (besides morphology, anatomy, behaviour and bioacoustics) to elucidate and reconstruct the evolutionary past and speciation of owls. We have already analyzed 35% of species and 68% of genera of owls. The phylogenetic relationships inferred from sequences of the cytochrome b gene are generally in a good agreement with the classical taxonomy of owls and the attribution of species to a given genus. However, paraphyly and polyphyly are evident in the genus *Otus* and in the *Bubo* complex and we have proposed to split *Otus* into three genera *Otus, Ptilopsis*, and *Megascops*, but to merge *Nyctea, Ketupa*, and *Scotopelia* in *Bubo*.

INTRODUCTION

In order to occupy the ecological niche of a nocturnal raptor, owls had to evolve several adaptations (Burton 1992, Hume 1991, Mikkola 1983, König *et al.*, 1999). Besides specialized hunting strategies, owls developed a sophisticated acoustical communication system. On the other hand their morphology is often invariant but the distinctive calls which are inherited and not learned are of considerable taxonomic value (König, 1991a,b, 1994a,b; Hekstra, 1982). If phylogenetic relationships are reconstructed on the basis of the morphological characteristics alone, wrong conclusions might be drawn since some of these characters may be convergent traits that are unrelated to the true phylogeny.

The amplification of marker genes by polymerase chain reaction (PCR) followed by DNA sequencing (overviews in Avise 1994; Hillis & Moritz, 1990; Mindell 1997) provide a powerful tool to elucidate phylogenetic and phylogeographic relationships. The molecular approach does not make the traditional analysis obsolete; just the opposite, it is rather complementary and the right questions can only be asked if we have a solid frame-work based on morphology, geography, behaviour, or acoustics. The analysis of mitochondrial genes is central today in most molecular studies on birds (Mindell 1997), since mtDNA evolves much faster than nuclear DNA. Among mitochondrial genes,

many studies use the cytochrome b gene, as this gene usually is a good marker at the species and genus level, but it loses resolution on divergence events which are more than 30-50 million years away. This is mainly due to multiple nucleotide substitutions at the same position, which can lead to homoplasy.

We have chosen the mitochondrial cytochrome b gene to study speciation and phylogeny of owls (Heidrich & Wink 1994, 1998, Heidrich *et al.*, 1995a,b, 1996, 1997, 1998; Wink & Heidrich 1999). In this communication we present results of our molecular investigation on phylogenetic relationships in the genera *Tyto, Phodilus, Otus, Bubo, Ketupa, Nyctea, Scotopelia, Strix, Pulsatrix, Glaucidium, Athene, Speotyto, Aegolius, Ninox, Asio,* and *Surnia*. So far we have covered 35% of species and 68% of genera of owls. The missing genera mostly belong to monotypic genera, so that a general picture on the phylogeny of owls becomes possible with the present data set. (We would be happy to receive blood, tissue or feather samples from species that are not included in our trees, since we hope to arrive at a complete tree of Strigiformes some day).

MATERIAL AND METHODS

Details on materials and methods used for DNA isolation, PCR, PCR primers, DNA sequencing, and tree reconstruction have been published (Heidrich 1998; Heidrich & Wink, 1994, 1998; Heidrich *et al.*, 1995, 1996, 1997, 1998; Wink 1995; Wink *et al.*, 1996). Sequences have been deposited with Gene Bank.

For most species we have determined cytochrome b sequences (1040 bp) from two and more individuals, so that the sequences used in this analysis are unequivocal and reliable (Heidrich, 1998). For the molecular analysis (Fig. 1) we have assembled a data set consisting of a single cytochrome b sequence per taxon in those cases, that significant haplotype differentiation was absent.

Distances (p-distance) are calculated as the proportion of nucleotide substitutions (in %) between pairs of taxa (Wink & Heidrich, 1999). Distances correlate with divergence time and a crude estimate equals 2% nucleotide substitution with 1 million years of separation (Shields & Wilson, 1987). This molecular clock provides a rough estimate for a temporal frame-work (Moore & DeFilippis, 1997), but needs to be interpreted with caution, since the clock was not calibrated for owls. Distances can be used to decide whether a taxon can be regarded as a distinct species; in owls a divergence of >1.5% is usually indicative for species level and in a case where morphological, acoustical, and geographic characters support a separation, we have advocated to treat such a taxon as a distinct species.

Phylogenetic trees were reconstructed using Maximum Parsimony, Maximum Likelihood (PAUP*; Swofford 1993) or Neighbour Joining methods (MEGA; Kumar *et al.*, 1993). Bootstrap values provide an estimate how good a furcation is supported by the sequence data. Although bootstrap values are discussed controversially by many authors, they can be helpful. Hillis & Bull (1993) concluded that nodes with calculated bootstrap values of 70% and higher actually occurred in 95% and more of the simulated phylogenies. This means that a bootstrap value of 70% can be regarded as evidence for a well-supported node (Moore & DeFilippis, 1997).

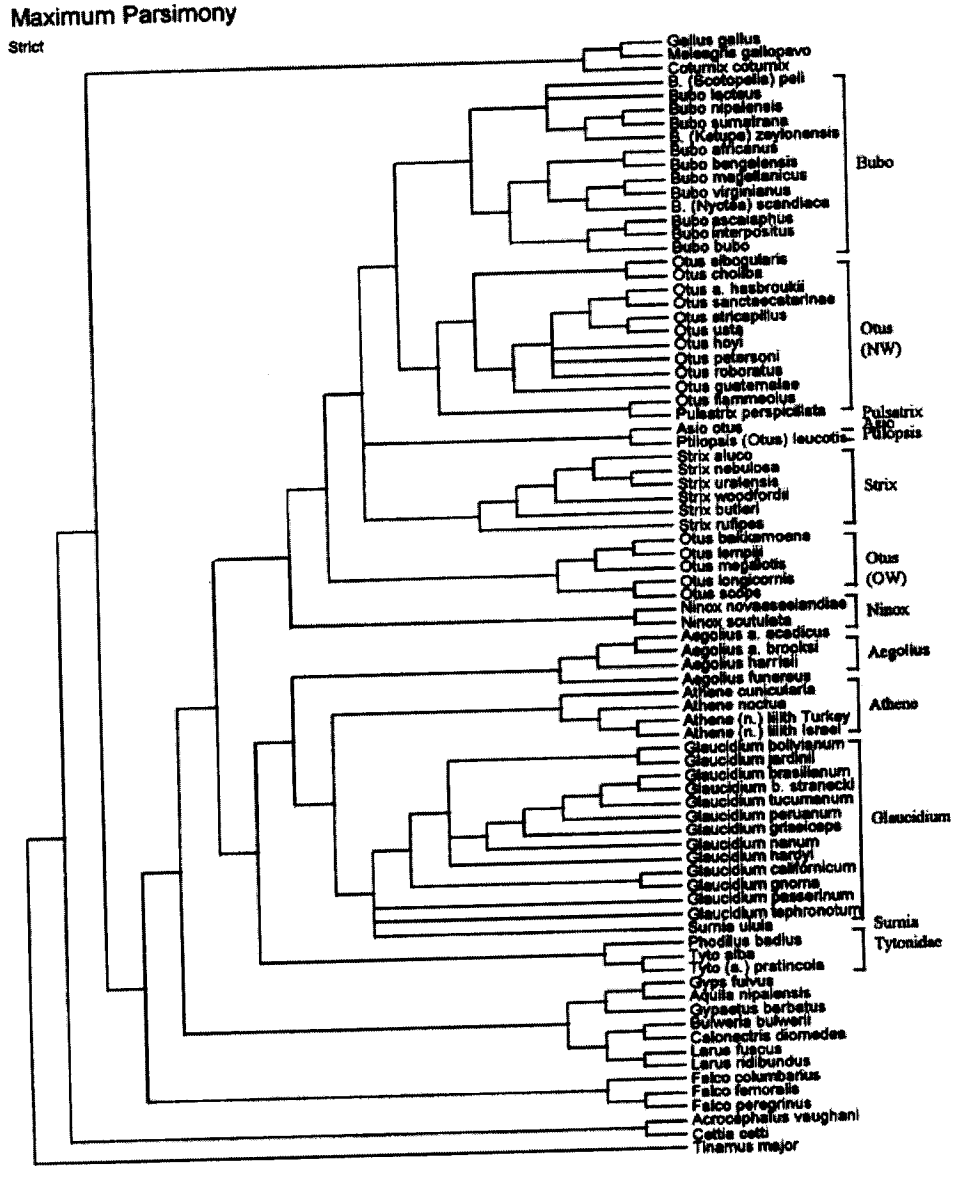
Phylogenetic relationships within the Strigiformes

The sequence dataset with one sequence per taxon was analyzed by Neighbour joining (NJ), Maximum Likelihood (ML), and Maximum parsimony (MP). A few non-related families of birds (such as Passeres, Falconidae, Accipitridae, Lariidae, Procellariidae, Phasianidae) were selected as ingroups and *Tinamus major* as a distant outgroup. The owl tree was stable and rather independent of different ingroups and outgroups. The resulting trees are congruent in most groupings (compare Fig. 1 A, B, and C). Differences were found in the placement of individual taxa within a few genera, e.g., in New World *Otus* and in the positioning of the genera *Strix, Asio*, and *Ptilopsis*.

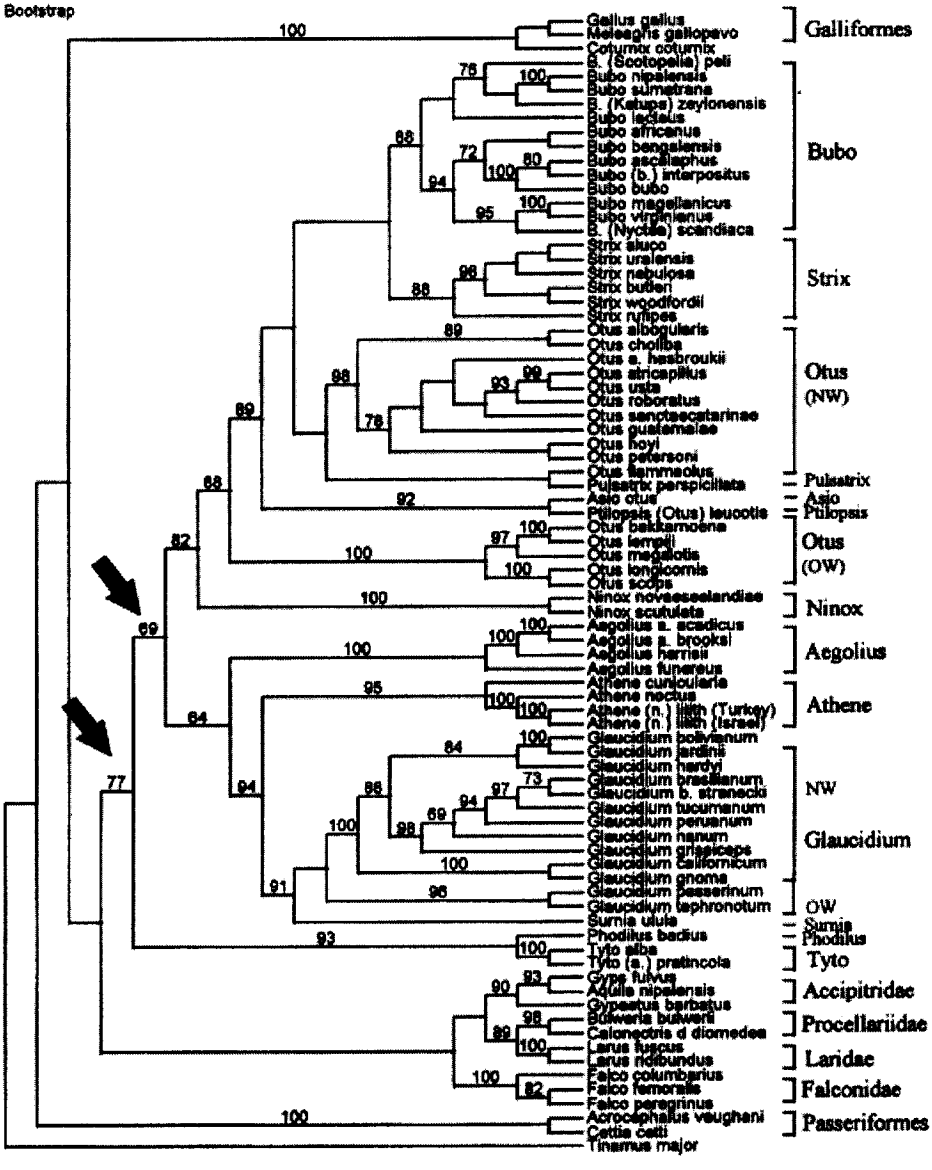
As can be seen from Fig. 1 the owl families Tytonidae and Strigidae form a monophyletic group, i.e. they share common ancestry. Passeriformes cluster at the base of the bird tree, a finding recently suggested when complete mitochondrial genomes were analyzed (Mindell & Sorenson, 1998). Falcons do not form a sister group to owls, as had been concluded from a cladistic analysis (Cracraft 1981).

Figure 1. Genetic relationships within the *Tytonidae* and *Strigidae* (based on 1040 nt of the cytochrome b gene)

A. Maximum parsimony strict consensus tree of 60 equally parsimonious trees. Conditions: Heuristic search (TBR branch swapping; tree length 5175 steps; consistency index CI= 0.217; retention index RI= 0.511).

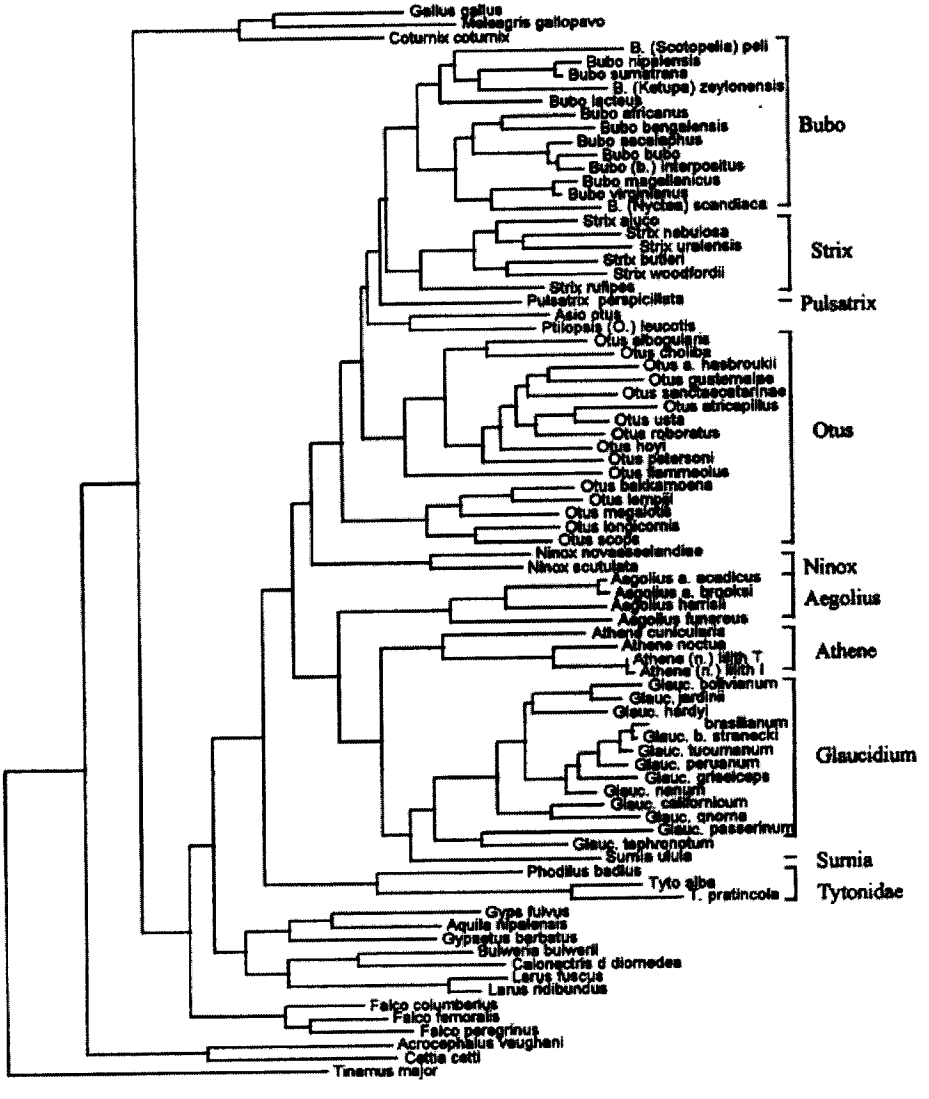


B. Neighbour Joining phylogram employing Jukes-Cantor as a distance algorithm (other algorithms, such as Kimura 2, Tamura-Nei) do not change tree topology). Bootstrap values from 500 replications (above 50%) above 70% are indicated. Arrows point to the branches leading to the Strigiformes and Strigidae.



C. Maximum Likelihood tree constructed by heuristic search with TBR branch swapping. Number of substitution types 2; all sites had same evolution rates; transition/transversion ratio 2; score of best tree was 26657; nucleotide frequencies: A= 0.269, C= 0.353, G= 0.136, T= 0.242. Branch lengths are proportional to genetic distances.

Maximum Likelihood



Sequence data were from our own laboratory, except *Meleagris, Coturnix, Gallus*, and *Tinamus* which were obtained from GeneBank.

OW= Old World; NW= New World

Relationships within the families *Tytonidae* and *Strigidae*

Tyto and *Phodilus*

Traditionally, two genera are distinguished within the Tytonidae: Tyto and Phodilus. This view is clearly supported by the sequence data (Fig. 1 B,C). the distances between both genera are large; i.e. they must have diverged from a common ancestor more than 9 million years ago.

Glaucidium

Pygmy owls of the genus *Glaucidium* which occur in the Old and New World show very similar plumage patterns, but can be distinguished by a unique repertoire of vocalizations (König, 1994b). Taxonomical classifications based on differing acoustic signals (König, 1994b) could be corroborated by DNA sequence data (Heidrich *et al.*, 1995). Fig. 1 clearly shows that Old and New World species cluster in separate monophyletic clades which share common ancestry but have diverged more than 7 to 8 million years ago. *G. gnoma/californicum* and *G. passerinum* are definitely not conspecific as sometimes assumed because of similar plumage patterns (Sibley & Monroe, 1990).

Within the South American *G. brasilianum* complex, several distinct haplotypes have been recognized (Fig. 1) that occur in different regions of Argentina, and Brazil. Because vocalisation and size also differ, *G. tucumanum* has been considered a distinct species (Heidrich *et al.*, 1995) and *G. b. stranecki* a new subspecies (König & Wink, 1995). *G. brasilianum* shares common ancestry with *G. peruanum, G. griseiceps* and *G. nanum* (Fig. 1); they form a monophyletic group. *G. bolivianum, G. jardinii*, and *G. hardyi* also cluster in a common, apparently monophyletic group in NJ and ML trees (Fig. 1 B, C). Both the *brasilianum* and the *bolivianum* complex are clearly separated from the small *Glaucidium* species, *G. californium* and *G. gnoma* which have distinctive calls and live in North America, especially the Rocky mountains, south to Central America.

Athene/Speotyto

Two species have been recognized in the genus *Athene*, i.e. *A. noctua* and *A. brama*. Within *A. noctua*, two genetic clusters are apparent which are supported by high bootstrap values; genetic differences between both groups account for 6.4% nucleotide substitutions suggesting species level. Birds from cluster I derive from Israel and Turkey [they could be treated as *Athene lilith* as suggested by Wink & Heidrich (1999)] whereas those from cluster II represent *A. noctua* from Europe.

Speotyto cunicularia represents the genus *Athene* in the New World, and this species has sometimes been considered as a member of the genus *Athene*. Since DNA-DNA hybridization suggested significant differences (Sibley & Monroe, 1990), a separation into a monotypic genus appeared justified. According to the sequence data, it is clear that *Speotyto* and *Athene* share common ancestry (divergence approximately 6 million years ago) and that they form a monophyletic group. Due to similarities in morphology, general outlook, vocal patterns, and behaviour, we suggest to merge *Speotyto* back in *Athene*. *Athene* always clusters as a sister group to the *Glaucidium/Surnia* complex (Fig. 1 A-C).

Surnia

The Northern Hawk Owl (*Surnia ulula*) of northern Eurasia and North America shares common ancestry and forms a monophyletic group with the *Glaucidium* complex of Old World origin but clusters at its base (Fig. 1 B,C). A separation as a monotypic genus could be debated, but might be justified because of morphological, behavioural and, last but not least, genetic differences.

Aegolius

Tengmalm's owls (genus *Aegolius*) can be found as a third major monophyletic group (Fig. 1) at the base of the branches leading to *Athene/Surnia/Glaucidium* (Fig. 1 A-C). The North American *A. acadius* diverges with 12.9% nucleotide substitutions from *A. funereus*, implicating a divergence time of more than 6 million years. Two geographically separated subspecies, *A.a. acadius* and *A.a. brooksii* can be recognized (distance 0.7%). As expected, the South American *A. harrisii* is closer related to the North American *A. acadius* than to *A. funereus* (Fig. 1), suggesting a common ancestor for the New World species.

Ninox

The genus *Ninox* comprises 19 species with Australasian distribution. *Ninox* clusters at the base to the branches leading to the genera *Otus/Strix/Bubo* in NJ,MP, and ML trees (Fig. 1 A-C). These groups apparently share common ancestry.

Strix

Tawny and wood owls (genus *Strix* with *ca* 21 species including the former genus *Ciccaba*) are always in a monophyletic clade (Fig. 1) and share ancestry with the *Bubo* complex (according to NL and MP reconstructions; Fig. 1 B,C). This sister group relationship is not supported by high bootstrap values (Fig. 1 B).

DNA data show that *S. butleri* is a distinct species (and not a subspecies of *S. aluco*) and rather related to the African *S. woodfordii* than to *S. aluco* (Heidrich & Wink, 1994). *S. uralensis* appears as a sister taxon of *S. aluco*, as suggested from behaviour and general appearance; genetic distances imply that both taxa diverged from a common ancestor more than 4 million years ago. The South American *S. rufipes* always clusters at the base of the *Strix* complex and could constitute a sister group of the Old World *Strix*, that was separated from a common ancestor 5-6 million years ago.

Pulsatrix

Four species are recognized in the Central and South American genus *Pulsatrix*, of which we could study *P. perspicillata* and *P. koeniswaldiana*. The phylogenetic position of *Pulsatrix* cannot be resolved with certainty: In NJ and MP trees (Fig. 1) it clusters with the *Otus flammeolus*, and in ML trees in the neighbourhood of *Strix* and *Asio* (Fig. 1C); but these relationships are not supported by high bootstrap values.

Otus, Mimizuka, Asio, and *Ptilopsis*

According to our genetic analysis (Fig. 1), members of the genus *Otus* appear in at least 3 different monophyletic clades, indicating that the presently recognized genus is polyphyletic. The Screech Owls of the New World represent a distinct group which is separated from Old World members of *Otus* by genetic distances between 12 and 16% (equivalent 6-8 million years). Within the Screech Owl complex, which has its radiation centre in South and Central America, several species have been recognized on account of different acoustic repertoires (König, 1994a). Sequence data could corroborate these findings (Heidrich *et al.*, 1995), stressing the importance of vocalization for speciation and taxonomy. *O. atricapillus* and *O. usta*, are distinct sibling species. *O. sanctaecatarinae*, *O. roboratus* and probably *O. guatemalae* and *O. asio* (from North America) also belong to this monophyletic cluster. *Otus hoyi* and *O. petersoni* appear as sibling species (in NL trees), as do *O. albogularis* and *O. choliba* (Fig. 1).

Of the large group of Old World Scops owls (overview in Sibley & Monroe, 1990) *O. scops, O. lempiji, O. megalotis, O. longicornis, O. brucei*, and *O. bakkamoena* have been included here as representatives for this group. As can be seen from Fig. 1 these Scops owls fall into a common clade which is very distinct from the New World Otus complex. Using 12S mt rDNA sequences, Mindell *et al.* (1997) showed that *O. mirus, O. mindorensis*, and *Mimizuku gurneyi* cluster together with *O. megalotis* and *O. longicornis*. As we also studied the latter two species we can conclude that *O. mirus, O. mindorensis*, and *Mimizuku gurneyi* are members of the Old World *Otus* group. Since *Mimizuku* clusters within this group we suggested merging *Mimizuku* in *Otus* (Wink & Heidrich, 1999).

The African *O. leucotis* (West Africa) and *O. granti* (South Africa) differ both morphologically and genetically from the other Old World *Otus* species and have been placed in the genus *Ptilopsis*. In all reconstructions (Fig. 1) *P. leucotis* figures as a sister group to the genus *Asio* in NJ, MP, and ML reconstructions (Fig. 1 A-C).

Genera should comprise monophyletic groups in phylogenetically orientated systematics. As polyphyletic groups are artificial, the present genus *Otus* needs a revision. Therefore, *Ptilopsis* and *Megascops* have been proposed as additional genera.

Bubo, Ketupa, Scotopelia, and *Nyctea*

Eagle Owls of the genus *Bubo* represent another prominent group of "eared owls". According to phylogenetic relationships and distances (Fig. 1), most taxa are distinct species, although some of them have been treated as subspecies of *B. bubo* (Sibley & Monroe, 1990). The southernmost taxon of South American Eagle Owls differs in size, vocalisations and DNA (Fig. 1) from *B. virginianus* and has been treated as a distinct species, *B. magellanicus* (König *et al.*, 1996).

B. (b.) ascalaphus, which occurs in North and West Africa, has been classified as a distinct species (Sibley & Monroe, 1990). In our analysis distances differ by 3.5% between *B. bubo* and *B. ascalaphus*. Also *B. b. interpositus*, which is morphologically distinct from *B. bubo* and lives in the Israelian desert, is also genetically distinct (distance 2.8%) (Fig. 1). As a sequence divergence of more than 1.5% is indicative of species level, it could be justified to treat both taxa as distinct species.

The Snowy Owl (*Nyctea scandiaca*) shares common ancestry with *Bubo* (Fig.1), especially with the New World species *B. virginianus* which would be in agreement with the arctic distribution of *N. scandiaca*. The separation from a common ancestor took place more than 4 million years ago. Since *Nyctea* represents a monotypic genus but unambiguously clusters within the *Bubo*-complex, the taxonomic consequences would be to merge *Nyctea* in *Bubo* and call the species *Bubo scandiaca*.

A similar situation can be found for *Ketupa*, of which 4 species have been described from Southeast Asia. *Ketupa zeylonensis* and *K. ketupa* cluster as close relatives of the Asian *Bubo* species, such as *B. nipalensis* and *B. sumatrana* (Fig. 1). Also the general appearance of *Ketupa* is similar to that of *Bubo*; because of genetic relationships (distance 9-10%) we agree with Amadon & Bull (1988) to merge *Ketupa* in *Bubo*. Three Fishing Owls (genus *Scotopelia*) are known which feed on fish as *Ketupa*. Both taxa are in a monophyletic clade with *Bubo lacteus*, *B. sumatrana* and *B. nipalensis*. We suggest to consider *Scotopelia* as a member of the monophyletic *Bubo* complex (Fig. 1 A-C).

CONCLUSION

Trees which are based on sequence data are not necessarily unequivocal and correct. If a dataset is incomplete (as in our study) and does not contain all related taxa for a comparison, errors are possible due to "long branch attraction". Nuclear copies of mitochondrial genes ("paralogous genes") can bias a phylogeny (Quinn 1997), although we found no evidence for this phenomenon in the owl data set. For mitochondrial genes it should be remembered that we can only trace maternal lineages ("gene trees") and that trees can be distorted by inbreeding and introgression. Some of these limitations have to be kept in mind when interpreting the phylogenetic trees presented in this communication which represents a form of progress report from an ongoing study.

As can be seen from Fig. 1 A-C the phylogenetic relationships inferred from sequences of the cytochrome b gene, are generally in a good agreement with the classical taxonomy of owls (Sibley & Monroe, 1990; Burton, 1992; Hume, 1991; König *et al*, 1999) and the attribution of species to a given genus. Paraphyly and polyphyly are evident in the *Otus* and in the *Bubo* complex and we have proposed to split *Otus* into three genera *Otus*, *Ptilopsis*, and *Megascops*, but to merge *Nyctea*, *Ketupa*, and according to this study also *Scotopelia* with *Bubo*.

Summarizing, sequence data of the mitochondrial cytochrome b gene provide a powerful tool (besides morphology, anatomy, behaviour and bioacoustics) to elucidate and reconstruct the evolutionary past and speciation of owls. As the analysis of a single gene only provides a window for a particular evolutionary period, we need to include more progressive or more conservative genes (i.e., both mtDNA and ncDNA) to solve other problems of microevolution or of higher level classifications.

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