Molecular Phylogeny of South American Screech Owls of the
*Otus atricapillus* Complex (Aves: Strigidae) Inferred from
Nucleotide Sequences of the Mitochondrial Cytochrome *b* Gene

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Z. Naturforsch. 50c, 294–302 (1995); received October 28/December 21, 1994

*Otus atricapillus* Complex, Bioacoustics, Speciation, Molecular Systematics,
Cytochrome *b* Gene

The cytochrome *b* gene of 6 South American screech owls of the genus *Otus* (*O. choliba*,
*O. atricapillus*, *O. usta*, *O. sanctaecatarinae*, *O. guatemalae*, and *O. hoyi*) and two Old World
species (*Otus scops* and *Otus leucotis*) was amplified by polymerase chain reaction (PCR)
and partially sequenced (300 nucleotides). *Otus atricapillus*, *O. guatemalae*, *O. hoyi*
and *O. sanctaecatarinae* which are morphologically very similar, have been treated as belonging
to a single species, *A. atricapillus* (Sibley and Monroe, 1990). Nucleotide sequences differ
substantially between these taxa (6.3 to 8.8% nucleotide substitutions) indicating that they
represent well established and distinct species which had been implicated already from eco­
logical and bioacoustical analyses (König, 1991, 1994). The importance of vocal and ecological
characters for the taxonomy of nocturnal birds is thus confirmed by our molecular analy­
sis. Phylogenetic relationships were reconstructed between Old and New World owls using
character state ("maximum parsimony": PAUP 3.1.1) and distance matrix methods (neigh­bour-joining; MEGA).

Introduction

The genus *Otus* (family Strigidae, order Strigiformes) consists, according to Sibley and Monroe
(1990) and Boyer and Hume (1991), of 53 and 41 species, respectively, occurring in the Old and
New World. Whereas half of the taxa are recognized as distinct species according to morphologi­
cal characters, the status of more than 20 others is still a matter of debate since morphology is often
invariant in this group in contrast to the distinctive calls (König, 1991, 1994; Hekstra, 1982). This is
especially true for South American screech-owls of the *Otus atricapillus* complex (Bond and Meyer
de Schauensee, 1994; Fitzpatrick and O’Neill, 1986; Haffer, 1987; Hardy et al., 1989; Hekstra,
Monroe (1990) classify *Otus atricapillus* and *O. watsonii* (these authors probably considered
*O. usta* which up to now has been treated as a southern subspecies of *watsonii*) as distinct species
and treat *O. atricapillus* (Temminck), *O. guatema­lae* (Sharpe), *O. hoyi* König et Straneck, and
*O. sanctaecatarinae* (Salvin) as belonging to a sin­gle species. However, König (1991, 1994) and
König and Straneck (1989) have provided evidence that these taxa occupy different ecological
niches and are distinguished by typical calls. Since in owls all vocalizations are inherited and not
learned, these authors have stressed the importance of vocal differences in nocturnal birds to
establish an efficient isolation mechanism as a pre­erequisite for speciation.

Using morphological and biological characters alone, the decision whether a taxon has the status
of a species or subspecies will remain difficult, even if the voice obviously seems to be the most
important factor in interspecific isolation mecha­nisms in owls. To evaluate the validity of the
ecological and bioacoustical approach to define species within the *O. atricapillus* complex, meth­ods
of molecular systematics might help to decide these issues (Hillis and Moritz, 1990; Hoelzel,
1992; Avise, 1994). Most resolution can be ob­tained by comparing the nucleotide sequences
of phylogenetically informative marker genes

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The mitochondrial cytochrome $b$ gene has often been selected as a marker gene in recent years, since its sequence has been found informative for many phylogenetic and taxonomic problems of animals, especially of bird taxa whose speciation took place within the last 20 million years (Kocher et al., 1989; Richman and Price, 1992; Helbig et al., 1993, 1994; Seibold et al., 1993, 1994a,b; Wink et al., 1993a,b, 1994; Heidrich and Wink, 1994; Avise, 1994).

In this study partial cytochrome $b$ nucleotide sequences of 6 South American and 2 Old World taxa of the genus *Otus* were determined and used to evaluate their degree of speciation in relation to acoustic differentiation and to reconstruct the phylogenetic relationship between them.

**Materials and Methods**

**Material**

The origin of owls of the *O. atricapillus* complex is documented in Table III. *O. choliba* was collected by C. König in B. de Irigoyen, Misones (Argentina) and at Imaya Cocha, Napo (Ecuador) by the Zoology Museum Copenhagen. *O. leucotis* came from Rwanda (East Africa) (collector F. Henning/B. Schottler) and *O. scops* from Crete (collector D. Ristow).

**DNA-methods**

Blood or tissues were stored in a modified EDTA-buffer (Arctander, 1988) at ambient temperatures in the field. DNA was extracted after digestion with proteinase K (Boehringer) according to Arctander (1988), Swatschek et al. (1993, 1994) and Heidrich et al. (1994).

Primer sequences used for PCR and direct sequencing were derived from Kocher et al. (1989) and are given in Seibold et al. (1994a), and Wink (1994). A 1026 bp portion of the cytochrome $b$ gene was amplified using 1 µg of total DNA as target, 25 pmol each of primers A and F, 1.5 mM MgCl$_2$ and 2 units Taq-polymerase (Promega or AGS). After initial denaturation (4 min at 94 °C), 30 cycles of 45 sec at 94 °C, 60 sec at 45 °C and 90 sec at 72 °C were performed on a Biometra thermocycler. After 30 cycles the reaction temperature was maintained at 72 °C for 4 min and then lowered to 4 °C for further storage. PCR products were run on a 1% agarose gel, excised and extracted using the Qiaex gel purification kit (Diagen). After elution, the amplified DNA was precipitated with isopropanol and sodium acetate. The pellet was redissoled in 7.5 µl H$_2$O. Direct sequencing of the double-stranded DNA was carried out by the chain termination method at 37 °C using α-^{35}S-dATP as a radioactive marker and Sequenase 2.0 (USB) or T7-polymerase (Pharmacia) according to the distributor’s specifications. Primer B was used as a sequencing primer. Products of the sequencing reactions were separated on a 6% polyacrylamide/7 M urea gel by electrophoresis at 65 W. After drying, the gel was exposed to an X-ray film for 3–4 days.

**Sequence analysis**

Sequences were aligned with the cytochrome $b$ gene of *Gallus gallus domesticus* (Desjardin and Morais, 1990). Phylogenetic trees were reconstructed using the maximum parsimony method (phylogeny program PAUP 3.1.1.; Swofford, 1993) and the neighbour-joining method (Saitou and Nei, 1987) (program package MEGA; Kumar et al., 1993). In the neighbour-joining analyses genetic distances were calculated based on the Kimura 2-parameter or the Tamura-Nei model (Kumar et al., 1993). With PAUP, heuristic algorithms were employed. Bootstrap analyses were performed to obtain confidence estimates for each furcation.

**Results and Discussion**

**Base composition and mode of substitutions**

Theoretically the abundance of each of the four DNA bases should be 0.25. Similar to the situation of mitochondrial genes in other animals (Irwin et al., 1991; Kocher et al., 1989; Edwards et al., 1991; Kornegay et al., 1993), guanine is significantly under and cytosine overrepresented (t-test, p < 0.001) probably reflecting a biased codon usage. This discrimination is even more expressed in the third codon position where G has an abundance of 4.1%, C 49.7%, T 13.7% and A of 32.6%.

Within the dataset (Table I) we found 108 variable sites, of which 82 were parsimony informative.
Table I. Partial nucleotide sequences of the cytochrome b gene of 8 Otus species. Only variable characters are illustrated; numbers above sites refer to position 15289 (= 1) to 14990 (= 300) (H strand), as determined for the cyt b gene of Gallus (Desjardin and Morais, 1990).

Table II. Pairwise genetic distances between sequences of the cytochrome b gene. Pairwise distances between taxa. Below diagonal: absolute distances; above diagonal: mean distances (adjusted for missing data).
About 83 nucleotide substitutions occur in the third position of a codon, which usually do not lead to amino acid substitutions. The resulting silent mutations are especially helpful for phylogenetic reconstructions since they are not adaptive as morphological characters. At the first and second position we observed 17 and 8 substitutions, respectively.

For most taxa (except *O. guatemalae* and *O. sanctaecatarinae*) at least 2–3 specimens were available. The degree of intraspecific variation which consisted of 1 to 4 base substitutions (= up to 1.4% difference) (Table II), was significantly smaller (p < 0.001, t-test) as compared to the differences encountered between species (substitution rate 6–16%) (Table II). Genetic distances between *O. atricapillus* and *O. usta* which are treated as distinct species by most authors (Sibley and Monroe, 1990; Hume and Boyer, 1991) are 3.1–3.2% and are also significantly higher than intraspecific variations (p < 0.001, t-test). Since *O. guatemalae*, *O. hoyi* and *O. sanctaecatarinae* differ by 6.3 to 8.8% from *O. atricapillus* and between each other and since they are not closely related phylogenetically (Figs 1,2) it is very likely that these taxa represent distinct species, as indicated from ecological and acoustical analyses (Table III). The coefficient between transitions/transversions (transitions are nucleotide substitutions from A to G or C to T and vice versa whereas transversion are changes from pyrimidine to purines and vice versa, i.e. G to C or T and A to C or T) is relatively low. In recently diverged taxa ns/nv ratios are high since transitions are 20 times more common as transversions. Because of multiple substitutions this difference becomes smaller with time and taxa which are separated by more than 10–30 million years have nv/ns ratios approaching 1 (Kornegay et al., 1993; Avise, 1994).

For comparisons between Old and New world *Otus* species a ns/nv coefficient of 1.5 ± 0.27 is obtained and 2.9 ± 0.74 for comparisons within

### Table III. Origin of the samples and comparison of morphological and other biological characters of the *O. atricapillus* complex.

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>O. atricapillus</em></th>
<th><em>O. watsonii</em></th>
<th><em>O. usta</em></th>
<th><em>O. hoyi</em></th>
<th><em>O. guatemalae</em></th>
<th><em>O. sanctaecatarinae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin of sample</td>
<td>Iguazu, Misiones (Argentina) No. 1–3</td>
<td>–</td>
<td>Rio Bení, La Paz (Bolivia) No. 1</td>
<td>Salta (Argentina) No. 1–3</td>
<td>Pasco (Peru) No. 1</td>
<td>Cerro tigre Misiones (Argentina) No. 1</td>
</tr>
<tr>
<td>Collector</td>
<td>C. König</td>
<td>LSUZM, Baton Rouge</td>
<td>C. König</td>
<td>LSUZM, Baton Rouge</td>
<td>C. König</td>
<td></td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iris colour</td>
<td>brown/amber</td>
<td>amber yellow</td>
<td>brown</td>
<td>yellow</td>
<td>yellow</td>
<td>yellow/hazel</td>
</tr>
<tr>
<td>Wing length</td>
<td>~175 mm</td>
<td>~175 mm</td>
<td>~175 mm</td>
<td>~175 mm</td>
<td>~165 mm</td>
<td>~190 mm</td>
</tr>
<tr>
<td>Length</td>
<td>~230 mm</td>
<td>~230 mm</td>
<td>~230 mm</td>
<td>~230 mm</td>
<td>200 mm</td>
<td>~245 mm</td>
</tr>
<tr>
<td>Crown</td>
<td>blackish</td>
<td>rather dark</td>
<td>very dark</td>
<td>not dark</td>
<td>not dark</td>
<td>rather dark</td>
</tr>
<tr>
<td>Voice</td>
<td>Male territorial Song (song A)</td>
<td>long trill up to 20 s</td>
<td>long sequence</td>
<td>long trill</td>
<td>rather long trill</td>
<td>medium long trill</td>
</tr>
<tr>
<td>Number of elements</td>
<td>14/s</td>
<td>7–8/s</td>
<td>2/s</td>
<td>11/s</td>
<td>14/s</td>
<td>14–15/s</td>
</tr>
<tr>
<td>Habitat</td>
<td>lowland rain-forest up to 600 m in tropical regions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Forests between 300 and 1000 m (mixed with <em>Araucaria</em>)</td>
</tr>
<tr>
<td>Distribution</td>
<td>SE-Brazil (north to Rio de Janeiro), NE-Argentina (northern Misiones), E-Paraguay</td>
<td>Surinam, E-Ecuador, Venezuela</td>
<td>Brazil, Peru, Bolivia</td>
<td>NW-Argentina, S-Bolivia</td>
<td>Mexico, south to N-Bolivia</td>
<td>SE-Brazil, Uruguay, NE-Argentina, Sierra de Misiones</td>
</tr>
</tbody>
</table>
the South American *Otus* taxa. As expected, the highest ratio (e.g., 5.1 ± 2.62) can be obtained between species of the *O. atricapillus* complex indicating that this group diverged later from a common ancestor than the other South American *Otus* species. Sequence differences suggest that the taxa of the *O. atricapillus* complex, assuming a constant molecular clock for mitochondrial genes (Quinn *et al*., 1991; Wilson *et al*., 1987), were separated from a common ancestor probably several (approximately 3 to 4) million years ago (Table II). It is highly unlikely that the species of the *O. atricapillus* complex were still interbreeding a few centuries ago as suggested by Burton (1992).

**Phylogenetic reconstructions**

The distribution of 1000 randomly produced trees is significantly skewed to the right ($g_1 = -0.895$) indicating that the data set contains a significant phylogenetic signal (Hillis and Huelsenbeck, 1992).

The character state method “maximum parsimony” implemented by PAUP 3.1.1. produced one
most parsimonious trees for the complete data set (Fig. 1). The shortest tree was 199 steps long (minimal length 145 steps, maximally 419 steps). Fig. 1 illustrates a phylogram of the Otus complex, in which figures correspond to the number of nucleotide substitutions between species. The distance matrix approach (MEGA 1.0) with the neighbour-joining method produced a phylogenetic tree (Fig. 2) which is highly congruent with maximum parsimony tree, only the positions of O. sanctaecatarinae and O. hoyi are exchanged. Variation of the outgroup changed the topology of the phylogenetic trees only slightly, as can be seen from Fig. 3 in which O. leucotis instead of Glaucidium was selected as an outgroup and the South American Otus taxa as an ingroup.

These analyses show that the Old world taxa (O. leucotis and O. scops) are not included in the clade of screech-owls of the New World, reflecting that both groups must have diverged a long time ago from a common ancestor (see distances in Table II). Therefore, it must be discussed whether both groups should be treated as belonging to a single genus.

The analyses show that O. atricapillus and O. usta (apparently we had only samples of O. usta and none of true O. watsonii) appear as closely related sister species. According to the common topology of the phylogenetic trees (Figs 1–3) O. sanctaecatarinae and O. hoyi seem to belong to the O. atricapillus complex, but the respective bootstrap values (52 or 53%) are not significant. In both MP and NJ analyses O. choliba clusters outside the O. atricapillus complex. The position of O. guatemalae cannot be resolved with certainty (Figs 1–3) and the NJ and MP reconstructions suggest that this taxon does not belong to the O. atricapillus complex which would agree with the concept outlined in Burton (1992), that O. guatemalae might perhaps form a superspecies including O. trichopsis, O. barbarus, O. marshalli, O. nudipes and O. clarkii.
Conclusions

This first analysis clearly indicates that the concept outlined in Sibley and Monroe (1990) to treat *O. atricapillus*, *O. sanctaecatarinae*, *O. hoyi* and *O. guatemalae* as a single species, i.e. as *O. atricapillus* cannot be supported by molecular evidence. On the contrary, the genetic data show that *O. atricapillus*, *O. watsonii* (apparently representing 2 taxa: *watsonii* and *usta*), *O. sanctaecatarinae*, *O. hoyi* and *O. guatemalae* represent independent and distinct species (Tables I–III; Figs 1–3).

It has been emphasized by several authors (König, 1991, 1994; König and Straneck, 1989) that ecological and bioacoustical characters are much more important for the classification of owls than morphological differences, since only the former characters can provide efficient isolation mechanisms in nocturnal animals to keep species separated. Because of the acoustical differences between species of the *O. atricapillus* complex (Table III), König (1991, 1994), König and Straneck (1989) had recommended to treat its members, i.e., *O. atricapillus*, *O. watsonii* (and *O. usta*), *O. sanctaecatarinae*, *O. hoyi* and *O. guatemalae* as distinct species. This conclusion can be supported by our molecular analysis confirming the importance of ecological and bioacoustical characters for taxonomy.

Acknowledgements

We thank Ute Kahl for technical assistance and D. Ristow, F. Henning, B. Schottler, J. Fjeldsä (Zoologisk Museum, Kobenhavn), and the Museum of Zoology, Louisiana State University (LSUZM), Baton Rouge for providing blood or tissue samples.


