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DNA Barcoding as a Tool for Elucidating Species Delineation in Wide-ranging Species as Illustrated by Owls (Tytonidae and Strigidae)

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The mitochondrial cytochrome *c*-oxidase subunit I (*cox1*) can serve as a fast and accurate marker for the identification of animal species, and for the discovery of new species across the tree of life. Distinguishing species using this universal molecular marker, a technique known as DNA barcoding, relies on the identifying the gap between intra- and interspecific divergence. One of the difficulties could be wide-ranging, cosmopolitan species that show large amounts of morphological variation. The barn owl *Tyto alba* is a case in point. It occurs worldwide and varies morphologically, leading to the recognition of many subspecies or, more recently, species. We analysed data from the *cox1* gene for 31 individuals of seven subspecies, and compared this with 214 sequences from 29 other owl species. Phylogenetic analysis of the *T. alba* samples gives very strong support for an Old World *alba*–clade (three subspecies) and a New World *furcata*–clade (four subspecies) that are genetically equidistant. The amount of intraspecific variation within each of these clades ranges from 0.66–0.99%, but variation among these clades ranges from 5.33–6.20%. Combined these data suggest that barn owl of the Old World is indeed best considered a separate species different from that of the New World. For combined dataset, sample size of owl species (n between 1 and 21 sequences) increased with geographic range size but we did not find significant relationships between interspecific divergence and sample size or between interspecific divergence and geographic range. For 21/24 species of owls with sample sizes of $n \geq 4$ the maximum interspecific divergences was ≤ 3.00%. However, similar to those found in barn owls, the largest amount of divergence (3.23–4.09%) was present in two other wide-ranging species (*Strix nebulosa* and *Aegolius funereus*) raising the possibility of multiple species in other wide-ranging owls as well.

Key words: Aves, DNA barcoding, phylogenetics, Tytonidae, taxonomy

INTRODUCTION

Recent studies suggest that sequences of the mitochondrial cytochrome *c*-oxidase subunit I (*cox1 or COI*) could serve as a fast and accurate marker for the identification of animal species, and for the discovery of new species across the tree of life (Hebert et al., 2003, 2010; Hajibabaei et al., 2007; Nijman and Aliabadian, 2010; Foerschler et al., 2010; Lijtmaer et al., 2012), a procedure for which the term DNA barcoding has been coined (Hebert et al., 2004). Variation of *cox1* sequences within species was an average of 20 times smaller than between species, and there was a clear gap between intra- and interspecific variation (Hebert et al., 2004). Utilizing this barcoding gap, Hebert et al. (2004) proposed a standard sequence threshold to define species boundaries of around 10 times the mean intraspecific variation for the group under study (in their case birds). While the barcoding gap appears to hold for overall comparisons among birds (Kerr et al., 2009) even if larger numbers of individuals are included (Kerr et al., 2007; Aliabadian et al., 2009; Pacheco et al., 2011), a more critical issue is that of distinguishing related combinations of species (Aliabadian et al., 2009), or wide-ranging, cosmopolitan species that show large amounts of morphological variation (e.g., Nijman and Aliabadian 2010; Bergsten et al., 2012).

Here we test the applicability of distance-based DNA barcoding within a wide-ranging species, the barn owl *Tyto alba*, comparing it with a large multi-species owl dataset. The owls comprise two families, the Tytonidae and the Strigidae. The Tytonidae can be separated into two genera, the barn and grass owls *Tyto* with 17 species and the bay owl *Phodilus* with two species (König et al., 2008). The barn owls have the largest distribution range of all the owls and occur worldwide, and even have been recorded in the sub-Antarctic (McCafferty and Lurcock, 2002). Geographically there is considerable amount of morphological variation, leading to the recognition of a large number of subspecies, and recently König et al. (2008), partially influenced by the work of Wink et al. (2008), elevated eight of these subspecies to species rank.

Wink et al. (2008, 2009) sequenced the mitochondrial

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protein coding cytochrome b gene [*cob*] and the nuclear RAG-1 gene from a large range of owl species. They found a large sequence divergence (up to ~5% in *cob*) between a New World clade (*furcata*, *n* = 4 sequences for RAG-1, *n* = 7 sequences for *cob*) and an Old World clade (*alba*, *n* = 4 and *n* = 6, respectively), however, bootstrap support for the splits were generally low (i.e., < 70%). An equally large amount of divergence (3.73%) but higher bootstrap values (99%) were found by Johnsen et al. (2010) who compared the cytochrome oxidase subunit 1 (*cox1*) between Scandinavian (*gutata*, *n* = 2) and North American barn owls (*pratincola*, *n* = 3). In light of the large divergence, Johnsen et al. (2010) concluded that *T. alba* deserved more detailed study. This ideally should involve larger sample sizes from a wider range of geographic localities.

Here, to resolve this issue of single versus multiple barn owl species, we compare mitochondrial DNA sequences from barn owls from the New World with barn owls from the Old World to estimate the level of interspecific genetic divergence between the two groups, and compare our results with an analysis of interspecific genetic divergence in a large range of other owl species. Through this large intergeneric comparison we evaluate the usefulness of distance-based DNA barcoding, in particular for wide-ranging species.

MATERIALS AND METHODS

We chose to sequence *cox1* as its amount of intra- and interspecific variation in birds is well-documented (Hebert et al., 2004, Kerr et al., 2007, 2009, Aliabadian et al., 2009; Pacheco et al., 2011) allowing us to evaluate the status of Old World and New World barn owls in comparison to other owls. Aliabadian et al. (2009), using a data set of 2776 *cox1* sequences from 566 bird species, established that intraspecific variation (average 0.24%) were some 24 times smaller than mean interspecific variation (average 5.97%). We agree with Meier et al. (2008) that comparisons using maximum interspecific distances are to be preferred above mean interspecific distances; for birds, as a rule of thumb, it appears that maximum interspecific distances are below 3.0% (Kerr et al., 2007; Aliabadian et al., 2009).

We follow the taxonomy of König et al. (2008); based on data presented by Prins et al. (2003, 2009) and C.S. Roselaar (pers. communication) barn owls from the Caribbean island of Bonaire are tentatively classified as *T. f. hellmayri* (cf. Flikweerd et al., 2007). Geographic range sizes (log transformed) of barn owls and other owl species included in the analysis (see below) were obtained from maps presented in König et al. (2008).

We sequenced 31 individuals from six taxa, three from Northern America and the Caribbean (*T. f. pratincola*, *T. f. hellmayri*, *T. bargei*) and three from Europe and western Asia (*T. a. alba*, *T. a. ernesti*, *T. a. erlangeri*). DNA was extracted from tissue or blood samples using DNeasy Tissue Kits (Qiagen, Valencia, California, U.S.A.) following the manufacturer's protocol. Polymerase chain reactions (PCR) and sequencing reactions follow protocols described by Aliabadian et al. (2007) which can be summarized as follows. A fragment of *cox1* was sequenced using two primer combinations that amplify a region of 612 bp starting from the 5′ terminus of the mitochondrial *cox1* gene: BirdF1 (5′-TTC TCC AAC CAC AAA GAC ATT GGC AC-3′), BirdR1 (5′-ACG TGG GAG ATA ATT CCA EET CCT G-3′), and Bird R2 (5′-ACT ACA TGT GAG ATG ATT CCG AAT CCA G-3′) (Hebert et al., 2003). PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen, Valencia, California, U.S.A.). Sequencing reactions were resolved on ABI 3100 or ABI 3730 automated DNA sequencers. The newly determined sequences have been deposited in Genbank, and their accession numbers are listed in Table 1.

Table 1. Geographic localities, Genbank accession and collection numbers of taxa included in the present study, numbers correspond to Figure 1. Taxonomy follows König et al. (2008)

Species/subspecies	Collection no.	Locality	Genbank Accession no.
Tyto bargei 1	ZMA 58966	Curaçao, Netherlands Antilles	KF432207
Tyto bargei 2	ZMA 55942	Curaçao, Netherlands Antilles	FJ465380
Tyto bargei 3	ZMA 55939	Curaçao, Netherlands Antilles	FJ465378
Tyto bargei 4	ZMA 55941	Curaçao, Netherlands Antilles	FJ465379
Tyto bargei 5	ZMA 55943	Curaçao, Netherlands Antilles	FJ465381
Tyto furcata hellmayri 1	ZMA 55945	Bonaire, Netherlands Antilles	FJ465375
Tyto furcata hellmayri 2	ZMA 58257	Bonaire, Netherlands Antilles	FJ465376
Tyto furcata hellmayri 3	ZMA 58259	Bonaire, Netherlands Antilles	FJ465377
Tyto furcata tuidara 1		Argentina	FJ028529
T. furcata pratincola 1		Ohio, USA	TAU91604
T. furcata pratincola 2	LSUMZ B49510	Florida, USA	KF432208
T. furcata pratincola 3		Florida, USA	DQ433249
T. furcata pratincola 4		British Columbia, Canada	DQ434212
T. furcata pratincola 5		British Columbia, Canada	DQ434213
T. furcata pratincola 6		Florida, USA	BOTW075
T. furcata pratincola 7		British Columbia, Canada	KKBNA048
T. furcata pratincola 8	LSUMZ B49509	Florida, USA	KF432209
T. furcata pratincola 9	LSUMZ B20485	Louisiana, USA	KF432210
T. furcata pratincola 10	LSUMZ B21784	Texas, USA	KF432211
T. furcata pratincola 11		British Columbia, Canada	KKBNA046
T. furcata pratincola 12	LSUMZ B16306	Louisiana, USA	KF432212
T. furcata pratincola 13	LSUMZ B20610	Louisiana, USA	KF432213
T. furcata pratincola 14	LSUMZ B49512	Florida, USA	KF432214
T. furcata pratincola 15	LSUMZ B49511	Florida, USA	KF432215
T. furcata pratincola 16	LSUMZ B29566	California, USA	KF432216
T. furcata pratincola 17	LSUMZ B44989	Louisiana, USA	KF432217
T. alba alba 1	ZMA 58237	The Netherlands	FJ465383
T. alba alba 2	ZMA 58235	The Netherlands	FJ465382
T. alba alba 3	ZMA 58844	The Netherlands	KF432222
T. alba alba 4	ZMA 58964	The Netherlands	KF432218
T. alba alba 5	ZMA 58963	The Netherlands	KF432219
T. alba alba 6	ZMA 58962	The Netherlands	KF432220
T. alba alba 7	ZMA 58965	The Netherlands	KF432221
T. alba ernesti 1	NHMC 8041089	Greece	KF432223
T. alba ernesti 2	NHMC 8041086	Greece	KF432224
T. alba ernesti 3	NHMC 8041087	Greece	KF432225
T. alba ernesti 4	NHMC 8041088	Greece	KF432226
T. alba erlangeri 1	MFUM 2007317	Iran	KF432227
T. alba erlangeri 2	MFUM 2007316	Iran	KF432228
Bubo virginianus		Ontario, Canada	HCBR161
Athene cunicularia		California, USA	BOTW069

Our data set was complemented by *cox1* sequences of owls from GenBank, as available on 1 August 2011 and the Barcode of Life Data Systems website (http://www.barcodinglife.org/, as accessed on 1 August 2011), adding one subspecies (*tuidara*) from South America, in addition to 197 sequences from 29 species of owls. Sequences were included provided they had a length of > 609 homologous to our sequences, with no more than 50 ambiguous or missing nucleotides. All sequences were aligned using Muscle which allows multiple sequence comparison by log-expectation (Edgar, 2004).

Sequence analysis

We carried out maximum likelihood (ML) and maximum parsimony (MP), analysis using PAUP* 4.0b10 (Swofford, 2002). ML models and parameters were determined by a hierarchical likelihood ratio test as implemented in Modeltest (Posada and Crandall, 1998).The estimated models were used in a subsequent ML heuristic tree search with 10 random addition sequence replicates, and TBR branch swapping. MP analysis was performed using heuristic searches with TBR branch swapping, stepwise addition starting tree, and random addition sequence with 10 replicates. To test the robustness of nodes, we ran 500 and 2000 bootstrap replicates under ML and MP, respectively, with a single random addition sequence replicate per bootstrap replicate.

We used two members of the Strigidae family (subfamilies Striginae and Surniinae) as outgroups. Genetic distances were calculated to quantify sequence divergences among individuals using K2P models, theta, as implemented in MEGA 5 (Tamura et al., 2011).

RESULTS

Maximum likelihood tree (_ln = 1702.92) obtained from the mitochondrial *cox1* gene under TVM + G model of sequence evolution (Fig. 1) revealed that of the 609 characters, 451 were constant and 79 were parsimony-informative. MP searches recovered 2616 equally most likelihood trees (198 steps) with a consistency index of 0.91 and a retention index of 0.96. A strict consensus of these trees shows a very strong support for two major clades, one from the Americas (henceforth referred to as the *furcata*-clade, which includes *T. bargei*) and one from Europe and western Asia (the *alba*-clade). Genetic variation (K2P distances) within these two clades ranged from 0–0.52% for the *furcata*-clade and 0–0.66% for *alba*-clade, whereas variation between clades ranged from 5.33–6.34%. With respect to *cox1*, *T. bargei* from Curaçao is identical or very similar to *T. f. hellmayeri*, *T. f. tuidara* and *T. f. praticola*, and we find no molecular support for recognition this taxon as a separate species. Furthermore, recognising *T. bargei* as a separate species renders *T. furcata* paraphyletic. We found no evidence of a correlation between genetic and geographic structure. For instance, barn owl sequences from North America, the Caribbean, northern South America and southern South America did not group themselves in latitudinal clusters. Likewise sequences from northwestern Europe, the Mediterranean, and the Middle East clustered together.

For 24 species, the average interspecific distance was less than 1% whereas for all species it was below 2% (Table 2). Maximum interspecific divergences were generally less than 3%. The interspecific variation within the *furcata*- and *alba* clades are typical for that of other owl species. Three species show relatively large average and maximum

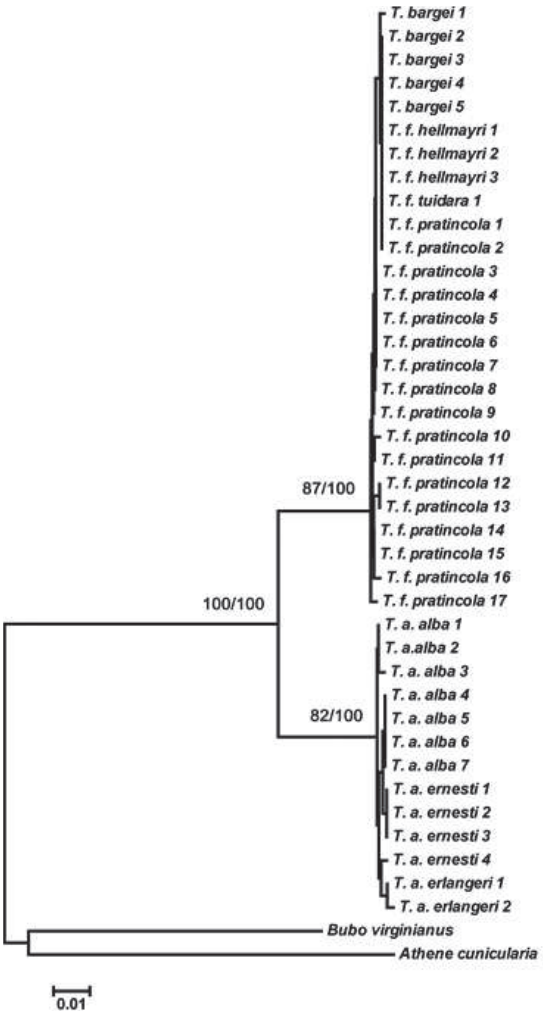

Fig. 1. Maximum likelihood tree (_ln = 1702.92) obtained from the mitochondrial cox1 gene under TVM + G model of sequence evolution; bootstrap values of the maximum likelihood and parsimony analyses are shown on the nodes, respectively.

divergences in combination with an uneven spread (signified by large standard deviations), i.e., western screech owl *Megascops kennicottii*, great gray owl *Strix nebulosa* and boreal owl *Aegolius funereus*.

For species with a sufficient sample size ($n \geq 4$ sequences, 22 owl species) there was no significant relationship between sample size and average or maximum divergence (Pearson's Product Moment Correlation Coefficient r = 0.19, P = 0.38, R^2 = 0.04, and r = 0.20, P = 0.37, R^2 = 0.12, respectively) suggesting a robust dataset. Species with larger geographic ranges were not represented by a larger number of sequences in our dataset, as there was no significant relationship between geographic range size and sample size (r = 0.39, P = 0.07, R^2 = 0.14). We found no significant relationship between geographic range size and maximum genetic divergence (r = 0.04, P = 0.85, R^2 = 0.03), that is, species with larger geographic ranges do not appear to have a larger amount of genetic diversity.

Table 2. Interspecific variation in 32 putative species of owls (average and maximum amounts of divergence between individuals; K2P distances in %) showing general low levels of interspecific variation.

Species	N (individuals)	N (comparisons)	average divergence	standard deviation	maximum divergence
Aegolius acadicus	14	91	0.1125	0.1311	0.5
Aegolius funereus	7	21	1.9371	1.927	4.09
Asio flammeus	15	105	0.4701	1.0886	3.39
Asio otus	18	153	0.5879	0.5086	1.35
Athene cunicularia	8	28	0.0247	0.0219	0.0501
Athene noctua	2	1	0	–	–
Bubo bubo	10	45	0.478	0.3515	1
Bubo scandiacus	10	45	0.0031	0.0022	0.0083
Bubo virginianus	9	36	0.0004	0.0007	0.0019
Glaucidium brasilianum	7	21	1.3971	0.8708	2.54
Glaucidium nanum	2	1	0	–	–
Glaucidium passerinum	2	1	0	–	–
Megascops asio	12	66	0.1048	0.135	0.5
Megascops choliba	6	15	0.11	0.161	0.33
Megascops kennicottii	10	45	1.5389	1.3447	3.04
Micrathene whitneyi	2	1	0.16	–	–
Ninox scutulata	7	21	1.3395	1.0692	2.52
Bubo scandiaca	2	1	0.34	–	–
Psiloscopus flammeolus	3	3	0	0	0
Otus lempiji	8	28	0.1518	0.1558	0.5
Otus lettia	2	1	0	–	–
Otus scops	4	6	0.08	0.0876	0.16
Otus sunia	4	6	0.08	0.0876	0.16
Strix aluco	6	15	0	0	0
Strix nebulosa	8	28	1.8357	1.5854	3.23
Strix occidentalis	7	21	0	0	0
Strix uralensis	5	10	0.132	0.1704	0.33
Strix varia	4	6	0.0044	0.0037	0.0089
Surnia ulula	3	3	0	0	0
Tyto alba	13	78	0.2994	0.1891	0.66
Tyto bargei	5	10	0.065	0.084	0.17
Tyto furcata	21	210	0.2081	0.1644	0.5

DISCUSSION

Our study added 31 new *cox1* sequences from the Caribbean, the southern United States, north-western Europe, the Mediterranean and the Middle East, to the existing North American ones. We found clear differences of at least 5.33% between the barn owls of the New World and the Old World for the mitochondrial *cox1* gene. Under both maximum likelihood and maximum parsimony, there were unequivocal levels of support for a New World – Old World split. The amount of variation between these two clades, i.e., > 5%, is well above the level proposed by Hebert et al. (2003) for recognising species, and is at least 2% larger than any other owl species for which *cox1* sequences are available.

Clear levels of support for the split between Old and New World barn owls were not found by Wink et al., (2008: 47), neither when analysing *cob* and RAG-1 sequences combined nor *cob* sequences only, but levels of divergence between the two clades were similar to that found in our study. Our results are in concordance with Johnsen et al. (2010), but are based on not only a larger number of sequences (31 vs. 5), but also from a much wider geographic range (large parts of the Old and New World vs Canada and Scandinavia). While Wink et al. (2008: cf. König et al., 2008) considered *T. bargei* as separate species, similar to our analysis they found the taxon neither to be monophyletic nor, genetically, markedly diverged. One of the challenges of analysing sequence divergence in wide-ranging species is obtaining good geographical coverage. While our analysis of barn owl sequences included samples from four continents, and geographic coverage is probably adequate for the purpose of the present study, especially samples from Africa (*T. a. affinis*), the Indian subcontinent (*T. a stertens*) and Southeast Asia (*T. a. javanica*) would add disproportionately to our understanding of genetic variation in barn owls.

Distance-based DNA barcoding used on a wide-ranging cosmopolitan species such as the barn owl elucidates the applicability of *cox1* as a marker for highlighting genetically distinct groups. Three species *M. kennicottii*, *S. nebulosa* and *A. funereus*, all with intermediate sample sizes, have the largest amount of average interspecific variation, the largest amount of variation between individuals and all have maximum divergences above 3%. *Megascops kennicottii* occurs in western North America and we suspect that the samples in GenBank or the BOLD database may include samples of its congener *M. asio* living to the east (or hybrids between the two species). Both *S. nebulosa* and *A. funereus* have wide Holarctic distributions (König et al., 2008) and similar to the barn owls may in fact comprise more than one species, with one confined to the Nearctic and one to the Palearctic.

As noted by Pacheco et al. (2011) while several studies have focused on the reliability of *cox1* for identifying species in different bird orders (Hebert et al., 2004; Baker et al., 2009; Kerr et al., 2009); limited efforts have been made to take into account the variance on its rate of evolution and rate heterogeneity. Pacheco et al. (2011), comparing substitution rates of mitochondrial genes of Neoaves taxa, found that *cox1* is a slow evolving gene, with a low variance among taxa and showing the least amount of rate heterogeneity of the 15 mitochondrial genes included in their study. They concluded that this low average, low variance and low heterogeneity of *cox1* supports its use in DNA barcoding. We agree with Kerr et al. (2009) that the original "10 × rule" as an appropriate barcoding gap proposed by Hebert et al. (2004) appears to be too conservative to recognise recently

diverged species. Using the dataset from Aliabadian et al. (2009), the amount of intraspecific variation to flag up potential species in birds is around 2–3% (i.e., 10 × the average interspecific variation of 0.24%), but Kerr et al. (2009) opted for a more liberal threshold of 1.6%. Owl species with large amounts of interspecific variation identified in the present study exhibit levels far beyond these thresholds and appear to be good candidates for further taxonomic research.

With a large dataset available against which to test levels of divergence (Kerr et al., 2007; Aliabadian et al., 2009) our study suggests that the amount of divergence in barn owls is considerably greater than what is the norm within other owls, and indeed other bird species, and exceeds proposed intraspecific levels of divergence. While we recognize the need for a more comprehensive analysis, including multiple (nuclear and mitochondrial) genes and including a larger range of taxa, it is clear that barn owls do indeed comprise multiple species. In addition, our study suggests that the amounts of genetic variation in other wide-ranging species may in fact be beyond what is the norm for the group under study. This may indicate that these indeed do comprise multiple species as well, or, in fact, may be a true reflection of the amount of interspecific variation present in the taxon under study. In the absence of other detailed analysis it is currently not possible to distinguish between these hypotheses, but it clearly indicates the usefulness of DNA barcoding as a fast and accurate marker for the identification of animal species, and for the discovery of new species across the tree of life.

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