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A mtDNA phylogeny of bustards (family Otididae) based on nucleotide sequences of the cytochrome *b*-gene

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Summary

Phylogenetic relationships of the bustard genera *Otis, Ardeotis, Afrotis, Chlamydotis, Eupodotis, Lophotis,* and *Tetrax* were inferred from nucleotide sequences of the mitochondrial cytochrome *b* gene (1143 bp). *Otis/Chlamydotis, Ardeotis/Eupodotis rueppellii,* and *Lophotis/Tetrax* cluster as sibling taxa both in MP and ML reconstructions. The genus *Eupodotis* appears to be polyphyletic. In the genus *Chlamydotis* two distinct groups are apparent, *C. u. undulata/C. u. fuertaventurae* and *C. u. macqueenii*. In the case of *C.u. macqueenii* birds from Sinai show a distinct haplotype. Because of substantial genetic, morphological and behavioural differences, it is suggested attributing species rank to *C. undulata* und *C. macqueenii*.

Keywords: Otididae, mtDNA, cytochrome *b*, phylogenetic analysis.

Zusammenfassung

Rekonstruktion der Trappenphylogenie (Familie Otididae) anhand von Nucleotidsequenzen des mitochondrialen Cytochrom-*b* Gens

Phylogenetische Beziehungen zwischen den Trappengenera *Otis, Ardeotis, Afrotis, Chlamydotis, Eupodotis, Lophotis*, und *Tetrax* wurden anhand von Nucleotidsequenzen des mitochondrialen Cytochrom b Gens (1143 Basenpaare) ermittelt. In Maximum Parsimony und Maximum Likelihood Rekonstruktionen clustern *Otis/Chlamydotis, Ardeotis/Eupodotis rueppellii*, und *Lophotis/Tetrax* als Schwestertaxa. Die Gattung *Eupodotis* bildet offenbar eine polyphyletische Gruppe. Innerhalb der Gattung *Chlamydotis* lassen sich zwei distinkte Entwicklungslinien erkennen, *C. u. undulata/C. u. fuertaventurae* und *C. u. macqueenii*. Innerhalb von *C.u. macqueenii* weisen die Trappen des Sinai-Gebietes einen eigenen Haplotyp auf. Da sich die beiden Entwicklungslinien durch klare genetische, morphologische und ethologische Merkmale unterscheiden, wäre es sinnvoll, *C. undulata* und *C. macqueenii* als distinkte Arten zu behandeln.

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	characters																			
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Tinamidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ardeidae	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Ciconiidae	0	0	1	01	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0
Threskiornithidae	01	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
Diomedeidae	0	0	0	1	0	1	1	1	1	1	0	1	0	1	1	1	0	0	0	0
Hydrobatidae	0	0	0	1	0	1	0	0	0	1	0	0	0	1	1	1	0	0	0	0
Procellariidae	0	0	0	1	0	1	0	01	0	1	0	1	0	1	1	1	0	01	0	0
Scopidae	0	1	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	1	0	0
Balaenicipitidae	1	1	2	1	1	0	0	1	1	0	1	0	1	0	1	0	0	1	0	1
Phaethontidae	0	0	1	1	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0
Fregatidae	0	?	2	0	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0
Pelecanidae	1	1	2	1	1	0	1	1	0	0	0	0	1	0	1	0	1	1	0	1
Phalacroc./Anhingidae	0	1	1	0	0	0	1	0	1	0	1	0	1	0	01	0	1	01	0	1
Sulidae	1	1	1	0	1	0	1	1	1	0	0	0	1	0	1	0	1	1	0	1

	characters													
	41	42	43	44	45	46	47	48	49	50	51	52	53	54
Tinamidae	0	0	0	0	0	01	0	0	0	0	0	0	0	0
Ardeidae	0	1	0	0	1	1	0	0	0	0	1	0	1	01
Ciconiidae	0	01	1	0	1	0	0	1	0	0	01	0	0	0
Threskiornithidae	0	0	1	0	0	0	0	1	0	0	01	0	0	0
Diomedeidae	1	0	0	1	0	0	0	2	1	0	0	0	0	0
Hydrobatidae	0	0	0	01	0	0	0	2	1	0	0	0	0	0
Procellariidae	1	0	0	1	0	0	0	2	1	0	0	0	0	0
Scopidae	1	1	1	0	1	0	0	1	0	0	0	0	1	1
Balaenicipitidae	1	1	0	0	1	1	0	0	0	0	0	0	0	1
Phaethontidae	1	?	?	0	1	0	1	2	0	1	0	0	0	0
Fregatidae	1	0	0	1	1	0	2	1	0	1	1	0	1	1
Pelecanidae	1	1	?	1	1	0	2	2	0	1	1	1	0	1
Phalacroc./Anhingidae	1	0	0	1	1	0	2	2	0	1	1	1	1	1
Sulidae	1	0	0	1	1	0	2	2	0	1	1	1	1	1

senbeck & Crandall 1997). ML trees were reconstructed by means of TBR branch swapping and the heuristic search option but with estimates of nucleotide substitutions, invariant sites and gamma parameters initially calculated from a neighbour joining (NJ) tree. A molecular clock was not enforced. Distribution of rates at variable sites = gamma (discrete approximation; shape parameter (alpha) = 0.5; number of rate categories = 4; representation of average rate for each category = mean. These settings correspond to the GTR+G model.

Results and Discussion

Genetic Distances

Mean genetic distances between the different genera of bustards (Table 2) range from 7.1 % (*Chlamydotis/Otis*) to 12.7 % (*Ardeotis/Lophotis*). The average mean distance between all genera analysed is 10.3 %. Nucleotide substitutions are most abundant at the third codon position (n = 260, 77.4 %); only 61 (18.2 %)

Table 2. Pairwise genetic distances (p-distance) in the cytochrome b data set (1.0 = 100 %).
Tab. 2. Paarweise genetische Distanzen (p-Distanz) im Cytochrom b-Datensatz (1.0 = 100 %).

		1	2	3	8	15	18	21
1	*Tinamus major*	–						
2	*Gallus*	0.22222	–					
3	*Afrotis afraoides*	0.20822	0.15748	–				
8	*Ardeotis arabs*	0.22163	0.17608	0.10072	–			
15	*Ardeotis kori*	0.22086	0.16613	0.09637	0.04424	–		
18	*Eupodotis senegalensis*	0.21128	0.16744	0.08067	0.10451	0.09927	–	
21	*Eupodotis rueppellii*	0.21435	0.17148	0.09799	0.10160	0.10336	0.10086	–
27	*Chlam. undulata*	0.21444	0.16457	0.09019	0.10511	0.10250	0.08513	0.09193
30	*Chlam. macqueenii*	0.21980	0.16815	0.09547	0.10872	0.10348	0.08954	0.09984
34	*Chlam. macqueenii* (Sinai)	0.22063	0.16987	0.09719	0.11129	0.10605	0.09211	0.10239
42	*Chlam. u. fuerta-venturae*	0.21522	0.16535	0.09099	0.10511	0.10251	0.08594	0.09274
43	*Lophotis ruficrista*	0.23097	0.18198	0.11461	0.12707	0.12634	0.11488	0.12861
48	*Otis tarda* (Hungary)	0.22242	0.16639	0.09808	0.10695	0.11053	0.08952	0.09719
52	*Otis tarda* (Spain)	0.22676	0.15552	0.09213	0.10506	0.10825	0.09141	0.09252
53	*Tetrax tetrax*	0.21785	0.16798	0.09711	0.10866	0.10158	0.09472	0.10061

		27	30	34	42	43	48	52
27	*Chlam. undulata*	–						
30	*Chlam. macqueenii*	0.01667	–					
34	*Chlam. macqueenii* (Sinai)	0.01929	0.00263	–				
42	*Chlam. u. fuerta-venturae*	0.00000	0.01664	0.01926	–			
43	*Lophotis ruficrista*	0.11031	0.11822	0.12079	0.11111	–		
48	*Otis tarda* (Hungary)	0.06572	0.07187	0.07444	0.06654	0.11477	–	
52	*Otis. tarda* (Spain)	0.07093	0.07680	0.07971	0.07183	0.11173	0.01097	–
53	*Tetrax tetrax*	0.09281	0.10160	0.10415	0.09361	0.11111	0.09369	0.08692

and 15 (4.5 %) exchanges occur at the first and second position, respectively. Given that the third position substitutions do not lead to an amino acid substitution ("silent" substitutions), they are selectively neutral, but might be subjected to homoplasy due to multiple substitution events (Mindell 1997).

Phylogeny Reconstruction

Phylogenetic relationships were investigated by the use of Maximum Parsimony (MP), and Maximum Likelihood (ML) analyses. Sequences of 2 individuals per taxon were selected for the analysis (the sequences of other individu-

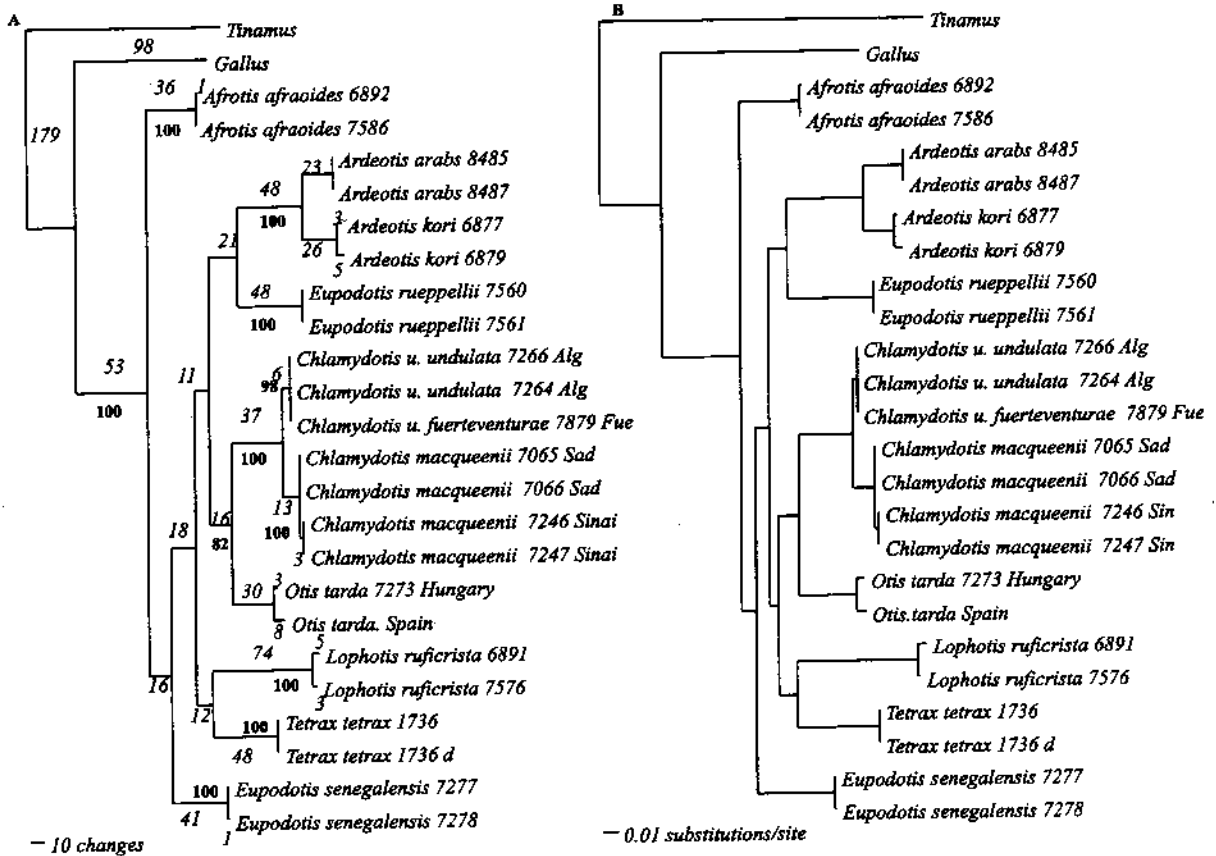

Fig. 1. A molecular phylogeny of bustards, inferred from nucleotide sequences of the mitochondrial cytochrome b gene. Reconstruction via Maximum Parsimony (A) and Maximum Likelihood (B); starting branch lengths obtained using least-squares method with JC distances. Nucleotide frequencies: A=0.282, C=0.344, G=0.122, T=0.252; tree length: 886 steps; CI=0.673, RI=0.784. Outgroups were *Tinamus major* (accession number NC_002781) and *Gallus gallus* (accession number NC_001323). Number of character changes are given at the corresponding bifurcations in MP tree; relevant bootstrap values (1000 replications) are shown in bold numbers.

Abb. 1. Eine molekulare Phylogenie der Trappen anhand von Nucleotidsequenzen des mitochondriellen Cytochrom b-Gens. Stammbäume wurden über Maximum Parsimony (A) und Maximum Likelihood (B) berechnet. Anfangs-Astlängen wurden über die "least-squares method" mit JC Distanzen berechnet. Nucleotidhäufigkeiten: A=0,282, C=0,344, G=0,122, T=0,252; Baumlänge: 886 Schritte; CI=0,673, RI=0,784. Außengruppen waren *Tinamus major* (accession number NC_002781) und *Gallus gallus* (accession number NC_001323). Im MP-Baum sind die Anzahl der Merkmalsänderungen normal und die Bootstrapwerte (1000 Wiederholungen) fett gedruckt.

Alg= Algeria; Fue= Fuerteventura; Sad= Saudi Arabia

Introduction

Despite their considerable body size and unique courtship display behaviour, our knowledge of the biology and systematics of bustards is sadly incomplete, mostly owing to their highly cryptic plumage and secretive behaviour. Bustards are adapted to temperate and tropical open grassy plains. They have colonised all major areas with such habitat in the Old World, ranging from dense bushland to near-desert and salt steppe. The family has an exclusively Old World distribution and encompasses a total of 11 genera and 25 species [number] (*Otis [1], Ardeotis [4], Afrotis [2], Lissotis [2], Chlamydotis [1], Eupodotis [5], Neotis [4], Lophotis [3], Sypheotides [1], Houbaropsis [1]* and *Tetrax [1]*) (del Hoyo et al 1996). Sibley & Monroe (1990), however, recognised only the five genera *Tetrax, Otis, Neotis, Ardeotis* and *Eupodotis*.

Current phylogenetic relationships within the Bustards as well as their systematic position within the class *Aves* are far from being resolved (Houde et al. 1997). It is difficult to distinguish ancestral characters in all members of the Otididae because of the wide ranging specialisation that might have evolved by convergence. The generic limits recognised within the family continue to fluctuate, as various attempts to simplify the situation come up against genuine and interesting levels of morphological and behavioural differences (Sibley & Monroe, 1990; del Hoyo et al. 1996, Houde et al. 1997).

The analysis of nuclear or mitochondrial marker genes has become a widely applied tool over the last 15 years in all fields of zoology, including ornithology for reconstructing phylogenies and phylogeographic relationships (overviews in Avise 1994, Mindell 1997). Molecular data have the great advantage that convergence does not impair the analysis to the same extent as morphological data.

In this work, a new attempt to elucidate phylogenetic relationships between 10 species of Bustards of 7 genera was undertaken in order to obtain an evolutionary framework for the interpretation of behavioural and ecological characteristics of this interesting bird family. Altogether, mitochondrial cytochrome *b* (1143 bp) sequences of 52 individual birds belonging to the genera *Otis, Ardeotis, Afrotis, Chlamydotis, Eupodotis, Lophotis*, and *Tetrax* were determined and analysed in a taxonomic and phylogenetic context. Our study complements another molecular analysis of bustards based on 444 bp of the cytochrome b gene that was published recently (Pitra et al. 2002).

Material and Methods

DNA isolation

Origin of samples is given in Table 1. Blood and tissues were either preserved in an EDTA buffer (0.1 M Tris, pH 7.4, 10 % EDTA, 1 % NaF, 0.1 % thymol) or in ethanol (Wink 1998) and stored at –20 °C until processing. Total DNA was extracted from the blood samples by an overnight incubation at 37 °C in lysis buffer (10 mM Tris [pH 7.5], 25 mM EDTA, 75 mM NaCl, 1 % SDS) including 1 mg of Proteinase K (Merck, Darmstadt), followed by a standard phenol/chloroform protein extraction. DNA was precipitated from the supernatant with 0.8 volume of cold isopropanol, centrifuged, washed, dried and resuspended in TE buffer.

PCR and sequencing

The mitochondrial cytochrome b gene was amplified by PCR with the aid of primers (L and H refer to light and heavy strand of mtDNA; numbers indicate the positions in the mt genome of *Gallus gallus*; Desjardins & Morais 1990). MT-A3 (L-14987 within cyt b): GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC TTC G, or L14857 (anchored on ND5): 5'-GGG TCT TTC GCC CTA TCA AT-3', or mT-F2 (H-16048 within tRNA thr): CTA AGA AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA GAC CAA TG. PCR was performed in 50 µl volume containing 1 unit of AMERSHAM PHARMACIA BIOTECH *Taq* DNA Polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 9. After an initial denaturing step for 5 min at 94 °C, 31 cycles were performed with annealing 52 sec at 60 °C, primer extension 80 sec at 72 °C, and denaturing 45 sec at 94 °C.

PCR products were sequenced directly by the dideoxy chain termination method with the cycle sequencing Kit (Amersham Life Science, RPN 2438/RPN 2538) in combination with internal CY-5 labelled primers (1.5 pmole). For cycle sequencing a two stage programme containing an initial denaturing step at 94 °C for 4 min and 25 cycles at 60 °C (40 sec), and 94 ° (30 sec) was used. The primers employed were mT-C2-CY(L-15298): XGA GGA CAA ATA TCA TTC TGA GG, mT-U2-CY: (H-15649): XGG GGT GAA GTT TTC TGG GTC, mT-C4-CY (H-15420): XAG TGT TGG GTT GTC TAC TGA, mT-V-cy (H-15421): XTGG AGG GGCR AAR AAT CGG GT, or mT-le-cy (L-15697): XTCA AAC CCG AAT GAT AYT TCC TAT T. CY-5 labelled fragments were analysed on an automated DNA sequencer (Amersham Pharmacia Biotech, ALF-Express II).

Alternatively, a cycle sequencing reaction (final volume 10 µl) was carried out after the initial PCR. Reaction buffer consisted of: 2 µl reaction mix with BigDye terminators (according to the BigDye Terminator Protocol; ABI Applied Biosystems), 10 pmole primer (smtA-5' -CAA CAT CTC AGC ATG ATG AAA CTT CG-3' and mt C-5' – TAY GTC CTA CCA TGA GGA CAA ATA TCA TTC TGA GG-3'). The cycle sequencing was carried out in 25 cycles at 96 °C for 10 sec, 52 °C 5 sec and 60 °C 4 min. Sequencing products were purified by precipitation: 1 vol reaction mix, 1/10 3 M NaAcetate (pH 4.6), 2.5 vol ethanol. After centrifugation for 15 min at 13,000 rpm, DNA pellets were washed in 70 % ethanol and taken up in 20 µl distilled water. The purified DNA was diluted 1:5 in water and applied to a 16 column automatic capillary sequencer (ABI 3100) with 50 cm capillaries and POP6 as a polymer.

Sequences of 1143 nt were obtained directly from the sequencer and aligned (GenBank accession numbers in Table 1). Deletions, insertions or inversions were not encountered. Despite the use of different PCR primers only a single product was encountered. As no stop codons were found and since nucleotide abundance corresponded to those of mtDNA, we conclude that we have amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes.

Phylogenetic and statistical analysis

The data were analysed by the use of maximum parsimony (MP) and maximum likelihood (ML) with PAUP* 4.0b10 (Swofford 2002). Genetic distances (uncorrected p-distances) were calculated from a dataset of 1143 bp. Unweighted MP analyses were performed by means of "tree-bisection-and-reconnection" (TBR) branch swapping and the heuristic search option. ML has proved to be powerful and is now widely applied (Swofford et al. 1996, Huel-

Table 1. Origin and number of individual birds sequenced. Nomenclature according to del Hoyo et al. (1996).
Tab. 1. Herkunft and Anzahl der untersuchten Trappen. Nomenklatur nach del Hoyo et al. (1996).

Family	Genus	Species/Subspecies	Origin	Number of individuals Accession number
Otididae	*Afrotis*	*A. afraoides*	Namibia	7; AJ511427-AJ511431
	Ardeotis	*A. arabs*	Saudi-Arabia	7; AJ511432-AJ511438
		A. kori	Namibia	3; AJ511439-AJ511440
	Chlamydotis	*C. undulata fuertaventurae*	Fuerteventura	1; AJ511463
		C. undulata macqueenii	Kazakhstan, Pakistan, Saudi-Arabia, Sinai	12; AJ511451-AJ511462
		C. undulata undulata	Algeria	3; AJ511448-AJ511450
	Eupodotis	*E. rueppellii*	Namibia	4; AJ511444-AJ511447
		E. senegalensis	West Africa	3; AJ511441-AJ511443
	Lophotis	*L. ruficrista*	Namibia	5; AJ511464-AJ511464
	Otis	*O. tarda*	Hungary, Spain	5; AJ511466-AJ511469
	Tetrax	*T. tetrax*	Spain	2; AJ511470-AJ511471

als/taxon (Table 1) were basically identical). The bustard data set contains 333 variable and 311 parsimony-informative positions (skewness $g_1 = -0.58$, $p < 0.01$). Since the phylogenies were inferred from a single mitochondrial gene and since not all bustard species could be sampled, results must be regarded as preliminary. A wider set of genes (additional mtDNA and nuclear genes) would be useful to corroborate the findings obtained. Despite these limitations some conclusions can be drawn from the present data set.

MP results in a single most parsimonious tree (Fig. 1A), which is identical with an ML reconstruction (Fig. 1B). Our tree differs from the phylogeny published by Pitra et al. (2002) based on a partial sequence of cytochrome b (444 bp instead of 1143 bp in our study). This could be due to the fact that our longer sequences provide more and better resolution. Pitra et al. (2002) had included 23 taxa, which can also influence tree topology.

Bustards apparently represent a monophyletic family (Pitra et al. 2002, M. Wink, unpublished results). Within the bustard tree *Otis* and *Chlamydotis* represent a well supported (82 % bootstrap) sister clade (also found by Pitra et al., 2002). A close affinity of this clade to Tetrax, Neotis and Ardeotis (Del Hoyo et al. 1996) can be excluded (this study; Pitra et al. 2002).

Lophotis/Tetrax cluster as sibling species both in MP and ML reconstructions. Pitra et al. (2002) also recovered this cluster, which appears to include the monotypic genera *Houbaropsis* and *Sypheotides*.

Ardeotis and *Eupodotis rueppellii* form a clade in both MP and ML reconstructions. In Pitra et al. (2002) *Eupodotis rueppellii* clusters as an isolated taxon at the base of the bustard tree. We analysed several individuals from blood samples and obtained identical sequences in all instances, whereas Pitra et al (2002) had relied on museum material that might have contained degraded DNA. The isolated position in Pitra et al (2002) may be an indication that either the sample was mislabelled or that a nuclear copy or degraded DNA had been amplified.

The genus *Eupodotis* appears to be polyphyletic since *E. rueppellii* and *E. senegalensis* do not cluster in a single clade but at unrelated positions in the bustard tree. *Afrotis* and *Eupodotis senegalensis* form basal groups inside the bustard monophylum (supported by a bootstrap value of 100 %) whereas they figured as a sister group to *Otis/Chlamydotis* in Pitra et al. (2002). *Eupodotis senegalensis* and *E. caerulescens* clustered as a sister group to *Afrotis afra* and *A. afroides* in Pitra et al. (2002), which would agree with our findings (Fig. 1). The genera *Neotis* and *Lissotis*, which were not analysed by us, have been recovered in the Ardeotis cluster by Pitra et al. (2002), which suggests that *Neotis* and *Ardeotis* are paraphyletic.

Sibley & Monroe (1990) had included *Lophotis ruficrista* and *Afrotis afraoides* in the genus *Eupodotis*. Fig. 1 clearly shows that the genus *Eupodotis* would become highly polyphyletic in the framework of Sibley & Monroe (1990); therefore a splitting of this genus into separate genera (as outlined by del Hoyo et al. 1996) appears to be appropriate. Our result implies that a further splitting of the genus *Eupodotis* might be required; *Eupodotis rueppellii* has already been placed in a genus *Heterotetrax* (Clancey 1966).

Morphological (e.g. development of ornamental feathers) and ethological (i.e. extensive courtship display) criteria support a sibling taxon relationship between *Otis* and *Chlamydotis*, (supported by a bootstrap value of 82 % in MP and 95 % in NJ), features found to this extent only in *Otis* and *Chlamydotis*. Within *Otis tarda* a genetic distance of 1 % was discovered between birds from allopatric populations in Hungary and Spain, which indicates that these birds belong to already separated genetic lineages. A similar conclusion has been reached by Pitra et al. (2000). Another monophylum is formed by the two *Ardeotis* species (100 % bootstrap support), considered as superspecies (del Hoyo et al. 1996, Sibley &

Monroe 1990). This finding is corroborated by the great morphological similarity as well as the male courtship display of the "balloon" type (Del Hoyo et al. 1996). An anatomical specialisation associated with display behaviour is the ability to inflate the neck until it turns into a giant white puffball. Genetic distances of 4.4 % support the species status of *A. arabs* and *A. kori* (Table 2).

Chlamydotis – phylogeography and taxonomical consequences

With regards the genus *Chlamydotis*, two monophyletic sister groups can be recovered, with *C. u. undulata/C. u. fuertaventurae* on the one hand, and *C. u. macqueenii* on the other (bootstrap support 100 %). The Houbara bustard thus represents a polytypic species which is, according to current taxonomy (Sibley & Monroe 1990, Del Hoyo et al. 1996), divided into three subspecies according to size, colour and distribution: *Chlamydotis undulata undulata, Chlamydotis undulata fuertaventurae* and *Chlamydotis undulata macqueenii.* Today, the subspecies show an allopatric distribution with *C. u. undulata* in northern Africa west of the Nile valley, *C. u. macqueenii* east of the Nile and *C. u. fuertaventurae* on the Canary islands.

The phylogenetic analysis of the cytochrome *b* sequences differentiate two distinct lineages, with *C. u. macqueenii* (populations analysed in this study are from Saudi-Arabia, Sinai, Kazakhstan and Pakistan) on the one hand, and *C. u. undulata* on the other. Inside the *macqueenii* population, the birds from Sinai show a distinct haplotype (3 differing nucleotide substitutions; bootstrap support 95 %) whereas sequences from birds of Saudi-Arabia, Kazakhstan and Pakistan were identical. While the genetic distance of *undulata* and *macqueenii* differ by 1.7–2.6 %, sequences of *undulata* and *fuertaventurae* are identical. These findings are in agreement with earlier studies (Granjon et al. 1994, Gaucher et al. 1996). As Gaucher et al. (1996) showed, the male courtship display of *undulata* differs significantly from that of *macqueenii* in many respects as regards visual signals as well as associated vocalisation. A preliminary mt-DNA analysis based on a 300 bp fragment of the cytochrome *b* gene showed a genetic divergence of 1 % between the two subspecies. With regard to the courtship behaviour of *fuertaventurae* little is known, but investigations performed by Hinz & Heiss (1989) show a close similarity to that of *undulata*. The fact that *undulata* together with *fuertaventurae* are distinct from *macqueenii* both by unique qualitative morphological and ethological characters as well as on the genetic level (both forms group as distinct monophyla) implies a reproductive isolation of the two forms. In addition, there are no hybrids known from nature. From the above considerations consistent evidence suggests a modification of the recent Houbara bustard taxonomy as already put forward by Gaucher et al. (1996): *undulata* as well as *macqueenii* should be given species rank. The genetic distance of 1.7–2.6 % between the two taxa falls within the range of acknowledged congeneric species (0.4 – 8.1 %; Avise & Zink, 1988, Shields & Helm-Bychowsky 1988). With respect to the nominate species, *Chlamydotis undulata*, the trinomial nomenclature *C. u. undulata* should be maintained to distinguish it from the Houbara bustards from the Canary Islands *C. u. fuertaventurae*.

Molecular clock and the origin of bustards

The oldest fossil records of bustards are from the area of Phosphorites du Quercy in France and date back to the late Eocene and early Oligocene about 38 million years ago (Mourer-Chauviré 1982, Olson 1985). Further records are known from the Miocene of Bavaria (23 million years ago) and the Pliocene of Asia (5 million years ago, Sanches Marco 1989–90). According to del Hoyo et al. (1996) bustards evolved more than 70 million years ago. We endeavoured to use the cytochrome b distances to estimate divergence times in bustards. A molecular clock model was constructed on the basis of non-synonymous nucleotide substitutions and of amino acid dis-

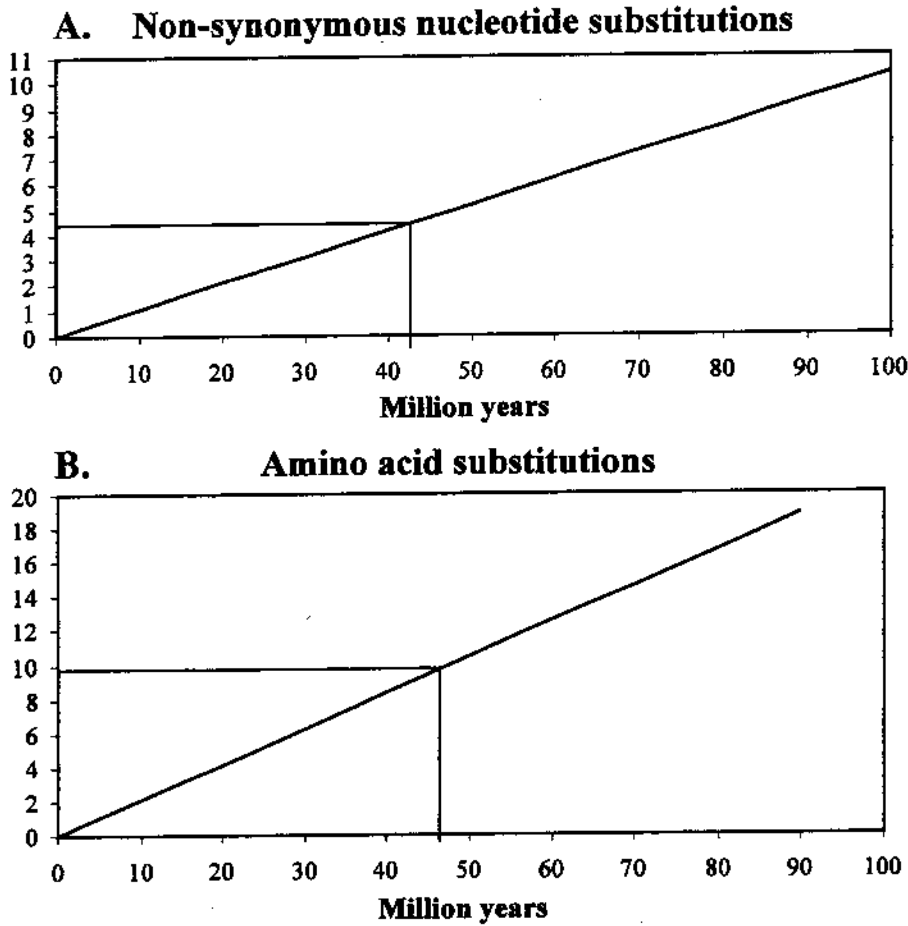

Fig. 2. Molecular clock: Calculation of the potential divergence date of the Otididae from their common ancestor. A: Non-synonymous nucleotide substitutions. 9.3 % ≅ 90 mio. years; distances of non-synonymous substitutions of *Struthio/Rhea* compared with 23 different bird families (Neognathae); mean distance within bustards = 4.3 %. B: Amino acid substitutions (derived from DNA sequences): 18.7 % ≅ 90 mio. years; mean distance within bustards = 9.9 %.

Abb. 2. Molekulare Uhr: Berechnung der potentiellen Divergenzzeit der Trappen von einem gemeinsamen Vorfahren. **A**: Nicht-synonyme Nucleotidsubstitutionen: 9,3 % ≅ 90 Millionen Jahre; Distanzen aus nicht-synonymen Nucleotidsubstitutionen von *Struthio/Rhea* mit 23 Vogelfamilien (Neognathae); mittlere Distanz innerhalb der Trappen = 4,3 %. **B**: Aminosäure-Substitution (Aminosäure-Sequenzen wurden von den DNA-Sequenzen abgeleitet): 18,7 % ≅ 90 Millionen Jahre; mittlere Distanz innerhalb der Trappen = 9,9 %.

tances (Fig. 2). Unlike the "classical" 2 % clock for mtDNA (Brown et al. 1979, 1982, Tarr & Fleischer 1993, Shields & Wilson 1987), this clock stays linear over long periods of time allowing estimates of divergence times of old taxa. Calibration of the clock was made by refence to the divergence date of the flightless ratites, about 90 million years ago (Hedges et al. 1996, van Tuinen et al. 1998) and by estimates of DNA divergence between ratites and Neognathae (Fig. 2).

According to Fig. 2 a divergence rate of 0.1 % per 1 mio. years is gained for non-synonymous nucleotide substitutions and 0.2 % per 1 mio. years for amino acid substitutions. The mean divergence within the family Otididae accounts for 4.3 % (non-synonymous substitutions) or 9.9 % (amino acid substitutions), which corresponds to 42 or 47 mio. years respectively. In the light of these values bustards appear to have originated about 40–50 million years ago. This dating agrees with the view

that many of the extant bird families originated during an explosive phyletic radiation at the Cretaceous/Tertiary boundary (Fedduccia 1995). Thus, our estimate of bustard evolution fits into the general picture of bird evolution.

Since bustards are mainly distributed in Africa (21 of 25 extant species) it is likely that bustards evolved in Africa (see also Pitra et al. 2002). This assumption is supported by the molecular data since African taxa take a basal position in all reconstructions (Fig. 1) whereas European/Asian taxa appear later. As regards non-synonymous distances, Eurasian taxa split from their African ancestors about 37 mio. years ago. We need a complete data set to settle these matters unambiguously.

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