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Out of Africa? Phylogenetic relationships between *Falco biarmicus* and the other hierofalcons (Aves: Falconidae)

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Abstract

The phylogeographic history of the lanner falcon (*Falco biarmicus*) and the phylogenetic relationships among hierofalcons (*F. biarmicus*, *Falco cherrug*, *Falco jugger* and *Falco rusticolus*) were investigated using mitochondrial (mt) DNA sequences. Of the two non-coding mt sections tested, the control region (CR) appeared more suitable as phylogenetic marker sequence compared with the pseudo control region (Ψ CR). For the comprehensive analysis samples from a broad geographic range representing all four hierofalcon species and their currently recognized subspecies were included. Moreover, samples of *Falco mexicanus* were analysed to elucidate its phylogenetic relationships to the hierofalcons. The sequence data indicate that this species is more closely related to *Falco peregrinus* than to the hierofalcons. In the DNA-based trees and in the maximum parsimony network all hierofalcons appear closely related and none of the species represents a monophyletic group. The close relationships among haplotypes suggest that the hierofalcon complex is an assemblage of morphospecies not yet differentiated in the genetic markers used in the present study and that the radiation of the four hierofalcon species took place rather recently. Based on the high intraspecific diversity found within *F. biarmicus* we assume an African origin of the hierofalcon complex. The observed pattern of haplotype distribution in the extant species may be due to incomplete lineage sorting of ancestral polymorphisms, and interspecific gene flow through hybridization.

Key words: *Falco biarmicus* – hierofalcons – genetic analysis – molecular phylogeny – phylogeography

Introduction

The term 'hierofalcon' is commonly used for a complex of so-called 'desert falcons' (Cade 1982; Tucker and Heath 1994; Eastham 2000). Currently four species are ascribed to the hierofalcon complex: the lanner falcon (*Falco biarmicus* Temminck, 1825), the saker falcon (*Falco cherrug* J. E. Gray, 1834), the gyr falcon (*Falco rusticolus* L., 1758) and the laggar falcon (*Falco jugger* J. E. Gray, 1834). Defined as subgenus *Hierofalco* by Kleinschmidt (1901), the hierofalcons form a group of ecologically and morphologically similar species substituting each other in various parts of the world (Glutz v. Blotzheim et al. 1971; Baumgart 1975; Cade 1982; Kemp and Crowe 1993; Eastham 2000). They share similarities in body proportions (broad-based long wings, long tails) and soft plumage structure, which are assumed to have evolved as convergent adaptations to similar hunting modes in dry open landscape. Typically they capture their prey (birds, mammals and reptiles) close to the ground.

With respect to systematics there were debates whether the subgenus *Hierofalco* should comprise more taxa. Kleinschmidt (1901) initially placed two additional species, namely the prairie falcon (*Falco mexicanus* Schlegel, 1851) from North America and the black falcon (*Falco subniger* G. R. Gray, 1843) from Australia, into the subgenus *Hierofalco*. Later, Meinertzhagen (1954) even united *F. biarmicus*, *F. cherrug*, *F. jugger* and *F. rusticolus* together with *F. mexicanus* into one species, designated as *F. rusticolus* based on the priority rule of nomenclature. Stresemann and Amadon (1979) excluded *F. subniger* and treated the 'traditional' five species as a super-species. Thus, the morphology-based systematics of the hierofalcons remained unsettled.

More recent studies on the phylogenetic relationships of falcons were based on nucleotide sequences of the mitochondrial (mt) *cytochrome b* (*cytb*) or on the *recombination-activating gene* (*RAG*), providing mainly information about

deeper nodes within the family Falconidae (Griffiths 1999; Griffiths et al. 2004) or the genus *Falco* in general (Seibold et al. 1993; Helbig et al. 1994; Wink and Sauer-Gürth 2000, 2004). The *cytb* data (Helbig et al. 1994; Wink and Sauer-Gürth 2000, 2004) suggested that *F. mexicanus* may be the sister taxon of *F. peregrinus* Tunstall, 1771, whereas the remaining hierofalcons form a monophylum of closely related species. Within the hierofalcons *F. biarmicus* and *F. cherrug* appear to be paraphyletic according to the tree presented by Wink et al. (2004), a study that focused on *F. cherrug* and *F. rusticolus*, whereas only a small number of *F. biarmicus* specimens and single individuals of *F. jugger* and *F. mexicanus* were included. Therefore, the positions of these species have to be further confirmed by additional specimens. Moreover, given the close relationships among the hierofalcons, the *cytb* gene might not be variable enough to resolve the radiation of this group.

The present study was carried out to elucidate the phylogenetic relationships of hierofalcons and especially the phylogeographic history of *F. biarmicus* in greater detail, employing presumably more variable sections of the mt genome. In other birds of prey the non-coding sections of the mt genome proved to be considerably more variable (two- to threefold) than *cytb*: in the accipitrid genera *Buteo* (Haring et al. 2001; Riesing et al. 2003), *Aquila* (Seibold et al. 1996; Väli 2002) and *Spizaetus* (Gamauf et al. 2005). Thus, the following two sequences were tested for their suitability as phylogenetic markers: the control region (CR) and the pseudo control region (Ψ CR), a second non-coding region in the mt genome of several birds of prey (e.g. Mindell et al. 1998; Haring et al. 1999, 2001; Väli 2002). In order to achieve a more complete sampling we investigated *Hierofalco* samples from a broad geographic range including all currently recognized subspecies as well as *F. mexicanus*. In the following we give a brief description of the characteristics and distribution of the hierofalcons *sensu stricto*.

Falco biarmicus is a widespread and common Afro-Tropical species with a small western Palaearctic distribution range (Fig. 1). Several subspecies, which inhabit a variety of habitats, can be distinguished by their plumage colour and pattern, as well as body size and proportions (Brown and Amadon 1968; Cramp and Simmons 1980; Brown et al. 1982; Kemp and Kemp 1998; Forsman 1999; Ferguson-Lees and Christie 2001; Leonardi 2001). Primarily *F. biarmicus* is a sedentary species, although under extreme climatic conditions, like in the Namib Desert or West Africa, it moves over short distances or is even nomadic. In general, juveniles show the highest mobility (Thiollay 1978; Brown et al. 1982). Currently, five subspecies are accepted: *F. b. biarmicus* Temminck, 1825 has the widest distribution, ranging from South Africa to Kenya in East Africa, with a smooth transition towards *F. b. abyssinicus* Neumann, 1904 in the adjacent north. *F. b. abyssinicus* inhabits tropical sub-Saharan Africa from Senegambia in the west to Ethiopia and Somalia in the east. Both subspecies use a broad variety of habitats ranging from dry open landscape, like deserts and savannas, to bare or fragmentary forested mountains up to 5000 m a.s.l. (Brown and Amadon 1968; Brown

et al. 1982). The palest form, *F. b. erlangeri* Kleinschmidt 1901, lives exclusively in semiarid and arid north-west Africa. A second desert form, *F. b. tanypterus* Schlegel, 1843, is distributed in north-east Africa and the Middle East. Some researchers consider the two subspecies to be indistinguishable (Clark 1999) or classify *F. b. tanypterus* as an intermediate form between *F. b. erlangeri* and *F. b. feldeggii* Schlegel, 1843 (Forsman 1999). The latter is the largest and most intensively patterned subspecies. *F. b. feldeggii* is found in the eastern Mediterranean between Italy, Asia Minor and the southern Caucasus (Weick 1980; Ortá and Meyburg in del Hoyo et al. 1994; Clark 1999; Clark and Davies 2000; Ferguson-Lees and Christie 2001; Dickinson 2003). All subspecies mainly hunt small- and medium-sized birds, but also reptiles, small mammals and large insects (Goodman and Haynes 1989; Bijlsma 1990; Yosef 1991; Leonardi 1994, 2001; Jenkins 1995; Morri-mando et al. 1997; Jenkins and Avery 1999; Hartley 2000).

The smallest hierofalcon is the monotypic *F. jugger*, which is very similar to *F. biarmicus* in plumage pattern (Ferguson-Lees and Christie 2001) and has been even considered as a subspecies of *F. biarmicus* by Sibley and Monroe (1990). *Falco*

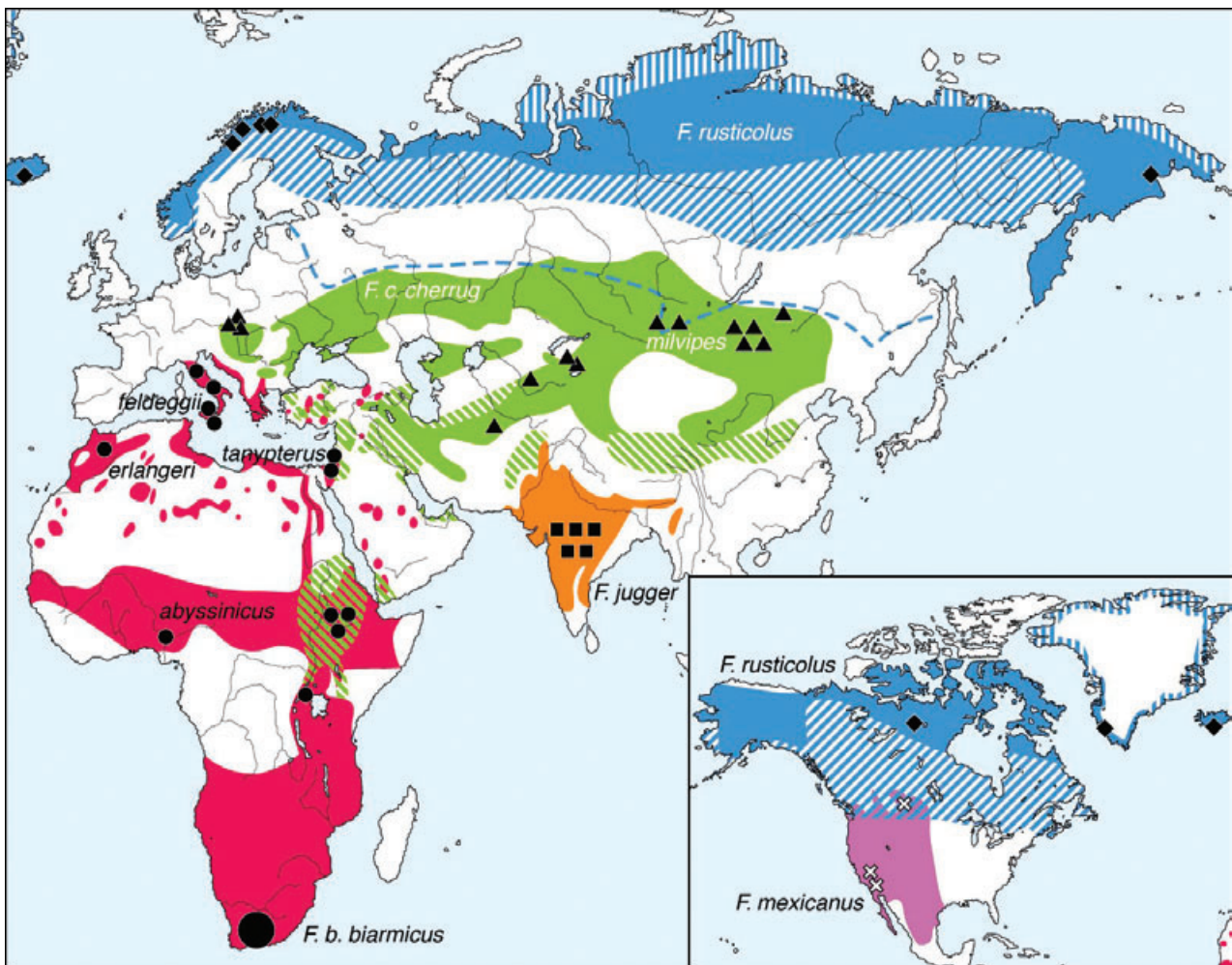


Fig. 1. Geographic distribution of the four hierofalcon species (*Falco biarmicus*, *Falco cherrug*, *Falco jugger* and *Falco rusticolus*) and *Falco mexicanus* (modified after Dementiev and Gladkov 1969; Ferguson-Lees and Christie 2001). Coloured areas are inhabited year-round, vertically hatched areas for breeding only, and diagonally hatched areas are wintering grounds. Dashed line indicates border of sporadic winter distribution of *F. rusticolus*. Symbols indicate collecting localities (see Table 1). Red: lanner falcon, *F. biarmicus* (ssp.: *abyssinicus*, *biarmicus*, *erlangeri*, *feldeggii* and *tanypterus*); green: saker falcon, *F. cherrug* (ssp.: *cherrug* and *milvipes*); orange: laggar falcon, *F. jugger*; blue: gyrfalcon, *F. rusticolus*; purple: prairie falcon, *F. mexicanus*

jagger is a sedentary species endemic to the Indian peninsula (Fig. 1), where it lives in dry open woodland and open habitat with scattered trees (Cade 1982).

The saker falcon *F. cherrug* is a breeding species of the Palaearctic avifauna favouring forest steppe, steppe and grassland, but in some regions inhabiting even open and dry country with scattered trees or electric pylons. The migrating part of the population winters south of that range (Fig. 1). Currently two subspecies are distinguished (Hartert 1912–1921; Vaurie 1961; Brown and Amadon 1968; Ortá and Meyburg in del Hoyo et al. 1994; Ferguson-Lees and Christie 2001): *F. c. cherrug* J. E. Gray, 1834 (eastern Europe to south-central Siberia) and *F. c. milvipes* Jerdon, 1871 (from south-central Siberia to north-eastern China).

The monotypic but polymorphic gyr falcon *F. rusticolus* occurs circumpolar (Fig. 1) inhabiting arctic wooded tundra, rocky outcrops near rivers, lakes and coasts, barren uplands and mountain crags above tree line. Most of the populations are migratory to nomadic (Vaurie 1961; Cade et al. 1998).

Materials and Methods

Samples

Samples of 63 individuals representing eight species were analysed (Table 1). Besides the hierofalcons (29 samples of *F. biarmicus*, 14 *F. cherrug*, eight *F. rusticolus* and five *F. jagger*), samples of *F. peregrinus* (peregrine falcon), *F. mexicanus*, *F. subbuteo* L., 1758 (hobby) and the outgroup species *F. tinnunculus* L., 1758 (kestrel) were analysed. With one exception all samples were obtained from wild populations of known origin, most of them from the breeding areas. As it was not possible to obtain fresh tissue (blood, muscle, moulted feathers) from all relevant taxa, in some cases samples from museum material (study skins) had to be analysed. In addition, the previously published sequence of *F. peregrinus* (Mindell et al. 1999; accession number AF090338) was included into the phylogenetic analysis.

DNA extraction

DNA from fresh tissue was extracted by overnight incubation at 55°C in extraction buffer [10 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaCl, 40 mM dithiothreitol, 1% sodium dodecyl sulphate (SDS), 0.5 mg ml⁻¹ proteinase K]. DNA extractions from museum material (skin from the foot pads of study skins) were performed in a 10% Chelex (Bio-Rad, Hercules, CA, USA) solution containing proteinase K (0.5 mg ml⁻¹). After incubation (4 h, 55°C, with agitation) solutions were heated to 95°C for 5 min and centrifuged for 1 min. The supernatant (both from extractions of fresh and museum material) was purified using the QIA Quick PCR Purification Kit (Qiagen, Hilden, Germany) with a final volume of 30–150 µl elution buffer (depending on the expected quantity and quality of DNA).

PCR amplification

Polymerase chain reaction (PCR) primers used to amplify the CR and Ψ CR, respectively, and their binding sites are listed in Table 2. The sections amplified in this study, primer combinations and their binding regions, fragment lengths and annealing temperatures are depicted in Fig. 2. As one attempt to detect amplification of nuclear sequences (numts), PCR was performed with different primer sets (Fig. 2). For PCR with DNA from fresh tissue approximately 100 ng were used as template DNA. Optimal amounts of template DNA from Chelex extractions were determined empirically (2–10 µl of the DNA solution). PCR was carried out with an Eppendorf Thermocycler in a volume of 20 µl, containing 1 unit Dynazyme DNA polymerase (Finnzymes, Espoo, Finland), 0.5 µM of each primer, and 0.2 mM of each dNTP. The solutions were heated to 94°C (3 min) and then put through 30 reaction cycles: 94°C (15 s), annealing temperature (15 s),

72°C (30–60 s), depending on expected fragment size, followed by a final extension at 72°C (7 min). To detect contaminations control extractions with pure extraction buffer (without tissue) were carried out and negative controls were included in the PCR experiments.

Cloning and sequencing

The PCR products were extracted from agarose gels using the QIA Quick Gel Extraction Kit (Qiagen) and cloned (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA, USA). Sequencing (both directions) was performed by MWG-Biotech (Ebersberg, Germany). To detect whether amplified sequences contained more than one variant (e.g. nuclear pseudogenes) selected PCR products were directly sequenced in both directions on an automated sequencer (performed by VBC Genomics, Vienna, Austria) using the BigDye Terminator Cycle Sequencing Kit version 2.0 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The sequences determined in the course of the present study are registered under the GenBank accession numbers included in Table 1.

Data analysis

Alignments of both mt marker sequences were produced manually. Sections in the Ψ CR sequences which cannot be unambiguously aligned were excluded from the analyses. Neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) were used to infer the phylogenetic analyses. All dendrograms were calculated with the software package PAUP*4.0b10 (Swofford 2002). Gaps in the alignment were treated as missing data. The appropriate substitution model for the two marker sequences was determined with MODELTEST version 3.6 (Posada and Crandall 1998; Posada and Buckley 2004). For the CR data set the Hasegawa-Kishino-Yano (HKY) + Γ model with a Γ -shape parameter of 0.302 was selected. For the Ψ CR data set the Tamura-Nei (TrN) + I model was selected with an estimated proportion of invariable sites of 0.199. For NJ trees uncorrected distances (p-distances) were used. Employing other models for the computation of distances did not alter the topologies of the trees. MP trees were generated with heuristic search using the tree bisection reconnection (TBR) algorithm and a random taxon addition sequence. The reliability of the nodes in each of the analyses was assessed by bootstrap analyses (1000 replicates). A parsimony network of the CR sequences was constructed using TCS 1.13 (Clement et al. 2000) to infer phylogeographic and potential ancestor-descendant relationships among haplotypes. Gaps were treated as missing data because length differences occurred only in a T-stretch of 8–10 bp.

Results

Data sets and sequence variation

We tested two non-coding mt sequences for their suitability as molecular markers on a set of 14 individuals (Table 1): the Ψ CR and the CR.

Amplification of the Ψ CR proved to be difficult. The non-repetitive part is rather short (from 154 to 212 bp). Moreover, its 3'-region which is adjacent to the repetitive section (up to 28 repeats of 27 bp) is not suitable for primer binding because it varies in length. Thus, although we were interested only in the non-repetitive part, the entire Ψ CR had to be amplified (Fig. 2). However, because of the long repetitive part this turned out to be rather difficult. Another problem arose from the presence of nuclear copies (numts) of the Ψ CR, in the hierofalcons as well as in *F. peregrinus* and *F. mexicanus*. These numts lack the repeats, which is possibly the reason why they are preferentially amplified in the PCR. Therefore, various primers had to be designed and tested (e.g. primer Ψ CR-rev) for specific amplification of the non-repetitive part of the genuine Ψ CR (designated Ψ CR-nr). The alignment of 14

Table 1. Specimens investigated.

Taxon	Code	Ti	Origin, Year	Source/Voucher	Marker	Acc. No.
<i>Falco biarmicus</i>						
<i>F. b. abyssinicus</i>	F.b.aby-2	fe	Ethiopia, Addis Abeba, 1969	NMW 94038	CR-ps	DQ144142
	F.b.aby-54	pa	Camerun, Yagoua, 1970	MRAC RG 73.15.A.386	CR-ps	DQ144162
	F.b.aby-55	pa	Ethiopia, Ambo Schoa, 1956	PMJ/FSU 6116	CR-ps	DQ144163
	F.b.aby-56	pa	Ethiopia, Ambo Fl., 1956	PMJ/FSU 7030	CR-ps	DQ144164
<i>F. b. biarmicus</i>	F.b.bia-4	fe	South Africa, 2001	O. Knotzinger	CR-ps	DQ144155
	F.b.bia-5	fe	South Africa, 2001	O. Knotzinger	CR-ps	DQ144158
	F.b.bia-6	fe	South Africa, 2001	O. Knotzinger	CR-ps	DQ144165
	F.b.bia-17	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144139
	F.b.bia-18	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144140
	F.b.bia-19	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144141
					ψCR-nr	DQ144205
	F.b.bia-20	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144143
	F.b.bia-21	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144144
	F.b.bia-22	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144145
	F.b.bia-23	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144146
	F.b.bia-24	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144147
	F.b.bia-25	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144148
	F.b.bia-26	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144149
	F.b.bia-27	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144150
	F.b.bia-28	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144151
	F.b.bia-29	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144152
	F.b.bia-30	bl	South Africa, 2001	A. Stephenson	CR-ps	DQ144153
	F.b.bia-33	pa	Uganda, Kissenye, 1910	NMW 4135	CR-ps	DQ144154
	<i>F. b. erlangeri</i>	F.b.erl-50	sk	Marocco, 2004	EBD	CR-ps
<i>F. b. feldeggii</i>	F.b.fel-40	fe	Italy, Tuskany, 2003	A. Frankoni	CR-ps	DQ144156
					ψCR-nr	DQ144206
	F.b.fel-44	pa	Italy, Foggia, 1928	RMNH No.26	CR-ps	DQ144157
	F.b.fel-51	pa	Italy, Sicily, 1987	W. Wurzinger	CR-ps	DQ144160
	F.b.fel-53	bl	Italy, Sicily, 1994	W. Wurzinger	CR-ps	DQ144161
<i>F. b. tanypterus</i>	F.b.tan-11	fe	Israel, Divshon, 2002	O. Hatzofe	CR-ps	DQ144137
					ψCR-nr	DQ144211
	F.b.tan-13	fe	Israel, Divshon, 2002	O. Hatzofe	CR-ps	DQ144138
<i>Falco cherrug</i>						
<i>F. c. cherrug</i>	F.c.che-1	bl	Austria, Haringsee, 2002	H. Frey	CR-ps	DQ144166
					ψCR-nr	DQ144204
	Fc.che-13	fe	Afghanistan, 1976	O. Knotzinger	CR-ps	DQ144170
					ψCR-nr	DQ144209
	F.c.che-72	mu	Slovakia, Bratislava, 2000	J. Chavko	CR-ps	DQ144178
	F.c.che-105	fe	Slovakia, Solsonica, 1982	J. Chavko	CR-ps	DQ144167
	F.c.che-236	bl	Kazakhstan, Almaty, 1993	R. Kenward	CR-ps	DQ144168
F.c.che-261	bl	Kazakhstan, Tersek, 1997	R. Kenward	CR-ps	DQ144177	
<i>F. c. milvipes</i>	F.c.mil-8	fe	Russia, Dauria, 2001	A. Gamauf	CR-ps	DQ144179
					ψCR-nr	DQ144208
	F.c.mil-158	bl	Mongolia, 1994	D. Ellis	CR-ps	DQ144171
	F.c.mil-162	bl	Mongolia, 1994	D. Ellis	CR-ps	DQ144174
	F.c.mil-190	mu	Mongolia, 1995	D. Ellis	CR-ps	DQ144175
	F.c.mil-192	mu	Mongolia, 1995	D. Ellis	CR-ps	DQ144172
	F.c.mil-199	bl	Russia, South Siberia, 1992	R. Pfeffer	CR-ps	DQ144173
	F.c.mil-277	bl	Mongolia, 1997	D. Ellis	CR-ps	DQ144176
F.c.mil-319	pa	Mongolia, Ulan Bataar, 1962	ZMMU 95280	CR-ps	DQ144169	
<i>Falco jugger</i>						
<i>F. jugger</i>	F.jug-2	fe	India, 1980	O. Knotzinger	CR-ps	DQ144181
	F.jug-6	fe	Captivity, 1989	H. Laueremann	CR-ps	DQ144182
	F.jug-7	pa	India, 1875	NMW 71294	CR-ps	DQ144183
					ψCR-nr	DQ144207
	F.jug-8	pa	India, 1875	NMW 71292	CR-ps	DQ144184
	F.jug-13	fe	India, 1985	O. Knotzinger	CR-ps	DQ144180
<i>Falco rusticolus</i>						
<i>F. rus-2</i>	F.rus-2	fe	Russia, Anadyr, 2001	R. Probst, R. Schmid, M. Pavlicev	CR-ps	DQ144192
	F.rus-4	fe	Canada, 1999	VUW	CR-ps	DQ144197
	F.rus-8	pa	Greenland, Gothaard, 1906	NMW 30007	CR-ps	DQ144195
	F.rus-10	fe	Iceland, Rekjavik, 1993	D. Hartmann, H. Laueremann	CR-ps	DQ144190
	F.rus-16	mu	Norway, Tranøy, 1980	Tromsø Museum	CR-ps	DQ144191
	F.rus-17	mu	Norway, Tromsø, 1996	Tromsø Museum	CR-ps	DQ144196
	F.rus-21	bl	North Sweden, 2001	P. Lindberg	CR-ps,	DQ144193
					ψCR-nr	DQ144210
	F.rus-22	bl	North Sweden, 2001	P. Lindberg	CR-ps	DQ144194

Table 1. Continued

Taxon	Code	Ti	Origin, Year	Source/Voucher	Marker	Acc. No.
<i>Falco mexicanus</i>	F.mex-1	fe	South West Canada, 1995	NMW 88524	CR-ps	DQ144185
	F.mex-5	mu	USA, California, 2000	AMNH PAC723	CR-ps ΨCR-nr	DQ144186 DQ144202
	F.mex-6	mu	USA, California, 1997	AMNH PAC479	CR-ps ΨCR-nr	DQ144187 DQ144203
<i>Falco peregrinus</i>	F.p.per-28	mu	Slovakia, 2002	J. Chavko	CR-ps	DQ144188
	F.p.per-33	bl	Sweden, 2001	P. Lindberg	CR-ps	DQ144189
<i>F. p. ssp.</i>	F.per-GB	–	–	Mindell et al. 1999	CR-ps ΨCR-nr ΨCR-nr	AF090338 AF090338
<i>Falco s. subuteo</i>	F.s.sub-1	mu	Russia, Dauria, 2001	A. Gamauf	CR-ps ΨCR-nr	DQ144198 DQ144201
<i>Falco t. tinnunculus</i>	F.t.tin-1	mu	Austria, Lake Neusiedl, 2000	J. Ortel	CR-ps	DQ144199
					ΨCR-nr	DQ144200

Abbreviations are according to species and subspecies names (taxonomy following del Hoyo et al. 1994).

GB, sequences obtained from GenBank; ti, tissue; bl, blood; fe, basal feather quill; sk, skin; pa, skin from foot pad; mu, muscle; YCR-nr, non-repetitive part of the pseudo control region; CR-ps, partial section of the CR.

Locality and year of collection according to information from specimen's labels or tissue databases.

Table 2. PCR primers used in the present study

Primer	Sequence (5'–3')	5'-binding site	Target gene
Thr+	AACRTTGGTCTTGTAACCC	14 878	tRNA ^{Thr}
CR1+	AGGGCCATAACTTGGTTAATCC	15 663	CR
CR10–	ATGAAAGATAAGATAACGG	16 099	CR
Pro–	GAGGTTTGAGTCCTCTTTTC	16 462	tRNA ^{Pro}
Glu+	GAGACCTACAGCTTGAAAAAC	17 070	tRNA ^{Glu}
ΨCR–rev	AGCTACGGGGACGTATCTC	18 062	ΨCR
H1067	ATAGTGGGGTATCTAATCCCAGTTT	512	12S

Binding sites correspond to the 5'-position of the reference sequence of *Falco peregrinus* (accession number AF090338). Primer H1067 is reverse to L1091 published by Knight and Mindell (1993).

PCR, polymerase chain reaction; ΨCR-nr, non-repetitive part of the pseudo control region; CR-ps, partial section of the CR.

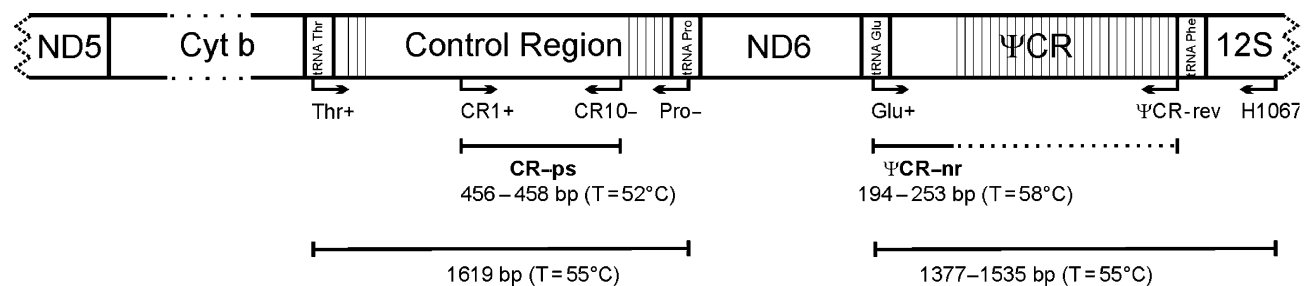


Fig. 2. Section of the mitochondrial (mt) genome including analysed regions [control region (CR), pseudo control region (ΨCR)]. Primer-binding sites, annealing temperatures and respective polymerase chain reaction (PCR) fragments are depicted. The non-repetitive part of the ΨCR (ΨCR-nr) and a partial section of the CR (CR-ps) were used for sequence comparisons. Hatched regions in the CR and ΨCR are repetitive sections

sequences, which included also the published sequence of *F. peregrinus*, had a length of 146 sites.

To select an appropriate section of the CR that is short enough for easy amplification from old material but still informative for phylogenetic analyses, the complete CR was isolated and sequenced from one individual of *F. cherrug* and compared with the published sequence of *F. peregrinus*. The fragment selected contains the most variable non-repetitive sections flanked by conserved primer-binding sites. This partial section designated as CR-ps fragment (length: 456–458 bp) extends from the central part of the CR to the region at the 5'-side of the repetitive part (Fig. 2). The alignment, which also includes the published sequence of *F. peregrinus*, comprises 418 sites.

Based on the alignments of 13 sequences distances in both marker sequences were determined (Table 3). Sequence variability was generally lower in the CR-ps than in the ΨCR-nr sequences. Between ingroup and outgroup species (*F. tinnunculus*) the range of p-distances was 21.5–23.0% for ΨCR and 8.0–8.5% for the CR. Within the hierofalcons distances are rather low (ΨCR: 0–3.6%, CR: 0–1.9%). Remarkably, pairwise distances within hierofalcon species are in some cases higher than those found between species. In contrast to our expectations, the substitution rates for the CR appear to be similar to those obtained for *cytb* by Wink and Sauer-Gürth (2004).

Nevertheless, we selected the CR as the more appropriate non-coding marker sequence for the subsequent phylogenetic

Table 3. Percentage sequence divergence (p-distance) between taxa derived from CR-ps (above diagonal) and from Ψ CR-nr (below diagonal)

Code	1	2	3	4	5	6	7	8	9	10	11	12	13
1 F.b.tan-11	–	1.7	1.0	1.9	0.7	1.0	0.7	1.0	5.1	4.8	5.1	5.4	8.5
2 F.b.bia-19	2.2	–	0.7	0.7	1.0	1.2	1.0	0.7	3.9	3.6	5.3	5.1	8.5
3 F.b.fel-40	2.2	1.5	–	1.5	0.2	0.5	0.2	0.5	4.1	3.9	5.1	4.9	8.7
4 F.c.che-1	3.6	1.5	2.9	–	1.7	1.9	1.7	1.5	4.6	4.3	5.1	4.9	8.5
5 F.c.mil-8	0.7	1.5	1.5	2.9	–	0.2	0.0	0.2	4.3	4.1	4.8	5.1	8.2
6 F.c.che-13	0.7	1.5	1.5	2.9	0.0	–	0.2	0.5	4.1	3.9	4.6	5.4	8.2
7 F.rus-21	0.7	1.5	1.5	2.9	0.0	0.0	–	0.2	4.3	4.1	4.8	5.1	8.2
8 F.jug-7	1.5	0.7	0.7	2.2	0.7	0.7	0.7	–	4.1	3.9	4.6	4.9	8.0
9 F.mex-6	19.4	17.9	17.9	19.4	18.7	18.7	18.7	19.4	–	0.5	4.6	6.1	8.5
10 F.mex-5	20.2	18.7	18.7	20.1	19.4	19.4	19.4	15.2	0.7	–	4.3	5.8	8.7
11 F.perGB	15.9	15.2	15.2	16.7	15.2	15.2	15.2	14.5	12.7	13.4	–	5.1	9.0
12 F.s.sub-1	22.2	22.2	20.7	23.0	21.5	21.5	21.5	21.5	16.5	17.3	11.0	–	6.6
13 F.t.tin-1	23.0	22.2	20.7	23.0	22.2	22.2	22.2	21.5	23.5	24.2	19.7	21.9	–

For designation of specimens see Table 1.

analysis for the following reasons. The length variable sections (indels up to 25 bp) in the Ψ CR-nr cannot be aligned unambiguously and thus would have to be excluded from the analysis. This would further reduce the length of the comparable section and the phylogenetic information, especially for the closely related taxa, would be limited drastically. Faced with the difficulties in PCR amplification and the problems with numts, this marker sequence seems not suitable for the genus *Falco*. In contrast, almost no length variation was observed in the CR-ps sequences (only the sequence of *F. tinnunculus* had a 2 bp deletion) and amplification proved to be straightforward.

Phylogenetic relationships among mtDNA sequences

A total of 64 CR-ps sequences were included in the calculations. Among the 56 individuals of the hierofalcon complex 31 haplotypes were found. The NJ and ML trees derived from the CR-ps sequences are depicted in Fig. 3. In both trees *F. mexicanus* stands closer to *F. peregrinus* than to the hierofalcons. Moreover, none of the hierofalcon species forms a monophyletic group. In the NJ tree the hierofalcons are divided into two mixed clades: clade A contains all four species, whereas clade B consists only of *F. cherrug* and *F. biarmicus* individuals. Representatives of four subspecies of *F. biarmicus* (*biarmicus*, *erlangeri*, *feldeggii*, *tanypterus*) are found in clade A, whereas in clade B only *F. b. biarmicus* and *F. b. abyssinicus* occur. In the ML tree the basal split within the hierofalcons separates two *F. jugger* individuals from the rest. In the cluster of the remaining species one distinct clade corresponds to clade B of the NJ tree. In general, relationships within the hierofalcon complex are only poorly supported in the bootstrap analyses.

In the MP analysis 24 shortest trees were obtained (length = 109, CI = 0.826, RC = 0.766, bootstrap values are included in Fig. 3). With respect to the hierofalcon complex the tree topology resembles the ML tree (clade B and various distinct branches). However, bootstrap support is generally low. In the 50% bootstrap consensus tree only clade B remained stable, whereas all the other nodes within the hierofalcon complex collapsed. In all MP trees *F. mexicanus* forms the sister group of *F. peregrinus* (bootstrap value 88%).

The trees obtained with the limited Ψ CR data set (14 sequences, trees not shown) are congruent with the CR trees,

confirming the close relationships among hierofalcons as well as the clustering of *F. mexicanus* with *F. peregrinus*. *Falco subbuteo* also clusters with the latter two species, but with a bootstrap support below 50%. The deeper nodes in those trees have weak bootstrap support, as can be expected considering the shortness of the sequence and the few variable sites. The congruence between the two trees is a further proof that authentic mt sequences were used.

In Fig. 4 the relationships among the haplotypes of the hierofalcons are depicted as a parsimony network. Most haplotypes of *F. biarmicus* form the centre of the network (cluster I) that also contains two individuals of *F. jugger*. Seven individuals share the main haplotype I. From this centre two clusters emerge. One (cluster II) comprises haplotypes of *F. cherrug* and *F. biarmicus*, with a main haplotype II shared by the two species. The other one (cluster III) contains individuals of *F. cherrug*, *F. rusticolus*, *F. biarmicus* and *F. jugger*. The central haplotype of the latter cluster, designated main haplotype III, is found in all four species. Leaving aside the paraphyly of *F. cherrug* with two highly distinct lineages, haplotype variability in this species as well as in *F. rusticolus* (both mostly migratory species) is rather low compared with *F. biarmicus* and *F. jugger* (both mostly sedentary species). Among 14 *F. cherrug* individuals five haplotypes are found, and four haplotypes among the eight *F. rusticolus* individuals. In contrast, 20 haplotypes occurred among the 29 *F. biarmicus* individuals, 15 of them possess a private haplotype. In *F. jugger* four of the five individuals possess their own haplotype.

The subspecies of *F. biarmicus* are not randomly distributed in the network. In the centre of the network most representatives of *F. b. biarmicus* are found, whereas two of them branch off from main haplotype II. The two *F. b. abyssinicus* haplotypes also are derivatives of main haplotype I. The link between the centre and main haplotype III is formed by a haplotype of *F. b. feldeggii* from which the only specimen of *F. b. erlangeri* is separated by a single substitution. One of the other two *F. b. feldeggii* individuals possesses main haplotype III, whereas the other one is separated from it by two substitutions. The two specimens of *F. b. tanypterus* also branch off from the main haplotype III and are not directly connected to the other *F. biarmicus* haplotypes. The two distinct groups of *F. cherrug* individuals in the network do not reflect any subspecific division, as *F. c. cherrug* as well *F. c. milvipes* are found in both clusters.

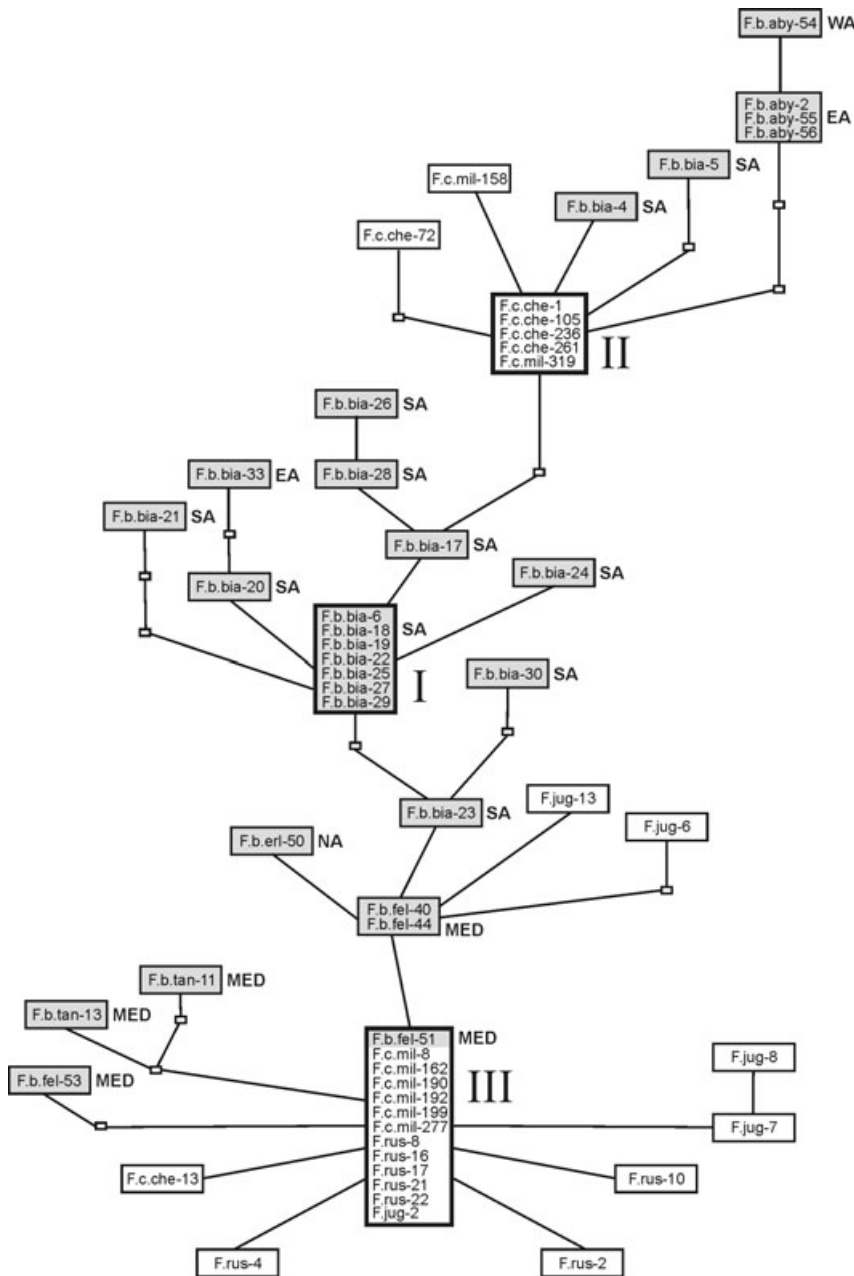


Fig. 4. Maximum parsimony network of control region (CR) haplotypes obtained from the four hierofalcon species. Abbreviations of specimens are according to Table 1. Specimens of *Falco biarmicus* are shaded grey. Main haplotypes I, II and III are indicated. Hypothetical haplotypes are depicted as small rectangles. Geographic origins of *F. biarmicus* haplotypes are indicated beside haplotypes: SA, South Africa; EA, East Africa; WA, West Africa; NA, North Africa; MED, Mediterranean region

obtained from the highly variable CR sequences confirm the assumption that the hierofalcon complex is an assemblage of morphospecies not yet differentiated in the genetic markers used in the present study. This corroborates the superspecies status of the hierofalcons postulated earlier by Ortá and Meyburg in del Hoyo et al. (1994) and Ferguson-Lees and Christie (2001).

Phylogeography of the hierofalcons

The observed haplotype pattern could be explained in two ways which are not mutually exclusive: (1) incomplete lineage sorting of ancestral polymorphisms and (2) interspecific gene flow through hybridization.

Haplotype diversity in our data set may provide valuable hints for the phylogeographic history of this group. Despite the small sample size for *F. cherrug*, *F. jugger* and *F. rusticolus*,

the specimens cover a large geographical area (Table 1) comprising the Holarctic region and the Indian subcontinent (Fig. 1). In contrast to *F. jugger*, sequence diversity within the two *F. cherrug*/*F. rusticolus* clusters (clusters II and III) is comparatively low, suggesting genetic bottlenecks in the population histories of these two species. The highest haplotype diversity is found in *F. biarmicus*. Fourteen haplotypes are represented in the 22 individuals originating from sub-Saharan Africa. This result, together with the haplotype distribution in the minimum spanning network, could be taken as an indication for an African origin of the hierofalcon complex. To support this hypothesis, larger samples of the other three hierofalcon species should be analysed.

The following evolutionary scenario may have led to the present situation. The ancestral population of the hierofalcon complex presumably had a more or less Pan-African distribution. In the course of glacial temperature oscillations the

southern boundary of the Sahara desert shifted to the south (Andersen and Borns 1997; Wilson et al. 2000; Hewitt 2004). Under these conditions the northern populations became isolated and probably inhabited mainly the Mediterranean area. During warmer periods of the Pleistocene three independent colonizations of Eurasia occurred. Whereas the African populations gave rise to the recent species *F. biarmicus*, the colonizers evolved into the species *F. jugger* (colonizing India from the eastern Mediterranean area), *F. rusticolus* (colonizing the northern Palaearctic from the western Mediterranean area) and *F. cherrug* (colonizing central Asia from eastern Africa). Especially the low haplotype diversity among *F. rusticolus* individuals corroborates the assumption of a fast expansion of a genetically deprived population. In the course of subsequent cold periods the three species remained isolated in refuge areas.

How can the present haplotype distribution be explained? The fact that the *F. cherrug* haplotypes, represented in our data sets by two clusters (II and III), appear as independent offshoots may be ascribed to incomplete lineage sorting. The same could be true for *F. rusticolus* and *F. jugger*. For the coexistence of two distinct haplogroups in *F. cherrug* another explanation involving secondary contact seems also plausible. Initially, the ancestors of *F. cherrug* carried only haplotypes of cluster II, whereas those of *F. rusticolus* possessed haplotypes of cluster III. The presence of cluster III haplotypes in recent *F. cherrug* individuals could be the consequence of a secondary contact between the two species and successive introgression of type III haplotypes into *F. cherrug*. Hybridization could have taken place at the end of the last glaciation, when both species probably expanded their distribution ranges rapidly north- and east-wards, making contact, e.g. in central Asia. This assumption is supported by the haplotype distribution in the network, where cluster II mainly contains individuals from the current western regions, whereas cluster III includes mainly eastern individuals. To confirm this, more individuals of *F. cherrug*, *F. jugger* and *F. rusticolus* should be investigated. Furthermore, the data obtained from mt sequences need to be complemented by additional data from nuclear markers. Preliminary experiments using genomic fingerprinting with inter-simple-sequence-repeats (ISSR)-PCR, a method which addresses changes in the nuclear genome and usually detects differences between species, revealed very little variation within the hierofalcon complex (M. Wink, unpublished data). Future investigations will include microsatellite markers in order to infer population structure and gene flow.

Palaeontology

In general, there are only relatively few fossil records of falcons available worldwide, mostly from Europe and North America (Brodkorb 1963; Janossy 1977; Hernandez Carasquilla 1993; Mourer-Chauviré 1993; Feduccia 1996; Döppes and Rabeder 1997; Tyrberg 1998; Mlíkovský 2002). No bones assigned to the genus *Falco* were found in the bird remains of the Plio-Pleistocene from the Rift Valley in East Africa (J. Mlíkovský, personal communication). In Europe, the first dated falcons are from the lower Pleistocene, with an age of about 1 million years. They are attributed to *F. peregrinus* (Victoria, Spain; Marco 2004). The lack of earlier fossils can be explained in two ways: either large falcons originated rather late, or they settled before the Pleistocene only in regions from where fossils are scarce, e.g. the southern hemisphere.

The oldest documented hierofalcon, recorded from Corsica with an age of > 34 000 years, was identified as *F. biarmicus* (Bonifay et al. 1998) supporting the idea that the ancestral species resembled this species. According to our hypothesis older fossils of this species should be found in Africa, but the fossil record of birds is generally rather poor. Our assumption that *F. cherrug*, *F. rusticolus* and *F. jugger* represent younger offshoots of a *F. biarmicus*-like ancestor is corroborated by their late appearance. The oldest dated fossils of *F. cherrug* are from Ohalo 2 (Israel, 19 400 years; Simmons and Nadel 1998). Late Pleistocene findings of *F. cherrug* are also known from Hungary, Croatia, Slovenia, Ukraine and southern Mongolia (Tyrberg 1998; Martynovich 2002). *Falco rusticolus* was found in excavations from the same period, covering a large area extending from Spain eastwards to Romania (Tyrberg 1998). No fossils of *F. jugger* have been documented so far. In North America the oldest large falcon representatives (*F. peregrinus*, *F. mexicanus*, *F. rusticolus*) from cave deposits in the western USA are from the late-Pleistocene (Emslie 1985, 1998; Emslie and Heaton 1987). The North American fossil record of the early- to middle-Pleistocene contains *Falco* sp. remains that were not further identified (Emslie 2004).

Summarizing the fossil data and the phylogeographic scenario deduced from the sequence data we conclude that the radiation of the hierofalcons was a rather recent event that probably took place not before the last interglacial period. Due to incomplete lineage sorting we observe a reticulate pattern of haplotype distribution in the extant species.

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Zusammenfassung

Ursprung in Afrika? Die phylogenetische Verwandtschaft von Falco biarmicus zu den übrigen Hierofalken (Aves: Falconidae)

Die phylogeografische Geschichte des Lannerfalken (*Falco biarmicus*) und die phylogenetischen Verwandtschaftsbeziehungen der Hierofalken wurden mittels mitochondrialer (mt) DNA-Sequenzen untersucht. Von den beiden getesteten nichtkodierenden Abschnitten des mitochondrialen Genoms erwies sich die Kontrollregion (CR) im Vergleich zur Pseudokontrollregion (Ψ CR) besser als phylogenetische Markersequenz geeignet. Für die umfassende Analyse wurden Proben aus einem weiträumigen geografischen Areal untersucht, wobei alle vier Hierofalkenarten (*F. biarmicus*, Sakerfalken, *F. cherrug*, Lugerfalken *F. jugger* und Gerfalken *F. rusticolus*) einschließlich ihrer derzeit anerkannten Unterarten erfasst wurden. Zusätzlich wurden auch Proben des Präfalken *F. mexicanus* analysiert, um dessen phylogenetische Verwandtschaft zu den Hierofalken zu klären. Die Sequenzdaten zeigen, dass diese Art dem Wanderfalken *F. peregrinus* näher steht als den Hierofalken. In den DNA-Stammbäumen und dem Maximum Parsimony Netzwerk erscheinen alle Hierofalken sehr nahe miteinander verwandt zu sein, wobei keine der vier Arten eine monophyletische Gruppe bildet. Die enge Verwandtschaft zwischen den Haplotypen bedeutet, dass der Hierofalkenkomplex eine Gruppe von Morphospezies darstellt, die zumindest in den hier verwendeten Markersequenzen noch nicht differenziert sind, und dass die Radiation der vier Hierofalkenarten vor relativ kurzer Zeit erfolgt sein muss. Die hohe innerartliche Diversität von *F. biarmicus* führt zur Annahme, dass der Ursprung des Hierofalkenkomplexes in Afrika zu suchen ist. Das Muster der Haplotypenverteilung innerhalb der rezenten Arten könnte auf den Weiterbestand anzealer Polymorphismen und auf zwischenartlichen Genfluss durch Hybridisierung zurückzuführen sein.

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