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Repeated Sequence Homogenization Between the Control and Pseudo-Control Regions in the Mitochondrial Genomes of the Subfamily Aquilinae

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ABSTRACT In birds, the noncoding control region (CR) and its flanking genes are the only parts of the mitochondrial (mt) genome that have been modified by intragenomic rearrangements. In raptors, two noncoding regions are present: the CR has shifted to a new position with respect to the ''ancestral avian gene order,'' whereas the pseudo-control region (ΨCR) is located at the original genomic position of the CR. As possible mechanisms for this rearrangement, duplication and transposition have been considered. During characterization of the mt gene order in Bonelli's eagle *Hieraaetus fasciatus*, we detected intragenomic sequence similarity between the two regions supporting the duplication hypothesis. We performed intra- and intergenomic sequence comparisons in *H. fasciatus* and other falconiform species to trace the evolution of the noncoding mtDNA regions in Falconiformes. We identified sections displaying different levels of similarity between the CR and ΨCR. On the basis of phylogenetic analyses, we outline an evolutionary scenario of the underlying mutation events involving duplication and homogenization processes followed by sporadic deletions. Apparently, homogenization may easily occur if sufficient sequence similarity between the CR and ΨCR exists. Moreover, homogenization itself allows perpetuation of this continued equalization, unless this process is stopped by deletion. The Pandionidae and the Aquilinae seem to be the only two lineages of Falconiformes where homology between both regions is still detectable, whereas in other raptors no similarity was found so far. In these two lineages, the process of sequence degeneration may have slowed down by homogenization events retaining high sequence similarity at least in some sections. *J. Exp. Zool. (Mol. Dev. Evol.) 312B:171–185, 2009.*

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In contrast to the nuclear genome, where duplications and rearrangements are an important driving force of genome evolution, the mitochondrial (mt) genome of vertebrates has a more or less conserved structure. Nevertheless, modifications and rearrangements were detected in several groups of vertebrates. For example, the exchange of the positions of tRNA genes has been found in marsupials (Pääbo et al., '91),

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reptiles (Kumazawa and Nishida, '95) and fish (Miya and Nishida, '99). Gene duplications were found in reptiles (Kumazawa et al., '96, '98; Macey et al., '97) as well as in fish (Inoue et al., 2001; Lee et al., 2001). In birds, however, the noncoding control region (CR) and its flanking genes are the only parts of the mt genome that have been involved in intragenomic rearrangements. Desjardins and Morais ('90) described a major deviation with respect to the mammalian gene order in the mt genome of galliform birds. Further studies revealed that this gene arrangement is also present in many other bird species and thus it was considered as the ''standard avian gene order.'' However, later it turned out that not all birds share this genomic arrangement. Considering the galliform standard gene order as the ancestral state in the avian lineage, from which the other rearrangements derived, we follow the concept of Gibb et al. (2007), who introduced the term ''ancestral avian gene order.''

The first deviations from this ancestral gene order were detected in the mt genomes of the two raptor species *Falco peregrinus* (Mindell et al., '98) and *Buteo buteo* (Haring et al., '99). Furthermore, the comparison of mt genomes in several bird lineages revealed that similar rearrangements are present in bird species belonging to six different orders: Cuculiformes, Falconiformes, Passeriformes, Piciformes, Psittaciformes and Procellariiformes (Mindell et al., '98; Haring et al., '99, 2001; Bensch and Härlid, 2000; Eberhard et al., 2001; Muñoz et al., 2001; Väli, 2002; Abbott et al., 2005; Gibb et al., 2007). As each of these bird orders is more or less undisputed as a monophylum, the sporadic occurrence of these rearrangements, sometimes only in subbranches of the different lineages, implies that they have originated independently several times during avian evolution. The common characteristic of this mt rearrangement is the existence of an additional noncoding region besides the CR. The functional CR has moved between the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes, and an additional noncoding section is located between the $tRNA^{Glu}$ and $tRNA^{Phe}$ genes at the original site of the CR in the ancestral gene order.

To explain the origin of this mt rearrangement, the ''duplication hypothesis'' has been favored by several authors (Moritz et al., '87; Quinn, '97; Mindell et al., '98; Bensch and Härlid, 2000). It assumes that the rearrangement was initiated by a tandem duplication of the original CR together with flanking sections (e.g., *tRNA-Thr*, *tRNA-Pro*, *nd6*, *tRNA-Glu*), followed by deletions or partial degeneration in both duplicated sections. Support for the duplication hypothesis came from the apparent similarity between the two noncoding sequences as observed in species of the order Passeriformes (*Smithornis*: Mindell et al., '98; *Phylloscopus*: Bensch and Härlid, 2000). Later, two almost identical copies of the CR were found in the genus *Amazona* (order Psittaciformes), where both paralogues contain the same conserved sequence motifs (Eberhard et al., 2001). This situation was interpreted to represent an early stage after CR duplication, previous to the degeneration of one copy. In most other cases the second noncoding sequence lacked the conserved motifs characteristic for a functional CR probably representing an eroding remnant of the original CR free of functional constraints. The various designations used so far for the second noncoding region detected in various species reflect in general the interpretation of the authors concerning this lack of function and/or the assumed origin via duplication of the authentic CR (e.g., pseudo-control region, ΨCR: Haring et al., '99; noncoding region, nc: Bensch and Härlid, 2000; CR(2): Gibb et al., 2007). For the sake of congruence with earlier studies (Haring et al., '99, 2001; Väli, 2002), we maintain the term ΨCR for the copy located downstream of the functional CR. This seems to be justified because in most cases among the falconiform species investigated so far this second copy lacked CR-specific sequence motifs.

In Falconiformes, until recently, no sequence similarity between CR and ΨCR has been found. All raptor species analyzed exhibited a ΨCR with a characteristic structure consisting of a 5′ nonrepetitive region without similarity to the CR followed by a large cluster of conserved tandem repeats (Mindell et al., '98; Haring et al., '99, 2001; Väli, 2002). Nevertheless, neither this structural characteristic nor the lack of sequence similarity necessarily contradicts the duplication hypothesis. It was assumed that the rearrangement was initiated by a single mutation event early in the Falconiformes lineage. Subsequently, the ΨCR degenerated completely while repetitive sequences accumulated, similarly as found, e.g., in the 3′ region of many CRs. A surprising finding was reported recently by Gibb et al. (2007): Two almost identical copies of the CR are present in the mt genome of the osprey *Pandion haliaetus*.

Furthermore, Gibb et al. (2007) concluded that both CR copies may be functional and, consequently, they designated the second copy as CR(2).

In this study, we present a striking intragenomic similarity between the two noncoding sequences in Bonelli's eagle *Hieraaetus fasciatus* (Accipitridae, Falconiformes), which provides strong support for the duplication hypothesis. To investigate this sequence similarity in more detail, we characterize the mt gene order in Bonelli's eagle in the region comprising the CR and ΨCR. We also describe the internal structure of both noncoding regions and the presence of conserved sequence blocks in the CR.

Furthermore, we trace the evolution of the CR and ΨCR sequences in Falconiformes. With this purpose, we perform intra- and intergenomic sequence comparisons in *H. fasciatus* and other falconiform species including published sequences as well as several new sequences determined in this study. In particular, we were interested in finding out to what degree the second CR copy is retained in the various lineages and whether sequence homogenization (through mechanisms such as gene conversion or recombination) between the paralogues may have played a role in the aquiline lineage, to which *H. fasciatus* belongs. On the basis of phylogenetic analyses of different sections of CR and ΨCR, we outline an evolutionary scenario that could explain the different levels of similarity observed in these sections and the underlying mutation events.

MATERIALS AND METHODS

Samples and DNA extraction

To determine CR and ΨCR sequences, five samples of *H. fasciatus* were analyzed, one of them consisting of cells frozen after plasma separation (sample 1360, Cádiz, Spain) and the other four consisting of blood, three preserved in ethanol (HFA, Murcia, Spain; PD1, Rabat, Morocco; HFC, Alicante, Spain) and one preserved in Seutin buffer (SA2, Morocco) (Seutin et al., '91). Furthermore, CR and ΨCR sequences from one sample of *Accipiter gentilis* (HAB-1A, bred in captivity, Austria) as well as CR sequences from *Aquila heliaca* (Ahel1, Lower Austria, Austria), *Aquila chrysaetos* (Achr1, St. Petersburg, Russia) and *Aquila pomarina* (Apom1, Slovakia) were determined for sequence comparison.

Genomic DNA was extracted using a slightly modified version of the proteinase K–LiCl method described by Gemmell and Akiyama ('96). Samples (100 μL of blood in ethanol or Seutin buffer) were incubated at 56°C overnight in 300 μL extraction buffer (100 mM NaCl, 50 mM Tris–HCl, 1% SDS, 50 mM EDTA, pH 8, 100 μg/mL proteinase K). Nucleic acids were extracted with 5 M LiCl and chloroform:isoamyl alcohol 24:1, and then precipitated with ethanol. For the *Aquila* samples, DNA was extracted from feather samples with the DNeasy Kit (Qiagen, Hilden, Germany).

PCR amplification, cloning and sequencing

Primer sequences are listed in Table 1. Primer binding sites and resulting polymerase chain reaction (PCR) fragments are depicted in Figure 1. For the determination of the CR sequence of *H. fasciatus*, either the complete CR was amplified with primers that bind in the flanking $tRNA^{Thr}$ and $tRNA^{Pro}$ genes (ThrF/ProR) or the sequence was amplified in two overlapping fragments (361 bp overlap) using the primer pairs Thr+/CSB− and CR2+/Pro−. Amplification of the ΨCR was performed using the primers nd6-1+/12S-1− binding in the genes for *nd6* and *12S rRNA*, respectively. A PCR fragment including the section from the 3′ part of the CR, $tRNA^{Pro}$, *nd6* to $tRNA^{Glu}$ was amplified with the primer pair Hier-CR4+/Hier-Glu2−. For *Ac. gentilis* it was possible to amplify and clone the complete section spanning from $tRNA^{Thr}$ to *12S rRNA* with primers Thr+/12S-1−, and sequencing was performed with primer walking. The CR sequences of Aquilinae species were obtained by amplification of two overlapping PCR fragments using the following primer pairs: Thr+/CSB− and SpiCR3+/Pro− (Table 1).

PCR amplification was performed in an Eppendorf thermocycler in a volume of 25 μL containing 2.5 μL of PCR buffer, 0.2 mM of each nucleotide, 1 μM of each primer, 1 unit Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) and 100 ng of DNA. The PCR reaction comprised an initial heating for 2 min at 94°C followed by 35 cycles: 10 s at 94°C, 15 s at annealing temperature and 60 s at 72°C. After the last cycle, a final extension of 5 min at 72°C was performed. PCR products were extracted from agarose gels with the QIAquick Gel Extraction Kit (Qiagen) and cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Sequencing of the clones (both directions) was performed by primer walking at MWG-Biotech (Ebersberg, Germany).

TABLE 1. Primers utilized to amplify the mt CR and ΨCR in Hieraaetus fasciatus, Accipiter gentilis and three Aquila species

Primer	Sequence	Annealing temperature (°C)	Region amplified	Reference
ThrF	TTGGTCTTGTAAACCAAARANTGAAG	62	CR	[1]
ProR	AATNCCAGCTTTGGGAGYTG	62	CR	[1]
Thr+	AACRTTGGTCTTGTAAACC	50	5′-Section CR	[2]
CSB−	ATGTCCAACAAGCATTCAC	50	5′-Section CR	This study
CR2+	AAACCCCTAGCACTACTTGC	54	3′-Section CR	This study
SpiCR3+	CGGACCGGTAGCTGTCGGAC	58	3′-Section CR	This study
Pro−	GAGGTTTGAGTCCTCTTTTTC	54	3′-Section CR	[2]
nd6-1+	ACCCGAATCGCCCCACGAG	57	ΨCR	[3]
12S-1−	ATAGTGGGGTATCTAATCCCAGTTT	57	ΨCR	[3]
Hier-CR4+	CACCCAAAACAACCTCTA	52	3′ End CR to $tRNA^{Glu}$	This study
Hier-Glu2−	TTTGGAGAGAAGCCAAGCA	52	3′ End CR to $tRNA^{Glu}$	This study

CR, control region; ΨCR, pseudo-control region.
[1]Godoy et al. (2004).
[2]Nittinger et al. (2005).
[3]Haring et al. ('99).

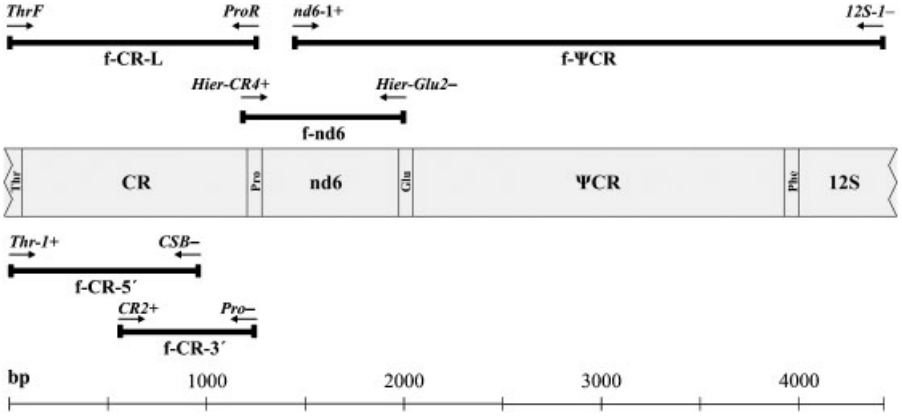

Fig. 1. Mt gene order in the region including the CR and ΨCR in *H. fasciatus*. Primer binding sites (arrows) and PCR fragments (f-CR-L, f-ΨCR, f-nd6, f-CR-5′, f-CR-3′) are indicated. Primer names are given in italics. CR, control region; ΨCR, pseudo-control region; PCR, polymerase chain reaction.

Sequence analysis

When targeting mt sequences, using blood as a source of DNA can be problematic, given that avian erythrocytes are nucleated but are relatively depauperate in mtDNA; therefore, PCR might favor the amplification of "numts" ("nuclear copies of mitochondrial genes"; López et al., '94). To make sure that we were amplifying mt fragments, we compared the blood-obtained sequences used in this study with partial CR and ΨCR sequences obtained afterwards from muscle and feather samples, which are mtDNA-richer tissues (Sorenson and Quinn, '98). These sequences were identical to those obtained using blood as a template, supporting the assumption that the sequences studied here are of mitochondrial origin (data not shown).

Sequences were aligned and edited manually with BioEdit 7.0.1 (Hall, '99). Distances (p-distances) were calculated by hand; all gaps were treated as one mismatch irrespective of their size. NJ trees (neighbour joining; based on p-distances) were calculated using the software package PAUP (version 4.0b10; Swofford, 2002) to illustrate the complicated pattern of varying sequence similarities between CR and ΨCR across different sections. These trees are not intended to provide a phylogeny of the taxa involved, even more as the alignments of these sections are very short. Instead, we use them to demonstrate the effect of chimerical (homogenized/nonhomogenized) sections. Thus, determining substitution models for such chimerical sequences as well as application of more sophisticated tree building algorithms is not useful in this case. Positions with gaps in pairwise comparisons were excluded from the analysis. For the tree in Figure 5B, all positions with gaps were excluded because of

several large deletions in various taxa. Bootstrap values (1,000 replicates) were also calculated with PAUP. Sequences determined for the phylogenetic comparisons are deposited under the following GenBank accession numbers: *H. fasciatus* (FJ627048), *Ac. gentilis* (FJ627047), *Aq. pomarina* (FJ627045), *Aq. heliaca* (FJ627046) and *Aq. chrysaetos* (FJ627044). For comparisons the following sequences from GenBank were used: conserved sequence blocks within CR: *B. buteo* (AF380305; Haring et al., 2001), *Neophron percnopterus* (AY542899; Roques et al., 2004), *Gypaetus barbatus* (AY542900; Roques et al., 2004), *F. peregrinus* (DQ144188; Nittinger et al., 2005), *Ciconia ciconia* (AB026818), *Alectoris barbara* (AJ222726; Randi and Lucchini, '98) and *P. haliaetus* (NC998550; Gibb et al., 2007); CR: *Spizaetus nipalensis* (AP008238; Asai et al., 2006), *Grus japonensis* (AB017620; Hasegawa et al., '99), *P. haliaetus* (NC008550; Gibb et al., 2007); ΨCR: *Aq. heliaca*, *Aq. pomarina*, *Aq. chrysaetos* (AF435096, AF487453.1, AF435099; Väli, 2002).

Taxonomic remarks

Although recent molecular investigations revealed that within Aquilinae the genera *Spizaetus*, *Aquila* and *Hieraaetus* as currently defined are paraphyletic groups (Helbig et al., 2005; Lerner and Mindell, 2005; Griffiths et al., 2007; Haring et al., 2007) and, accordingly, several species mentioned in this article should be renamed, a thorough taxonomic revision comprising all representatives of this subfamily is still lacking. Thus, we follow Dickinson (2003) and use the conventional names in this article (also concerning orders, families and subfamilies).

RESULTS

Gene order of CR, ΨCR and flanking genes of H. fasciatus

From three samples of *H. fasciatus* (1360, HFA and SA2), the complete CR was amplified using primers that bind in the flanking tRNA genes (PCR fragment f-CR-L in Fig. 1). From two additional samples (HFC, PD1), the complete CR was obtained by isolating two overlapping fragments (PCR fragment f-CR-5′, f-CR-3′). Successful amplification of the whole ΨCR (f-ΨCR) was only achieved in one sample (HFC). This fragment includes regions flanking the ΨCR (5′ side: part of *nd6* and $tRNA^{Glu}$; 3′ side: $tRNA^{Phe}$ and part of *12S rRNA*). The sequence analysis confirmed that in *H. fasciatus* the CR is flanked by the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes, whereas the ΨCR is located between the $tRNA^{Glu}$ and $tRNA^{Phe}$ genes. To investigate if the mt gene order in the CR/ΨCR section of *H. fasciatus* is the same as in other birds of prey (e.g., Mindell et al., '98; Haring et al., '99, 2001; Roques et al., 2004; Nittinger et al., 2005; Gibb et al., 2007), we amplified the interjacent sequence (PCR fragment f-nd6). The expected PCR product of 688 bp was obtained from all five individuals. From one sample (HFC) the respective fragment was sequenced and thus the complete sequence spanning from $tRNA^{Thr}$ to *12S* was determined in this individual. This sequence was used for all subsequent analyses.

Structure of the CR and ΨCR

The complete CR of *H. fasciatus* (sample HFC) is 1,158 bp long. The usually distinguished three domains (DI–DIII) of the CR were recognized in *H. fasciatus* (Fig. 2). To identify previously described conserved sequence blocks in the CR of *H. fasciatus*, we aligned the sequence with those of two other accipitrid species: *S. nipalensis* (Asai et al., 2006) and *Ac. gentilis* (this study), which is shown in the electronic supplement (ES.1). The alignment was readily achieved for DII. However, in parts of DI and DIII it proved difficult to accomplish because of length variation owing to repeats, which are located at different positions and vary in length, sequence and number. Furthermore, we constructed an alignment of the conserved sequence boxes described by Randi and Lucchini ('98) and detected in *H. fasciatus* (ETAS1, F box, E box, D box, C box, CSBa, CSBb, CSB1), using several raptor species as well as *C. ciconia* and *A. barbara*. We also included the mammalian consensus sequence (Sbisà et al., '97). The alignment and a detailed description of sequence boxes are given in the electronic supplement (ES.2). The existence of various repeats has been reported for the DIII of birds (see also Table 6 in Haring et al., 2001). However, *H. fasciatus* lacks long tandem-repetitive sequences present in other species (Wenik et al., '94; Yamamoto et al., 2000; Haring et al., 2001; Roques et al., 2004). It only harbors two kinds of smaller repeats: one comprises five imperfect repeats of unequal length (9–11 bp) in a tandem array, and the other is a perfect repeat of 13 bp present in two copies, one upstream and one downstream of CSB3 (Fig. 2).

The general structure of the ΨCR of *H. fasciatus* (Fig. 2) is similar to that found in other genera of

birds of prey, such as *Falco* (Mindell et al., '98; Nittinger et al., 2005), *Buteo* (Haring et al., '99), *Aquila* (Väli, 2002), *Spizaetus* (Asai et al., 2006) and *Accipiter* (this study). It starts with a 5′ nonrepetitive part (nr-ΨCR, 882 bp) followed by a cluster of tandem repeats, each 49 bp in length (r-ΨCR). Concerning the r-ΨCR, it was not possible to span the whole cluster of tandem repeats within one sequence reaction (from the 3′ end of the nr-ΨCR to the 5′ end of the $tRNA^{Phe}$ gene). Only three repeat copies from the 5′ end and eight copies from the 3′ end were sequenced. The actual number of 21 tandem repeats was estimated from the size of the PCR fragment in an agarose gel. The first repeat (adjacent to the nr-ΨCR) differs from the following copies by one substitution. The last repeat (adjacent to the $tRNA^{Phe}$ gene) is incomplete (23 bp).

Homology between CR and ΨCR in H. fasciatus

An intragenomic alignment between the CR and the nonrepetitive part of the ΨCR of *H. fasciatus* revealed that a major part of the ΨCR (732 bp) is homologous to parts of the CR. The CR-homologous section extends from the middle part of DI to almost the end of DIII (Fig. 3) except for a stretch of 145 bp, which is missing in the ΨCR, probably as the result of a deletion (intervening section in ES.3). The 5′-section (119 bp) of the

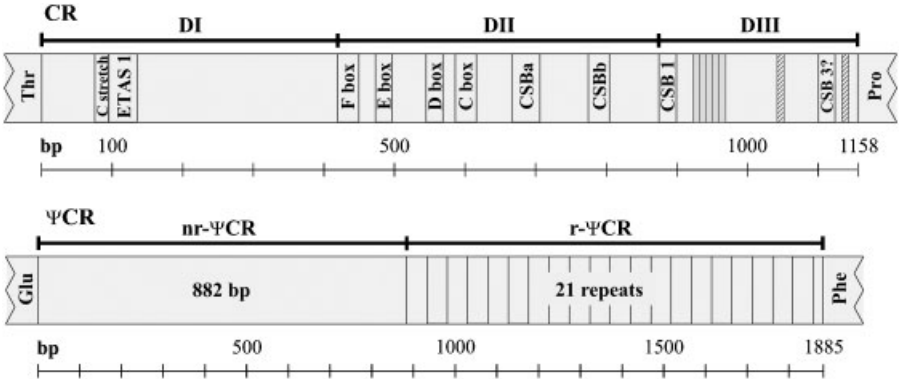

Fig. 2. Structure of CR and ΨCR in *H. fasciatus*. Positions of the conserved boxes and the division into the three domains DI, DII and DIII are shown. Repetitive sequences in DIII are depicted as dark gray and hatched bars. We considered DI to comprise the section between $tRNA^{Thr}$ and the first conserved sequence box (F box). We placed the boundary between DII and DIII upstream of the conserved sequence box CSB1, following Sbisà et al. ('97) and Roques et al. (2004). The nonrepetitive section (nr-ΨCR) is followed by a tandem-repetitive section (r-ΨCR) consisting of 49 bp repeat units. The length of the ΨCR is based on the number of repeats estimated from an agarose gel. CR, control region; ΨCR, pseudo-control region.

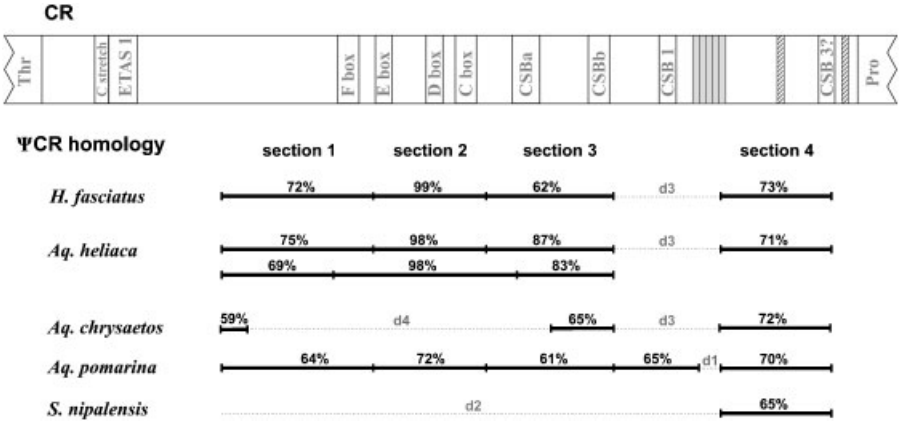

Fig. 3. Homology of various ΨCR sequences to CR sections found in *H. fasciatus* and other accipitrids. The bars indicate homologous sections of the ΨCR sequences of *H. fasciatus*, *Aq. heliaca*, *Aq. chrysaetos*, *Aq. pomarina* and *S. nipalensis*. Sequence similarity values (in %) refer to intragenomic comparisons between CR and ΨCR in the respective species. For *Aq. heliaca* the CR-homologous sections are shown as two bars. The upper bar displays the sections as determined in *H. fasciatus*, whereas the lower bar shows the central section homogenized in *Aq. heliaca* (extended section 2) and the remaining parts of sections 1 and 3. Presumed deletions (d1–d4) mentioned in the text are depicted as dotted lines. CR, control region; ΨCR, pseudo-control region.

ΨCR, upstream of the CR-homologous region, cannot be aligned unambiguously with the CR; nevertheless, the characteristic C-stretch motif can be recognized. In addition, in the nonrepetitive downstream region of the ΨCR (82 bp) no clear CR-homology is found. Within the homologous region sections with different similarity values become apparent (Fig. 3). In section 1 (length in ΨCR 204 bp) the similarity with the CR is 72% (each gap in the alignment was treated as one mismatch regardless of the gap size). The adjacent section 2 (159 bp) is almost identical (99.4%) to the corresponding CR sequence, differing only by one 1 bp deletion. This section includes the conserved boxes E, D and C. It is followed by section 3 (172 bp) with 62% similarity. Separated by a 145 bp intervening section not present in the ΨCR, the last homologous section (section 4; 146 bp) has 73% similarity. The boundaries of these sections relate to the situation found in *H. fasciatus* where section 2 proved to be almost identical between CR and ΨCR. The regions flanking this section were designated as 1 and 3, respectively. The 3′ end of section 3 is determined by the following 145 bp deletion. The region downstream of this deletion was designated as section 4 (see Fig. 3).

Inter- and intraspecific comparisons

Two almost identical CRs have been detected so far in *P. haliaetus*, which represents the monotypic family Pandionidae (Gibb et al., 2007). Thus, among raptors, *H. fasciatus* is the second case in which two CR regions with obvious intragenomic homology were found. To answer the question whether the two noncoding sections of *H. fasciatus* evolved independently or whether homogenization events may be assumed as in *P. haliaetus* (Gibb et al., 2007), we constructed a NJ tree based on sections 1–4 of both species. As an outgroup, we first tested the CR of *F. peregrinus*, which represents the family splitting off from the basal node in the phylogeny of Falconiformes. However, it turned out that this species—like, e.g., *Ac. gentilis* and *B. buteo*—possesses a highly derived CR that cannot be aligned unambiguously over the entire length. In a tree based on the more conserved section 2 only, the long branches of these three species illustrate the elevated substitution rates in their CR as compared with *Pandion*, *Hieraaetus* and *Spizaetus* (Fig. 4A). In contrast, the Japanese crane *G. japonensis* proved to possess a rather conserved CR compared with *P. haliaetus* and *H. fasciatus*, and was therefore used to root the trees (Figs. 4 and 5). Assuming the independent evolution of the two noncoding sequences after a duplication event in the common ancestor, we would expect two clearly separated clades, one joining the CRs and the other the ΨCRs of *P. haliaetus* and *H. fasciatus*, respectively. If homogenization had occurred only in the *Pandion* lineage, the CR of *H. fasciatus* should cluster with the two *Pandion* sequences. The tree (Fig. 4B), however, clearly shows intragenomic clustering of the two CR copies in both *P. haliaetus* and *H. fasciatus*. This indicates that in each species the duplicated CR sequences evolved in a concerted manner.

We also investigated other raptor species with respect to intragenomic (CR vs. ΨCR) as well as intergenomic (CR vs. CR and ΨCR vs. ΨCR) similarity. So far, the presence of two noncoding sequences in the mt genome has been published

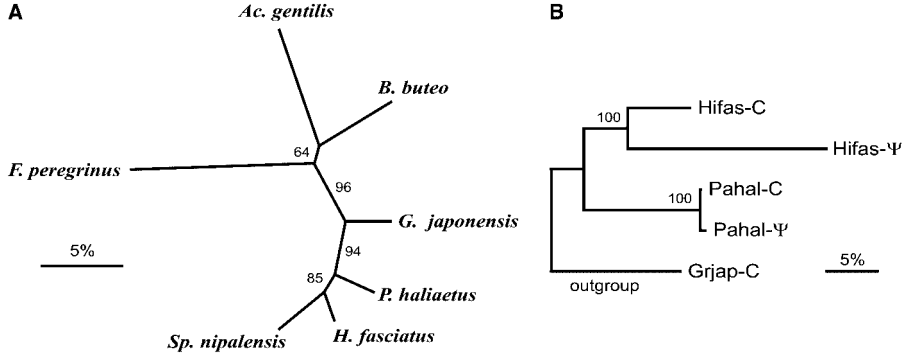

Fig. 4. (**A**) Unrooted NJ tree based on section 2 of the CR of several falconiform species as well as of *G. japonensis*. The long branches in the CRs of *F. peregrinus*, *Ac. gentilis* and *B. buteo* indicate elevated substitution rates. Therefore, these sequences were excluded from further phylogenetic reconstructions. (**B**) NJ tree based on CR and ΨCR sections 1–4 of *P. haliaetus* and *H. fasciatus*. *G. japonensis* was used as an outgroup. In each species CR and ΨCR cluster together indicating that the duplicated CR sequences were homogenized independently in both lineages. CR, control region; ΨCR, pseudo-control region.

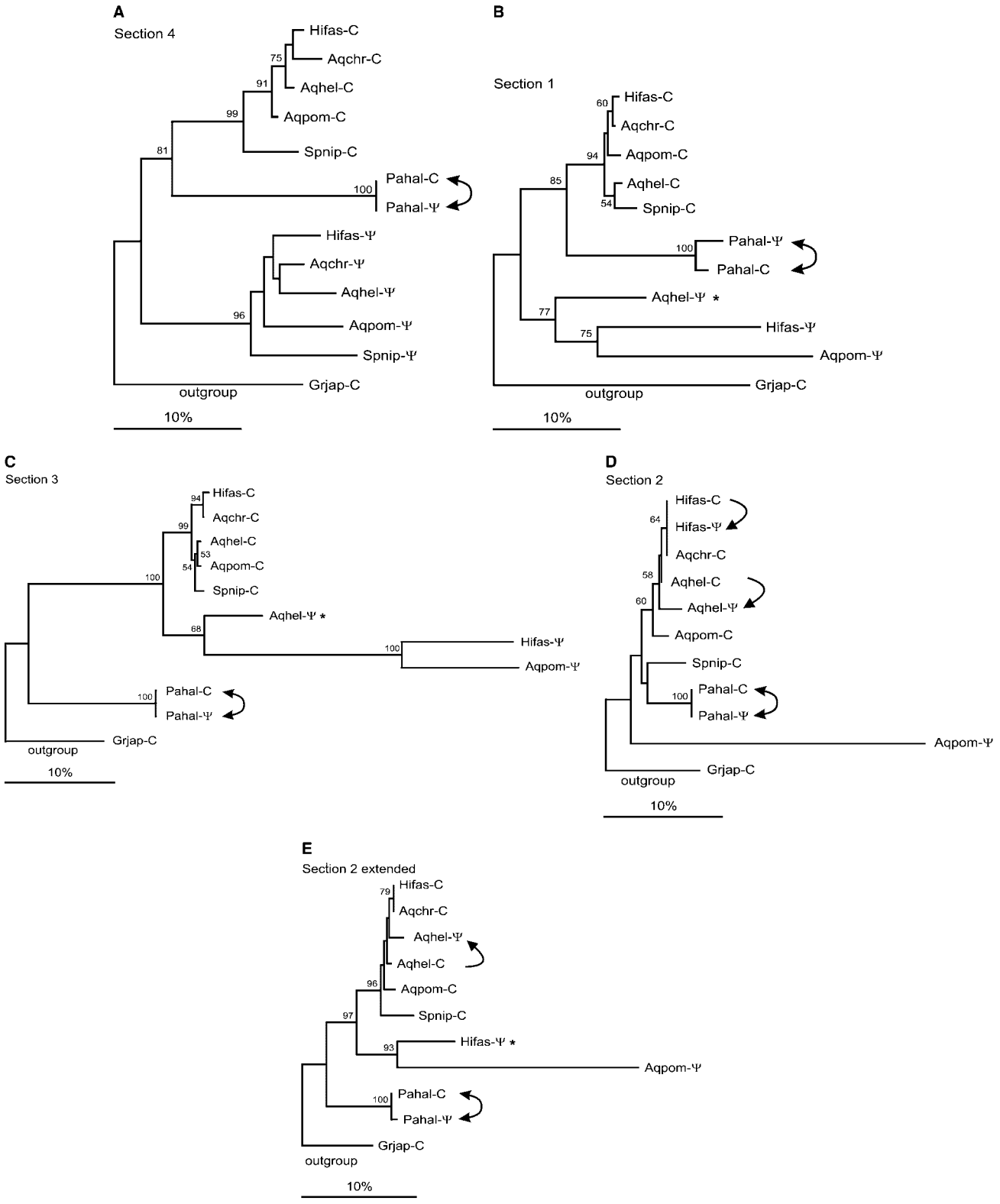

Fig. 5. NJ trees based on the four sections illustrating sequence similarities between sections of CR and ΨCR in Falconiformes: (**A**) section 4 (length of the alignment: 158 bp); (**B**) section 1 (157 bp); (**C**) section 3 (190 bp); (**D**) section 2 (160 bp); (**E**) extended section 2 (247 bp), corresponding to the homogenized part in *Aq. heliaca*. The ΨCRs of *Aq. chrysaetos* and *S. nipalensis* are found in the tree of section 4 only as in these species the other sections are deleted. Bootstrap values are indicated at the nodes. CR, control region; ΨCR, pseudo-control region.

for a few species only (*B. buteo*: Haring et al., 2001; *S. nipalensis*: Asai et al., 2006; *Ac. gentilis*: this study). In *Ac. gentilis* and *B. buteo* (which are members of the related subfamilies Accipitrinae and Buteoninae) no similarity between CR and ΨCR is found and no reasonable alignment was possible between their ΨCR and that of *H. fasciatus*. In *S. nipalensis* (like *H. fasciatus*, a member of the Aquilinae), however, a short region (147 bp) in the ΨCR corresponding to section 4 (Fig. 3) shows clear intragenomic similarity to the CR (although the overall sequence similarity of 63% is rather low owing to several gaps, the alignment is straightforward). In the interspecific comparison between *S. nipalensis* and *H. fasciatus*, the ΨCR sequences differed considerably in length (1,158 vs. 340 bp), but there are shared sections that turned out to be quite similar, especially in section 4 which showed 84% similarity. The 5′- and 3′-sections of the ΨCRs of both species (lacking CR-homology) can be aligned but are less conserved and more variable in length.

The genera *Hieraaetus* and *Spizaetus* belong to the subfamily Aquilinae, which forms a well-supported clade in the molecular phylogeny of Accipitridae established by Lerner and Mindell (2005). The similarity between CR and ΨCR found in *H. fasciatus* and *S. nipalensis* raised the question whether such an intragenomic homology is present in other Aquilinae species too. Several ΨCR sequences of species of the genus *Aquila* have been published (Väli, 2002) and the corresponding CR sequences were determined in this study. We selected three species representing three distinct lineages of the genus *Aquila* (*Aq. pomarina*, *Aq. heliaca* and *Aq. chrysaetos*) and aligned their CR and ΨCR sequences with those of *H. fasciatus* and *S. nipalensis*. Interestingly, in all three *Aquila* species we identified CR-homologous sections. They are also depicted in Figure 3. *Aq. pomarina* possesses the longest CR-homologous section that extends also into the region that is missing in the ΨCR of *H. fasciatus* (between sections 3 and 4). In the ΨCR of *Aq. chrysaetos* there is a large deletion with respect to the CR. After a short (38 bp) homologous region at the beginning of section 1, there is a big gap spanning to the middle of section 3. In the rest of section 3 as well as section 4 there is again homology to the CR. At first sight *Aq. heliaca* seems to have exactly the same CR-homologous sections as found in *H. fasciatus*. It shares also the same deletion between sections 3 and 4. Nevertheless, closer inspection of the alignment reveals a large section of high sequence similarity that includes the last 42 bp of section 1, the entire section 2 and 45 bp of section 3. Sections 1–4 of CR and ΨCR of *H. fasciatus*, *S. nipalensis,* and the four *Aquila* species are aligned in ES.1. The distance values between CR and ΨCR of the various species are shown in Figure 3. Whereas for most sections the similarity values are around 70%, exceptionally high values are found in section 2 of *H. fasciatus* (99.4%) and *Aq. heliaca* (97.5%). These different levels of similarity are illustrated by trees based on sections 1–4 (Fig. 5). The tree in Figure 4B showed that homogenization must have occurred in the lineage of *H. fasciatus*, however the trees based on the four sections illustrate that the pattern is much more complicated and several homogenization events have to be assumed. Some of the nodes in these trees are not well supported in the bootstrap analyses. Several factors may be responsible for this: the shortness of the sequences (especially section 2), differences in substitution rates and the effect of chimerical (homogenized/nonhomogenized) sections. Although a robust phylogeny may not be deduced from such short sequences, it should be emphasized that these trees should only serve to exemplify our hypotheses by illustrating the sequence similarities among sections and species.

In the tree based on section 4 (Fig. 5A) two clusters can be distinguished. One consists exclusively of ΨCR sequences and the other one of CR sequences, except for the ΨCR sequence of *P. haliaetus*, which is identical to its paralogous CR. This identity reflects the presumed recent homogenization of the two sequences in *P. haliaetus* (which comprises almost the entire CR; Gibb et al., 2007). With respect to the other sequences there is no indication for recent homogenization. Within each cluster (CRs and ΨCRs), the topology reflects roughly the phylogenetic relationships among the Aquilinae species. The tree based on section 1 (Fig. 5B) has a similar topology (CR and ΨCR clusters). As in Figure 5A the CR and ΨCR of *P. haliaetus* cluster closely, but the Aquilinae CR cluster is not clearly resolved. Section 1 of the ΨCR is missing in *Aq. chrysaetos* and *S. nipalensis* owing to partial or complete deletion of this region.

Figure 5C shows a tree based on section 3. In this case the CR and ΨCR sequences of Aquilinae are sister groups to the exclusion of CR and ΨCR of *P. haliaetus* (bootstrap support 100%). This can be interpreted as an indication for the

homogenization of the ΨCR in a common ancestor of Aquilinae. After this event the two noncoding sequences started again to diverge in the course of the cladification of the various species. It cannot be assessed whether this event happened before or after the split of *S. nipalensis* as in this species section 3 of the ΨCR is deleted and thus not available for comparison.

A different picture becomes apparent in the tree based on section 2 (Fig. 5D), where the ΨCR clade consists of one very long branch only, representing *Aq. pomarina*. The ΨCRs of *P. haliaetus*, *H. fasciatus* and *Aq. heliaca* are included in the CR clade, each being (almost) identical to its paralogous CR counterpart. Within this cluster the branches are short as section 2 contains functional motifs and thus is the most conserved region of the CR. The branching order is not clearly resolved as indicated by the low bootstrap values, which are most probably owing to the shortness of section 2 and the long branch leading to the ΨCR of *Aq. pomarina*. However, it is clearly visible that the ΨCR sections of *H. fasciatus* and *Aq. heliaca* are close to the CR sequences, whereas only the ΨCR of *Aq. pomarina* is highly differentiated. This pattern can be interpreted straightforward by two further homogenization events. One led to homogenization of section 2 in the lineage of *H. fasciatus*. The other took place in the lineage of *Aq. heliaca*. However, in *Aq. heliaca* the homogenized section comprises a larger region (247 bp) extending beyond section 2 at both sides (including 42 bp of section 1 and 45 bp of section 3; see Fig. 3, ES.3). In this section the similarity between ΨCR and CR of *Aq. heliaca* is 97.6%. In the tree based on this extended section 2 (Fig. 5E), the ΨCR of *Aq. heliaca* is still found in the CR cluster, whereas the ΨCR of *H. fasciatus* has shifted to the ΨCR clade. This is owing to the fact that this extended section 2 of *H. fasciatus* contains flanking sequences of 87 bp (42+45 bp), which have not been homogenized and thus are more similar to the ΨCR sequences.

The position of the ΨCR of *Aq. heliaca* in the trees based on sections 1 and 3, respectively, can also be explained by the large homogenized central section in this species. The last 42 bp of section 1 (length: 157 bp) and the first 45 bp of section 3 (length: 189 bp) are part of the homogenized region in *Aq. heliaca* and thus almost identical to the CR. Thus, in *Aq. heliaca* sections 1 and 3 of the ΨCR are composites of homogenized and nonhomogenized parts resulting in rather short branches in the trees and low bootstrap values of the nodes defining the ΨCR clades.

DISCUSSION

We analyzed the organization and structure of the main regulatory region of the mt genome, the CR, along with another important noncoding mt section, the ΨCR, in *H. fasciatus*. Concerning the CR, birds of prey are similar in their content of conserved boxes and motifs, many of them shared with other birds or even with mammals, although sequence comparisons over a wide taxonomic range revealed that in several cases the flanking sections are more conserved than the classical boxes. This finding suggests that the selective constraints might extend beyond the described boxes or that the functionally important positions have even shifted along the CR sequence. Regarding the ΨCR, the basic structure in *H. fasciatus* with a nonrepetitive part followed by a cluster of tandem repeats is the same as in the raptor species studied so far. The repetitive section seems to be a characteristic of the ΨCR of Falconiformes as no repetitive region is found in the ΨCR of other bird species (e.g., *Smithornis*: Mindell et al., '98; *Phylloscopus*: Bensch and Härlid, 2000).

Origin of CR and ΨCR in Falconiformes

It is highly probable that all falconiforms share the same gene order, as it is present in three distantly related families: Falconidae, Accipitridae and Pandionidae, i.e., in all representatives where the gene order has been determined so far (Mindell et al., '98; Haring et al., '99, 2001; Nittinger et al., 2005; Asai et al., 2006; Gibb et al., 2007). Within Accipitridae, sequence information of CR and ΨCR is available for representatives of the subfamilies Haliaeetinae (*Haliaeetus*), Buteoninae (*Buteo*), Aquilinae (*Hieraaetus*, *Aquila* and *Spizaetus*) and Accipitrinae (*Accipiter*). The fact that the CR in *N. percnopterus* and *G. barbatus* is flanked by $tRNA^{Thr}$ and $tRNA^{Pro}$ (Roques et al., 2004) indicates that also the subfamilies Gypaetinae and Aegypiinae may share the same gene order with the other birds of prey. Furthermore, with the exception of *P. haliaetus*, the ΨCR of all birds of prey studied so far is characterized by a structural peculiarity: a nonrepetitive part followed by a large cluster of tandem repeats. Thus, despite the fact that the same mt rearrangement obviously originated several times independently in various other groups of the avian order, its presence in Falconiformes is most parsimoniously explained by a single ancestral mutation event. The intragenomic similarity between CR and ΨCR observed in some

falconiform species strongly supports the hypothesis that the two noncoding sequences originated via duplication as proposed repeatedly in the past for various bird species (Moritz et al., '87; Desjardins and Morais, '90; Quinn, '97; Mindell et al., '98; Bensch and Härlid, 2000; Eberhard et al., 2001). The fact that a similar gene rearrangement was also detected in fish species of the order Anguilliformes (Inoue et al., 2001) implies that at least in vertebrates similar mechanisms might favor the generation of rearrangements in this mt region.

Maintained intragenomic similarity through homogenization

Gene conversion of a duplicated CR has been reported for parrots (Eberhard et al., 2001) and albatrosses (Abbott et al., 2005) as well as several nonavian species (e.g., reptiles: Kumazawa et al., '96; ticks of the familiy Ixodidae: Black and Roehrdanz, '98). Although the mechanisms are not understood yet, some studies imply that recombination (homologous as well as nonhomologous) does occur in animal mtDNA (for a review, see Rokas et al., 2003). Intramolecular recombination of mtDNA was shown to take place in vitro (Tang et al., 2000) and the ability of the CR to self-recombine was demonstrated by Lunt and Hyman ('97). As the mechanisms that lead to maintenance of sequence similarity between paralogous sections are still unclear we prefer the more general term ''homogenization.''

The results of *P. haliaetus* (Gibb et al., 2007) and the evidence provided in this study indicate that homogenization occurred in the Falconiformes too. It could be argued that the two almost identical CRs in *P. haliaetus* might have originated via a recent duplication instead of homogenization of formerly duplicated CRs. However, in the case of a recent tandem duplication event as observed, for instance, in albatrosses (Abbott et al., 2005), duplication of the flanking genes (*nd6*, $tRNA^{Thr}$, $tRNA^{Pro}$, $tRNA^{Glu}$) would also be expected, which is not the case in *P. haliaetus*. Trying to explain the situation found in *P. haliaetus* with a recent duplication would require the assumption of a replicative transposition into an eroded ΨCR (the assumption of an ancestral duplication of the whole region is required to explain the positions of the *nd6* and *tRNA* genes; see Gibb et al. 2007). The result would be the same, but up to now no evidence of such an event has been found in birds.

The fact that so far no homology between CR and ΨCR was found in other birds of prey has formerly been taken as support for the assumption that the duplication has occurred already at the base of Falconiformes. In the absence of a selective pressure conserving a second copy of the CR, it is expected that sequence degeneration should have blurred all traces of homology after such a long period of time. Although the exact dating is still disputed, there is general agreement that diversification of Falconiformes started in the late Cretaceous (Ericson et al., 2006; Pereira and Baker, 2006; Brown et al., 2007), relating to a period from 100 to 65 MYA. However, the sequence similarity between CR and ΨCR in *P. haliaetus*, *H. fasciatus* and the other Aquilinae seems to contradict the assumption of such an early divergence. Why should sequence similarity have remained preserved over such a long period only in some particular lineages? It is plausible that homogenization prevented sequence degeneration in some falconiform lineages, whereas in other lineages this process was stopped earlier, most probably through large deletions (see below). In *P. haliaetus* the two CRs apparently have been homogenized over the entire length rather recently. For the Aquilinae lineage we assume three homogenization events between CR and ΨCR, which occurred during the cladogenesis of this subfamily. Thus, we regard homogenization as the most likely explanation. Nevertheless, alternative explanations have to be considered.

It might be possible that the different levels of similarity could be the consequence of selective pressures acting differently on the various sections of the ΨCR. However, phylogenetic considerations clearly reject this idea: Assuming that after duplication in a common ancestor (giving rise to CR and ΨCR) both paralogues evolved independently without sporadic homogenization, the distances between CRs and ΨCRs in orthologous as well as metalogous comparisons among the extant species should be in the same range. This should be also true for comparisons of each of the respective sections (1–4). The observed values of sequence similarity do not support this possibility. Then, one might argue that the presence of two functional copies of the conserved sequence boxes, which are located within the highly conserved domain II of CRs (Randi and Lucchini, '98), would provide a selective advantage. In fact, section 2 is located within domain II and contains three of these boxes (Figs. 2 and 3). However, assuming selection as the only responsible force, e.g., the

observed >99% similarity of section 2 between the two paralogues in *H. fasciatus*, would require to propose a tremendous selective constraint after duplication, preventing substitutions only in section 2 and not in the other conserved boxes. Moreover, this constraint would act only in some species (e.g., *H. fasciatus*, *Aq. heliaca*), but not in other closely related species, as, e.g., in *Aq. pomarina*, where the corresponding similarity is only 72% (statistical significance: $\chi^2 = 48.2$, $df = 1$, $P < 0.001$). The fact that this section of the ΨCR is completely deleted in *S. nipalensis* and *Aq. chrysaetos* (a close relative of *H. fasciatus*) demonstrates a lack of functional constraint on section 2 in the ΨCR (or on the ΨCR in general) at least in these species. Thus, we consider it more plausible that repeated homogenization events at different times have generated the various levels of similarity found in intragenomic comparisons. However, we do not rule out the possibility that in some species selection might have played a role after a homogenization event, favoring the maintenance of two highly similar copies of section 2 within one genome. First, the mitochondria carrying two CR copies must outcompete the other mitochondria at the intracellular level; second, this new haplotype has to become fixed in the population. Both processes might be driven by positive selection, although this probably depends on particular circumstances where the possession of two CRs provides a selective advantage as the general evolutionary trend in mt genomes favors the elimination of redundant sequences.

Evolution of CR and ΨCR in Falconiformes

On the basis of the patterns of sequence similarities between the four sections (Fig. 5) and the presence/absence of large deletions (Fig. 3, ES.3), we propose a possible scenario for the evolution of CR and ΨCR within Falconiformes (Fig. 6). This tree is an illustration of the hypothesis outlined below, which we think is the most plausible one, although there may be alternative models. The order of deletion events visible in Figure 3 can be traced on a phylogenetic tree of Falconiformes (according to Lerner and Mindell, 2005) including those taxa where sequence information of both noncoding sequences is available. A small deletion (d1) of the region upstream of section 4 should have occurred already in the common ancestor of Aquilinae. Subsequently, the entire upstream region was deleted in the lineage of *S. nipalensis* (d2). Independently, another deletion (d3) occurred in the common ancestor of *H. fasciatus*, *Aq. heliaca* and *Aq. chrysaetos* (spanning the sequence between sections 3 and 4). Finally, in the lineage of *Aq. chrysaetos* a fourth deletion occurred in the anterior part of the CR-homologous section comprising parts of sections 1 and 3 as well as section 2 (d4).

Concerning the homogenization events, the phylogenetic analyses (Fig. 5) have to be considered in connection with sequence similarities. Similarity values observed between the CR and the ΨCR of the various species in sections 1 and 4 are quite low, mostly ranging between 61 and 75% (Fig. 3, ES.3). This is in accordance with the

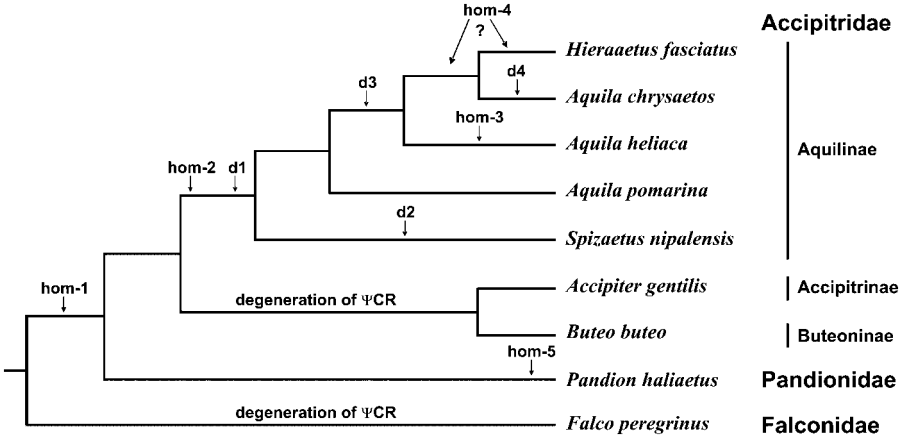

Fig. 6. Evolutionary scenario proposed for the CR and ΨCR in Falconiformes. The cladogram shows the phylogenetic relationships according to the tree of Lerner and Mindell (2005). Only those species from which sequence data of CR and ΨCR are available are shown. hom, homogenization event; d, deletion in the ΨCR; CR, control region; ΨCR, pseudo-control region.

assumed independent evolution of CR and ΨCR in these regions as exemplified in the trees based on sections 4 and 1 (Fig. 5A and B). The separated CR and ΨCR clusters are expected if independent divergence after an initial duplication in an early stage of falconiform evolution is assumed. Although it is clear that no recent homogenization event occurred in these sections, it is possible that homogenization happened earlier in a common ancestor before the split of *Pandionidae* (hom-1). Another homogenization has to be assumed in a common ancestor of Aquilinae as suggested by the tree based on section 3 (hom-2). The high similarity values found in section 2 of *H. fasciatus* and *Aq. heliaca* have to be ascribed to additional, more recent homogenizations. The fact that the two homogenized sections have different boundaries indicates that two independent events (hom-3, hom-4) took place in the lineages of *H. fasciatus* (section 2) and *Aq. heliaca* (extended section 2), respectively. Finally, the most recent homogenization occurred in *P. haliaetus* (hom-5).

Although it is not possible to detect precisely all incidents of homogenization and deletion, the information we have so far enables us to trace the evolutionary events that have occurred in this part of the mt CR in much more detail than it was previously possible. It seems that homogenization may easily occur as long as there is sufficient sequence similarity between the CR and the ΨCR. Moreover, homogenization itself allows perpetuation of this continued equalization. A major factor that may stop this process is obviously deletion. Large deletions (e.g., as those found in *S. nipalensis*) destroy homology and bring the process to a halt. In such a lineage a ''point of no return'' is reached and CR and ΨCR may further diverge independently. This point was obviously reached in the Falconidae and in some Accipitridae, as in these species the ΨCR is rather short and homology to the CR can no longer be detected (e.g., subfamilies Buteoninae, Accipitrinae).

CR and ΨCR as molecular markers

Our results have also consequences with respect to phylogenetic analyses. The ΨCR as a molecular marker for phylogenetic and population genetic studies was considered as extremely useful because of its high substitution rate (Väli, 2002; Riesing et al., 2003; Kruckenhauser et al., 2004). In the phylogenetic analyses of the genus *Buteo* (Riesing et al., 2003; Kruckenhauser et al., 2004), the results based on ΨCR sequences are in accordance with those from another mt marker sequence (*nd6* gene) and the absence of intragenomic homology with the CR (as in the other members of Buteoninae) allows the conclusion that the ΨCR trees are based on orthologous sequence comparisons. In a ΨCR-based phylogenetic tree of five *Aquila* species presented by Väli (2002), only those sections were analyzed, which are present in all the investigated species. Therefore, owing to the large deletion in *Aq. chrysaetos*, the section involved in recent homogenizations detected in our study was not included. Nevertheless, for future analyses the danger of metalogous comparisons should be considered, especially when the sequence relationships between CR and ΨCR and possible intragenomic exchanges are not known.

CONCLUSIONS

Sequence comparisons of CR and ΨCR in *H. fasciatus* and other birds of prey support the assumption that the mt gene order in Falconiformes originated from a duplication of a section containing the CR. In most representatives of this bird group, the homology is no longer detectable. In the lineages of Pandionidae and Aquilinae, however, the process of sequence degeneration was slowed down by homogenization events retaining the sequence similarity at least in some sections. Further research on this topic is needed to clarify the complex sequence evolution in this region of the mt genome in more detail. In particular, additional sequence data of the CR from additional representatives of the Aquilinae (e.g., genera *Hieraaetus*, *Polemaetus*, *Spizaetus*) as well as Sagitariidae and other subfamilies of Accipitridae are required. Tracing the effects of mtDNA recombination by sequence comparisons can, on the one hand, provide further insights into these evolutionary processes and, on the other hand, have important implications for phylogenetic studies.

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