LETTER

Bird Mitochondrial Gene Order: Insight from 3 Warbler Mitochondrial Genomes

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Two main gene orders exist in birds: the ancestral gene order and the remnant control region (CR) 2 gene order. These gene orders differ by the presence of 1 or 2 copies of the CR, respectively. Among songbirds, Oscines were thought to follow the ancestral gene order, with the exception of the lyrebird and *Phylloscopus* warblers. Here, we determined the complete mitochondrial genome sequence of 3 non-*Phylloscopus* warblers species and found that the blackcap (*Sylvia atricapilla*) and the reed warbler (*Acrocephalus scirpaceus*) have 2 almost identical copies of the CR, whereas the eastern orphean warbler (*Sylvia crassirostris*) follows the remnant CR 2 gene order. Our results contradict previous studies suggesting that *Acrocephalus* and most sylvioid warblers exhibit the ancestral gene order. We were able to trace this contradiction to a misidentification of gene order from polymerase chain reaction length determination. We thus suggest that passerine gene order evolution needs to be revised.

Introduction

Mitochondrial gene rearrangements are considered to be rare evolutionary events, and as such the existence of a shared derived gene order between taxa is often indicative of a common ancestry (Boore 1999). Mindell et al. (1998) have shown that, in birds, 2 gene orders exist. By mapping these 2 gene orders on the avian phylogenetic tree, they have shown that one of these gene orders is ancestral (fig. 1*A*), whereas the second (fig. 1*D*) is a derived form, which evolved independently more than once. It is assumed that the derived gene order evolved from the ancestral one by a tandem duplication of the T/P/ND6/E/CR region (fig. 1*B*), followed by a deletion of the P_1/$ND6_1$/E_1 and T_2 regions (Gibb et al. 2007). This resulted in a duplicated control region (CR) (fig. 1*C*; duplicated CR gene order). In the derived form, the 5′ region of the CR_2 region is deleted as well. Thus, CR_2 in the derived gene order is a noncoding (NC) region and hence this gene order is termed the remnant CR_2 gene order (fig. 1*D*). This hypothesis is supported by the discovery of a duplicated $tRNA^{Thr}$–CR gene order in albatrosses (Abbott et al. 2005; Gibb et al. 2007) as well as by an intermediate gene order found in parrots, in which degenerate copies of the ND6 and $tRNA^{Glu}$ genes exist (Eberhard et al. 2001). Further support for this evolutionary scenario is provided by the discovery of the duplicated CR_2 gene order in osprey and aracari (Gibb et al. 2007). We note that the duplicated CR and the remnant CR_2 gene orders share, in fact, the same gene arrangement but differ in their length and functionality of the CR_2. However, we consider them as 2 different gene orders, following the nomenclature of Gibb et al. (2007).

The Passeriformes or songbirds form the largest bird order. It is traditionally divided into 2 major clades, the Oscines and the Suboscines, based on differences in the syringeal anatomy (Müller 1878). Debate exists, however, concerning the placement of the New Zealand wrens (Acanthisittidae), which have been considered to belong to a separate suborder (Ericson et al. 2002). In their comprehensive study of avian mitochondrial gene order, Mindell et al. (1998) concluded that "the alternative mitochondrial gene orders distinguish the 2 primary groups of songbirds." Analyzing gene orders in 106 Oscines and 9 Suboscines, they suggested that Oscines follow the ancestral gene order, whereas Suboscines follow the remnant CR_2 gene order. Exceptions to this rule have been described for 3 Oscine genera, *Menura* (lyrebird), *Phylloscopus*, and *Seicercus* (warblers) (Bensch and Härlid 2000; Slack et al. 2007), all of which were found to follow the remnant CR_2 gene order. Almost all these gene orders were determined based on polymerase chain reaction (PCR) fragment lengths. For example, Bensch and Härlid (2000) amplified a fragment from the center of the CR till the 5′ end of the 12S rRNA gene in order to determine gene orders in warblers. In this case, a short fragment indicates the presence of the ancestral gene order, whereas a long PCR fragment indicates the remnant CR_2 gene order. Similarly, Mindell et al. (1998) amplified a fragment from the 5′ end of the ND6 gene to the center of the CR in order to detect gene orders in Passeridae.

In this study, we applied the PCR fragment length method in order to determine the mitochondrial gene order in 3 species of Passeriformes (songbirds). We amplified the same mitochondrial fragment as that used by Bensch and Härlid (2000), using similar primers (see Supplementary Material online). The PCR fragment lengths obtained are shown in figure 2. Because the distance between the center of the CR and the 5′ end of the 12S rRNA is short (~440 bp) in the reed warbler *Acrocephalus scirpaceus* and the blackcap *Sylvia atricapilla*, they are inferred to possess the ancestral gene order. This finding is in agreement with the conclusions of Bensch and Härlid (2000) who studied other *Sylvioidea* species and suggest that most of them follow the ancestral gene order. In contrast, the long fragment (~1,550 bp) obtained for the eastern orphean warbler *Sylvia crassirostris* suggests that it possesses the remnant CR_2 gene order. This result was highly surprising as *S. atricapilla* and *S. crassirostris* have different gene orders, despite being closely related species that belong to the same genera.

Key words: *Sylvioidea*, *Sylvia*, *Acrocephalus*, complete mitochondrial genome, control region, concerted evolution, gene duplication.

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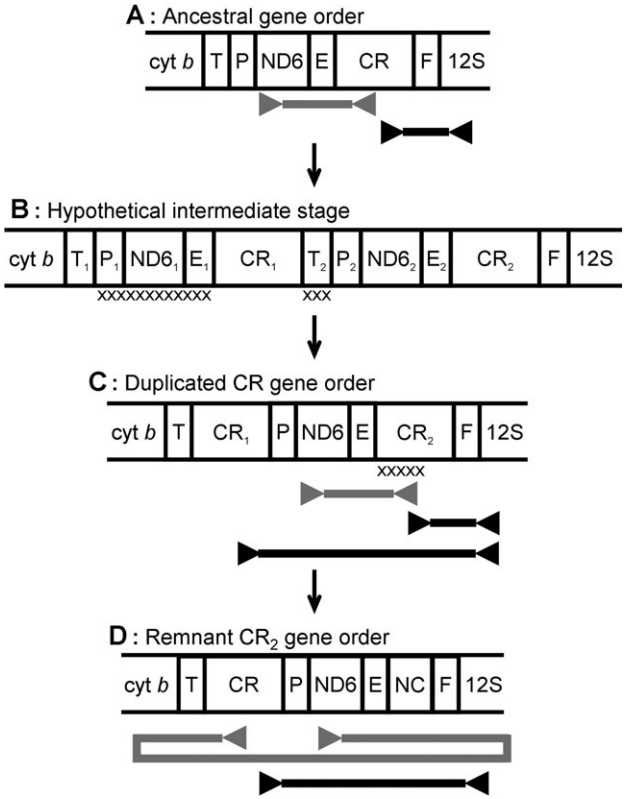

FIG. 1.—Avian mitochondrial gene orders. Arrows indicate putative scenario of gene order evolution. Gene order names follow Gibb et al. (2007). Xs indicate putative deleted regions between 2 gene orders. Triangles and bars indicate the location of primers and the region amplified for gene order determination in Passerines. The PCR fragments amplified by Mindell et al. (1998) and Bensch and Härlid (2000) are indicated in gray and black, respectively. For the duplicated CR gene order, 2 fragments are expected when using the primers of Bensch and Härlid (2000). However, the shortest fragment is always preferably amplified by PCR. Consequently, similar PCR fragment lengths are expected for the ancestral gene order and the duplicated CR gene order.

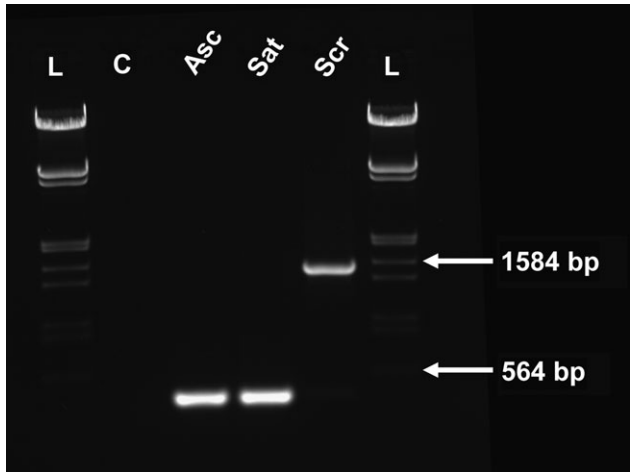

FIG. 2.—Gel photo of PCR amplification of the region from the center of the CR until the 5′ end of the 12S rRNA gene. L, size marker (*Hind*III/*Eco*RI digested lambda DNA); C, negative PCR control; Asc, *Acrocephalus scirpaceus*; Sat, *Sylvia atricapilla*; and Scr, *Sylvia crassirostris*.

To confirm this surprising result, we sequenced the complete mitochondrial genomes of the 3 above-mentioned species (detailed methods are provided as Supplementary Material online). The complete mitochondrial genome sequences have been deposited in the EMBL nucleotide database under accession numbers (AM889139–AM889141). Unexpectedly, the complete mitochondrial genome sequences did not confirm the gene order inferred based on PCR amplification. It transpired that *A. scirpaceus* and *S. atricapilla* have neither the ancestral gene order nor the remnant CR_2 gene order; but, rather, they have the duplicated CR gene order, in which the 2 CRs share a high level of sequence similarity (table 1). It is this duplicated CR region that misled the PCR fragment length analysis: 2 fragments should have been amplified, a long and a short one. Because shorter fragments are favorably amplified during the PCR, the same fragment lengths are obtained for both the duplicated CR gene order and the ancestral gene order (fig. 1). We note that amplifying the fragments with different *Taq* polymerases can produce faint bands for the long fragments. Nevertheless, these bands can only be obtained with some polymerases and can easily be overlooked (see Supplementary Material online for more details). Thus, the difference between the gene orders of *S. atricapilla* and *S. crassirostris* is now simpler to explain: It is easier to derive the remnant CR_2 gene order from the duplicated CR gene order than from the ancestral gene order.

In *A. scirpaceus*, the CR_1 and CR_2 sequences were identical for over 1,000 bp (only the first ~100 bp of both CRs and the last ~100 bp that are present only in the CR_2 were not identical; table 1, and sequence alignment provided in Supplementary Material online). This pattern was also found for *S. atricapilla* (table 1). This high level of similarity suggests either a recent and independent duplication of the CR in both species or a concerted evolution in both species. The latter explanation is more parsimonious and was also suggested and discussed by Eberhard et al. (2001), Abbott et al. (2005), and Gibb et al. (2007). Assuming that the CR duplication occurred before the divergence of the *Acrocephalus* and *Sylvia* genera, the remnant CR_2 gene order in *S. crassirostris* is inferred to be a derived state. It is reasonable to assume that, in this species, the duplicated CR_2 accumulated numerous substitutions and deletions, which prohibited concerted evolution and thus transformed CR_2 into an NC region.

Bensch and Härlid (2000) studied mitochondrial gene order in *Sylvioidea*. Analyzing PCR fragment lengths, they determined the gene order in 11 genera and suggested that 2 of these genera (*Phylloscopus* and *Seicercus*) follow the remnant CR_2 gene order. In this work, we show that the fragment length method they used to determine gene order was probably erroneous. Our data, combined with the data of Bensch and Härlid (2000), clearly show that representatives of 3 distant *Sylvioidea* families (Phylloscopidae, Acrocephalidae, and Timaliidae) possess either the duplicated CR gene order or the remnant CR_2 gene order (Supplementary Material online). It is thus most parsimonious to assume that the duplication of the CR occurred early on in *Sylvioidea* evolution and that both copies of the CR evolved via concerted evolution in most species. Interestingly, the

Table 1
***P* Distance between the CRs of Asc, Sat, and Scr**

	Asc (CR_1)	Asc (CR_2)	Sat (CR_1)	Sat (CR_2)	Scr (CR_1)
Asc (CR_2) (1,066 bp)	**0.000**				
Sat (CR_1) (1,071 bp)	0.247	0.247			
Sat (CR_2) (1,071 bp)	0.247	0.247	**0.000**		
Scr (CR_1) (1,072 bp)	0.271	0.271	0.175	0.175	
Scr (NC) (436 bp)	0.504	0.504	0.460	0.460	**0.451**

NOTE.—The first ~100 bp of both CRs and the last ~100 bp that are present only in the CR_2 were removed from the comparison. The number of base pairs involved in the computations is indicated for each sequence. Asc, *Acrocephalus scirpaceus*; Sat, *Sylvia atricatilla*; and Scr, *Sylvia crassirotris*. Distances between the two CRs within each species are indicated in bold.

fact that both *Sylvia* species have a different gene order suggests that once concerted evolution stops, the divergence between the 2 CRs dramatically increases (table 1). To conclude, our study suggests that the gene order needs to be reassigned in *Sylvioidea* and possibly in all Passerines.

Supplementary Material

Detailed Materials and Methods and Supplementary analyses are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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