Conflicting Character Distribution within Different Data Sets on Cardueline Finches: Artifact or History?

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Cardueline finches (Passeriformes: Fringillidae, Carduelinae) provide an example of unresolved species relationships despite decades of extensive study of the group. Existing morphological studies suffer from numerous cases of assumed parallel evolution due to a conflicting character distribution in different lineages. In this study, results of cytochrome b sequence analysis are compared with species relationships suggested by morphological and behavioral evidence. In the molecular analyses, species clusters mutually excluding each other were observed, lowering the statistical support of the internodes, i.e., the branches could not be resolved convincingly. Despite these difficulties, some phylogenetic signal was present in the molecular data as well as in the other approaches. In particular, any species or genus relationship suggested by cytochrome b sequence analysis was reflected by some other evidence. Based on this general congruence of the different data sets and on a considerable cytochrome b tree stability observed independent of species combination, choice of outgroup and tree-generating method, the short internodes are interpreted to reflect a historical reality. A model of cardueline evolution is proposed which assumes a population of cardueline ancestors with considerable polymorphism concerning the mitochondrial DNA and morphological characters alike. Retention of ancestral character states in different lineages and a subsequent rapid radiation are suggested to explain the conflicting character distributions observed in different fields of investigation.

Introduction

While some phylogenetic relationships can be settled and become a consensus view of the majority of taxonomists, others seem to resist convincing resolution independent of the method of analysis. In view of various aspects, cardueline finches (Passeriformes: Fringillidae, Carduelinae) provide an example for both. On the one hand, they are commonly recognized as a well-defined and closely related group (Marten and Johnson 1986), which is also reflected by their extensive cross-breeding ability (Panov 1989; Fehrer 1993). On the other hand, the relationships of their species and genera remain unclear (Björklund and Merilä 1993), although they have been well studied in various ways. In particular, the relationships of the Old World species are poorly understood and in need of further investigation (Marten and Johnson 1986). Morphological and behavioral studies on carduelines include: Tordoff (1954), Hinde (1956), Niccoli (1959), Ziswiler (1965, 1967), Wolters (1967), Güttinger (1978), Raikow (1978), Zusi (1978), van den Elzen (1985), van den Elzen and Nemeschkal (1991). In addition, there are serological comparisons (e.g., Mainardi 1957), two studies of allozyme variability (Marten and Johnson 1986; Stempel 1987), and a DNA–DNA hybridization study (Bledsoe 1988). A major obstacle to phylogenetic resolution seems to be the parallel evolution of many characters, i.e., a distribution of characters that leads to conflicting or mutually excluding species clusters, which is generally interpreted in terms of parallel gains and losses of those characters. While some “parallelisms” can be related to adaptive constraints, e.g., feeding specializations, others cannot easily be explained. Dealing with these difficulties, the question can be raised whether cardueline relationships can be elucidated by further data or whether this might be a general problem reflecting part of the concrete history of this group. To address this question, a molecular- genetic approach was performed using mitochondrial DNA sequences as a data set independent of previous studies.

Because of its high variability and comparably easy amplification with conserved polymerase chain reaction (PCR) primers (Kocher et al. 1989), the mitochondrial cytochrome b gene has proven to be particularly useful in phylogenetic studies at or near the genus level in recent years (e.g., Edwards and Wilson 1990; Meyer et al. 1990; Arctander 1991; Smith et al. 1991; Ritt et al. 1992; Richman and Price 1992; Helm-Bychowski and Cracraft 1993; Kornegay et al. 1993; Wayne 1993; Årason and Gullberg 1994). A 307-bp segment of the cytochrome b gene from eight cardueline and four non-cardueline species was sequenced and analyzed by different tree-constructing methods. The cardueline taxa were chosen according to a gradation of similarity and presumed relationships (i.e., different individuals of the same species, congeneric species, representatives of similar and dissimilar genera). Outgroup taxa ranging from the neighboring fringilline subfamily and other

Key words: cardueline finches, speciation, molecular systematics, mitochondrial DNA, cytochrome b, birds, phylogenetic analysis, evolution.

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finch families to an insect-eating songbird were included.

The aims of this study are (1) to compare the pattern of sequence divergence with cardueline relationships presumed by previous studies and (2) to find an explanation for the contradictory character distributions leading to difficulty in resolving species relationships.

Materials and Methods

Taxa Examined

Eight cardueline and four non-cardueline taxa were examined: a wild and two domestic Canaries (Serinus canaria), a Serin (Serinus serinus), a Yellow-fronted Canary (Serinus mozambicus), a Goldfinch (Carduelis carduelis), two Greenfinches (Carduelis chloris), a Redpoll (Acanthis flammea), a Common Bullfinch (Pyrrhula pyrrhula), a Hawfinch (Coccothraustes coccothraustes), two Chaffinches (Fringilla coelebs; Fringillinae), two Yellowhammer (Emberiza citrinella; Emberizidae), a Paradise Whydah (Vidua paradisaea; Viduidae), and two European Robins (Erithacus rubecula; Muscicapidae). The nomenclature of the species names is according to Peter's check-list (Mayr and Paynter 1964; Paynter 1968, 1970).

Sampling and DNA Isolation

Small drops of blood were obtained by puncture of the wing vein and stored in isotonic solution containing citrate to prevent coagulation or, alternatively, in 50% ethanol. Tissue samples (liver or muscle) were cut into small pieces and stored in 0.5 M EDTA or 50% ethanol. Cells were washed and lysed by detergent (Kawasaki 1990); proteins were removed by Proteinase K and subsequent salt/chloroform extraction (Müllenbach, Lago-da, and Welter 1989). Total genomic DNA was ethanol-precipitated and dissolved in water. The quality of the isolated DNA was examined on an agarose gel. A few nanograms of genomic DNA were used for PCR.

PCR, Cloning, and Sequencing

A 307-bp segment of the mitochondrial cytochrome \( b \) gene corresponding to positions 14991–15297 of the chicken sequence (Desjardins and Morais 1990) was amplified using standard primers and standard reaction conditions (Kocher et al. 1989). The quality and quantity of the amplified fragment were estimated by comparison with a DNA standard. Without purification, PCR products were blunted, phosphorylated, and cloned into the Smal-site of pUC18 using equimolar amounts of PCR product and vector. Transformation was performed in the strain DH5\( \alpha \) of Escherichia coli (Gibco BRL, Md). Recombinant clones were determined by blue-white-screening of the bacterial colonies and restriction endonuclease digestion of the isolated plasmids. Sequencing was done by the dideoxy chain-termination method with the Sequenase system (USB, Cleveland). Both strands were sequenced completely using standard primers against the vector sequence as well as the PCR primers. Three independent clones from each individual were sequenced unless the first two clones were identical.

Sequence Analysis

Since there seems to be no unequivocal superiority of a single method of phylogenetic analysis (Saitou 1988; Cracraft and Helm-Bychowski 1991; Sidow 1994; Takezaki and Nei 1994), three major tree-constructing methods (parsimony, distance matrix, and maximum likelihood [ML]) were employed (PHYLIP package, version 3.5, Felsenstein 1993). Because overall variability was low, i.e., below the 15%–20% limit where transitions at third codon positions tend to reach saturation (Meyer 1994), and most of the transitions belong to the categories that are known to saturate most quickly, i.e., third codon positions and first codon positions of leucine codons (Irwin, Kocher, and Wilson 1991), all substitutions were included in the different tree-building analyses. In particular, the exclusion of transitions at third codon positions can be misleading when closely related species are investigated (Irwin, Kocher, and Wilson 1991). Furthermore, transition/transversion differences did not suggest that transitions were approaching saturation. All positions were given equal weight, because different weighting also is only recommended with higher evolutionary rates (Hillis, Huelsenbeck, and Cunningham 1994). Analyses were performed using F. coelebs, E. citrinella and V. paradisaea as outgroup taxa, separately as well as in every possible combination. Additionally, the two Emberiza specimens were included with F. coelebs and V. paradisaea. Erithacus rubecula was only tentatively used because of its extremely biased base composition (table 1) and its low transition/transversion ratio (table 2), either as the only outgroup species or in combination with the three other non-carduelines. In every analysis, the input order of the species was randomized.

The results of bootstrap analyses (Felsenstein 1985) are represented by majority rule consensus trees. Additionally, branches that were not included in the consensus tree but that were similarly supported are considered for evaluation of the variability pattern. One hundred or 1,000 bootstrap replicates were performed in every analysis. Replicate number hardly influenced the bootstrap percentages, neither of the branches included in the majority rule consensus tree, nor of those not included, indicating that fewer replicate numbers give roughly the same picture. A single parsimony analysis including all sequences except that of E. rubecula was performed with 10,000 replicates. Since the bootstrap resampling
methodology has recently met with some criticism (Klug and Wolf 1993), it is used here solely as an index of support for certain groups and branching patterns.

Parsimony Analyses

Because the parsimony program of PHYLIP (DNAPARS) performs a heuristic search and therefore does not guarantee finding the most parsimonious solution, the chance of ending up in a local optimum was substantially decreased by running the program 30–100 times with randomized input order of the species. When several equally parsimonious trees were found, a majority rule consensus tree was built, but the particular nature of the alternative branchings was examined. To rule out the possibility of the tree topology depending strongly on the particular taxa included, parsimony analyses were done (100 bootstrap replicates each) with every single cardueline species sequentially omitted to assess the stability of the branching pattern.

Distance Analyses

Distances were calculated according to the Kimura two-parameter model. Other distance-calculating models (i.e., Jukes-Cantor and ML) gave very similar distances for every branch independent of the transition/transversion ratio assumed (varied between 2 and 20 according to the observed range), and the choice of model did not affect the topology of the tree. Although the Kimura two-parameter model presumes equal base frequencies, an assumption which does not hold for the data examined (see table 1), the distances are not substantially different from those obtained by ML estimates that use empirical base frequencies. Pairwise distances were analyzed by the Neighbor-joining (NJ) and Fitch algorithms of the PHYLIP package. Considerations of the branch lengths as well as a different topology obtained under the assumption of a molecular clock (KITSCH program) suggested that this strong assumption is inappropriate for the present data set.

ML Analyses

ML analyses (DNAML routine in PHYLIP) were run 30 times with randomized input order of the species using empirical base frequencies. In bootstrap analyses, only 100 replicates were performed because of the immense computational effort.

Results

Nucleotide Distribution

The nucleotide distribution pattern of the cytochrome b segment of the birds under study (table 1) was very similar to previous analyses of the entire cytochrome b gene in birds and mammals (Irwin, Kocher, and Wilson 1991; Kornegay et al. 1993) indicating that the 307-bp segment can be considered representative for the entire gene. At the first codon position, the four bases were equally distributed, at the second position, fewer G residues and a higher amount of T are seen. At the most variable third codon position, the bias against G and T was as strong as previously found for other bird species (Edwards, Arctander, and Wilson 1991; Birt et al. 1992; Kornegay et al. 1993). There was also a high preference of C over A up to the double percentage for C. The strongest bias so far observed was in the third codon position of the European Robin (Erithacus rubecula) with no G at all (out of 103 codons) and addi-
The reading frame starts with the second nucleotide.)

The asterisk marks an amino acid residue uncommon for birds but occurring in most other metazoan taxa studied so far. Abbreviations

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Within-species variability of the cytochrome b segment was very low for the species examined: The Yellowhammers (Emberiza citrinella) stemming from the same location, but from different years (1980 and 1988, respectively), differed by a single nucleotide. The two specimens of Greenfinches, Chaffinches, and European Robins from locations up to 500 km apart had identical sequences. A wild and a domestic Canary (a yellow bird from the stock of the Univ. of Kaiserslautern) also showed no difference. They had a single substitution compared to an English Yorkshire Canary, a race with marked morphological variation (wild and domestic stock: about 20 g, 13.5 cm; Yorkshire: 29 g, 19.5 cm).

These examples as well as observations on bird mitochondrial DNA in general (average of 0.3% intraspecific sequence divergence; Avise and Ball 1991) suggested that within-species variability was low enough that it was unlikely to interfere with interspecific comparisons. Sequence divergence between cardueline species was 7.5% on average (±2.5, range of 2.3%-11.4%) with an average transition/transversion ratio of 8.2 (±5.7, range of 2.0-23.0). Transversions increased linearly with over-

tionally very low T content, resulting in nearly two thirds of C and one third of A at this position. Because the bias in base composition of the sequences compared should be similar when phylogenetic analyses are to be performed, E. rubecula was not a suitable outgroup species for the present study.

**Cytochrome b Variability**

In the species studied, 97 of the 307 sites (nearly one third) of the cytochrome b gene segment (fig. 1) were variable (17/173 at 1./2./3. codon position, respectively) and 57 were “phylogenetically informative” (3/2/50 at 1./2./3. codon position, respectively) in the sense that a given base is shared by at least two sequences while two or more other sequences share another base at the same position. The majority of differences were silent substitutions as is expected for a protein-coding gene, particularly for close relatives, such as species within a genus (Kocher et al. 1989). Here, this finding held for all species under study even including the outgroup taxa, although it was more pronounced for the cardueline subfamily.

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### Figure 1

Sequence alignment of the cytochrome b segment for the taxa under study. Above the nucleotide sequences, the deduced amino acid sequence shared by *Serinus canaria*, *Serinus serinus*, *Serinus mozambicus*, *Carduelis carduelis*, and *Carduelis chloris* is given in the one-letter code. The asterisk marks an amino acid residue uncommon for birds but occurring in most other metazoan taxa studied so far. Abbreviations of the amino acids are placed above the second nucleotide of the corresponding codon. (The reading frame starts with the second nucleotide.)

The Canary nucleotide sequence is given in the next line; below, only differences to the Canary sequence are shown. A (2) after a species name indicates that this sequence is shared by two species. Below the sequences, the positions of three supposed membrane-spanning helices of the corresponding protein are shown by lines, the second of them is thought to contribute to heme-binding. All sequences have been deposited in the GenBank database under accession numbers U18853-U18866.

#### Table 1

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all variability (table 2). Also, transition numbers generally rose with increasing sequence divergence. Only the data for *E. rubecula* suggested that saturation of transitions had been reached (table 2), yielding further evidence to exclude this species for outgroup comparisons.

Most of the transitions fell into two categories, which are known to saturate most quickly: transitions at the third codon position (Meyer 1994) and transitions at the first codon position of leucine codons (Irwin, Kocher, and Wilson 1991). Especially among carduelines, few or even no other kinds of transitions occurred, so that these substitutions contribute most to the phylogenetic signal. According to properties of their cytochrome *b* sequences (overall variability, transition/transversion ratios, and the kinds of substitutions observed), cardueline species of different genera appeared to be even more closely related than species of the same genus in other bird groups (e.g., Edwards and Wilson 1990 [*Pomatosomatus*], Smith et al. 1991 [*Laniarius*], Birt et al. 1992 [*Amazona*], Richman and Price 1992 [*Phylloscopus*]).

### Amino Acid Sequences

The 102 amino acid residues derived from the 307 nucleotides of the cytochrome *b* gene cover a region of the protein which includes the first three of eight predicted membrane-spanning helices (Howell 1989; Beattie, Jenkins, and Howton 1994). This region represents the most conserved part of the protein which shows only little variation among species (degli Esposti et al. 1993). A maximum of four amino acid replacements was found among the cardueline species (table 2), one particular sequence was shared by *Serinus canaria*, *Serinus serinus*, *Serinus mozambicus*, *Carduelis carduelis*, and *Carduelis chloris* (fig. 1). Comparison of carduelines with outgroup taxa yielded 3-6 amino acid differences to *Fringilla coelebs* and *Emberiza citrinella*, 5-8 to *Eri-thacus rubecula*, and 8-10 to *Vidua paradisaea* (table 2). It is worth noting that *V. paradisaea*—although being a seed-eater normally placed in a finch family—displayed the most substitutional differences compared to all other “finches” under study, especially at the protein level, instead of the insect-eating *E. rubecula*.

Comparison of the deduced amino acid sequences with the cytochrome *b* of other metazoan taxa (degli Esposti et al. 1993) confirmed that the variability pattern of the sequences in the present study fitted well into that of the metazoan spectrum. The residues known as (almost) entirely unvariable across all taxa were the expected ones in the birds under study. Nevertheless, the N residue at position 71 (according to the yeast sequence [Nobrega and Tzagoloff 1980]) previously thought to be typical for birds (degli Esposti et al. 1993)
FIG. 2.—Unrooted trees obtained with the different methods. *a,b:* Parsimony analyses; *c,d:* distance analyses; *e–h:* maximum likelihood (ML) analyses. *a,e,f:* Summarized branching patterns of the majority rule consensus trees obtained with different combinations of outgroup taxa. Broken lines indicate the various positions found for *A. flammea.* Only the topologies are given; branch lengths do not have any meaning. (a) Simple parsimony analyses (30-100 runs), positions (i)-(iv) possible for *A. flammea,* in bootstrapped analyses (100 or 1,000 replicates), only positions (ii) and (iii) occurred. (b) Parsimony analysis with bootstrap percentages indicated at the internodes (1,000 replicates). (c) Neighbor-joining (NJ) tree with proportional branch lengths; the bar gives the scale for the distances. (d) Fitch tree with indicated bootstrap percentages (1,000 replicates). (e) General topology found in maximum likelihood analyses; brackets indicate that *C. chloris* and *P. pyrrhula* shared a common branch when *A. flammea* was at positions (iii) or (iv). (f) General topology in bootstrapped ML analyses (100 replicates). The *C. chloris/P. pyrrhula* clade was found with most of the outgroup combinations and was only untied when *E. citrinella* and/or *V. paradisaea* were included, yielding positions (i) or (ii) for *A. flammea.* (g) ML tree with proportional branch lengths. The bar gives the scale for the distances. (h) ML analysis with 100 bootstrap replicates. Percentages are given at the internodes.
was only present in *E. rubecula*. Any other species showed the negatively charged D residue (labeled in fig. 1) at that position like most other metazoan taxa. (Exchange of N and D residues can occur by a single transition [A→G] at the first codon position.)

The second membrane-spanning segment involved in heme-binding and the adjacent regions protruding from the membrane and contributing to the reaction centers showed little variation compared to other parts of the sequence (fig. 1). However the fact that the number of silent substitutions was also considerably lower in that part of the gene cannot be fully explained by selection at the protein level only.

Phylogenetic Analyses

**Parsimony**

On average, seven equally parsimonious trees were found (1–12 trees) in analyses with different outgroup taxa. Only minor modifications were observed, e.g., two taxa either clustering or branching off one after another indicating that the tree topology was fairly stable independent of the particular outgroup chosen. An exception was the position of *A. flammea*, which could not be resolved (summarized in fig. 2a, i–iv). Bootstrapping yielded the same general topology but constrained the possible positions of *A. flammea* (fig. 2a, ii–iii). Figure 2b shows a particular tree, representative concerning topology as well as bootstrap percentages. The single internodes were always poorly supported; the lowest values occurred near *A. flammea*, which was likely with respect to its unconstrained position. Only the grouping of the two emberizine specimens was supported at a statistically significant level (100%, not shown). The cluster of the two Canary specimens only reached bootstrap values of about 80%, although their sequences differ by only a single nucleotide. The next best supported was the *C. chloris/P. pyrrhula* group (about 50%). Branches not included in the consensus trees but repeatedly found with similar or lower percentages than the least supported branches included have been summarized in table 3. They indicate smaller differences of topology than the low bootstrap values alone might suggest and even contribute to explaining them (see Discussion).

The branching pattern turned out to be quite independent of the cardueline species combination. Omitting *A. flammea* from the analysis led to considerably higher bootstrap values for every remaining internode (about 50%). Omitting *C. coccothraustes* separated the carduelines most clearly from the outgroup taxa (95%). Additionally, the *C. chloris/P. pyrrhula* clade was more strongly supported (87%), which is obvious from table 3: the *P. pyrrhula/C. coccothraustes* clade, substantially lowering the support for the *P. pyrrhula/C. chloris* clade, did not compete any longer.

**Distance Matrix**

The branching pattern turned out to be completely independent of the chosen outgroup. The position of *A. flammea* was always between *C. carduelis* and the *C. chloris/P. pyrrhula* branch. A particular NJ tree is given in figure 2c. Fitch and NJ methods always resulted in the same topology. Bootstrap analysis generally yielded higher percentages for any internal branch compared to the parsimony studies (fig. 2d). The only difference in

### Table 3

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<th>Branches Not Included in the Majority Rule Consensus Trees</th>
<th>Parsimony</th>
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**Note.**—Internodes similarly or less supported compared to the least supported branches included in the consensus trees are shown with the range of bootstrap percentages found in the analyses with different combinations of outgroup taxa. For parsimony and maximum likelihood (ML) analyses, the bootstrap values were generally low for the least supported branches (compare fig. 2), i.e., about 30%. In Fitch and Neighbor-joining (NJ) trees, the internal branches generally showed higher bootstrap percentages, about 50% with Fitch and about 40% with Neighbor-joining, respectively. For appropriate comparison, only branches with more than half the support of those included in the tree have been listed, i.e., >15% for parsimony and ML, >20% for NJ, and >25% for Fitch. The numbers of analyses with the different outgroup combinations (total of eight) in which bootstrap values above the cut-offs have been found are indicated in brackets. “*S. canaria*” represents the branch leading to the two Canary specimens.

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**Conflicting Character Distribution in Carduelines 13**

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Fig. 3.—Hypothesis of cardueline relationships inferred from cytochrome b sequences. The branching pattern of the unrooted tree diagram summarizes the results of different phylogenetic analyses (see text). Exchanged positions of S. serinus and S. mozambicus with the different methods (fig. 2) could be resolved by subset analysis. Branch lengths have no meaning.

In figure 2e, the topologies obtained by the different outgroup combinations have been summarized. The variations concerned mainly the position of A. flammea. The situation with bootstrap analysis has been summarized in figure 2f. Depending on the particular outgroup taxa, the indicated positions for A. flammea were found. Although the trees in figure 2e and 2f do not differ greatly, every particular outgroup combination yielded a different placing of A. flammea with or without bootstrapping. However, the general topology and additional consideration of the branches not included (table 3) in the majority rule consensus trees showed considerable similarity to the results obtained in the parsimony analyses. For example, S. mozambicus was placed next to the Canaries in every analysis (fig. 2a–b, e–h), but bootstrap support for the S. serinus/S. canaria cluster was hardly lower (table 3). Figure 2g shows a particular ML tree with indicated branch lengths and fig. 2h gives an example of a bootstrapped ML tree.

Comparison of the Different Analyses

The branching patterns obtained by the different methods were nearly identical with the exception of A. flammea, whose position remained uncertain. The positions of S. mozambicus and S. serinus depended on the method employed: in parsimony and ML analyses, S. mozambicus clustered with the Canaries instead of S. serinus in the distance analyses. Examination of the nucleotide states of these taxa showed that out of their 18 variable positions, S. canaria shared ten character states with S. serinus and four with S. mozambicus (fig. 1). At three sites, S. serinus shared the same nucleotide with S. mozambicus, and at the position differing between the Canary specimens, the states of S. serinus and S. mozambicus were each supported by one of the Canaries. Thus, it seems that the cluster supported best (S. canaria/S. serinus) becomes obscured by the addition of more taxa. To clarify this, subsequent analyses were performed including only the questionable taxa and each of the remaining cardueline species. The S. canaria/S. mozambicus cluster only appeared when C. carduelis was included, even in distance analysis (Fitch). With any other cardueline however, S. canaria/S. serinus were found, even in parsimony and ML analyses. So, inclusion of the C. carduelis sequence seemed to be the cause for the formation of the S. canaria/S. mozambicus group, at least concerning the four-taxon case.

Cardueline Relationships

Comparison of the species relationships inferred from the cytochrome b sequences with those obtained with other studies (see Introduction) posed some difficulties. While there is no general agreement on cardueline relationships, alternative species arrangements exist and thus prevent the reliable construction of a single “overall evidence” tree. Additionally, the various studies usually involve different assortments of taxa as well as different methodological approaches. Therefore, it seemed most reasonable to discuss either applicable species relationship derived from these studies in comparison to those derived from the cytochrome b data (fig. 3).
3). A helpful compilation of earlier studies (Desfayes 1971) was additionally used for simplification.

From all species examined, the lowest divergence occurred between S. serinus and S. canaria (2.3% nucleotide substitutions). These species had also been assumed to be very closely related according to behavioral and allozyme characters (Nicolai 1959; Stempel 1987). Serinus mozambicus generally clustered with the other Serinus species, which is in agreement with a cladistic analysis of behavioral and plumage characters (van den Elzen and Nemeschkal 1991). However, C. carduelis also grouped to a certain extent with S. serinus/S. canaria, especially when distance methods were applied (table 3), and also in equally parsimonious trees (not shown). On the basis of frequently reported hybridizations, Nicolai (1959) suggested an even closer relationship of C. carduelis to these two Serinus species than of S. mozambicus. Substitution numbers of S. mozambicus and C. carduelis in comparison with the other Serinus species were almost equal (table 2). Based on cytochrome b sequences, C. carduelis was more similar to the Serinus group (including S. mozambicus) than to C. chloris (table 2, figs. 2 and 3). In contrast, close affinities of C. carduelis and C. chloris have been proposed based mainly on plumage and behavioral characters (Desfayes 1971; van den Elzen and Nemeschkal 1991).

In the most detailed study on Goldfinches and Greenfinches (Güttinger 1978), they were suggested to represent different lineages. Currently, C. carduelis and C. chloris never clustered in phylogenetic analyses, not even with lower bootstrap percentages (table 3). Instead, C. chloris frequently grouped with P. pyrrhula in the cytochrome b trees. The combination of these taxa was perhaps the least expected result and likely reflects their lower substitutional differences (table 2). Nevertheless, there is also support from morphological data, for Karlow (1978) clustered them because of presumed myological synapomorphies. Although not represented in the consensus tree (though indicated by suggestive orientation of the C. chloris/P. pyrrhula branch; fig. 3), the cytochrome b data also suggested an affinity of Pyrrhula and Coccothraustes (table 3). Both genera are divergent in morphology and behavior compared to the rest of the carduelines studied here (Nicolai 1959) and were placed together by Desfayes (1971). Also, when the sequences of the cardueline taxa were analyzed separately, a Pyrrhula/Coccothraustes cluster formed with C. chloris as sister taxon (not shown). Coccothraustes proved to be the most aberrant cardueline in this study with the highest divergence (9.8%–11.4%) and a double amount of transversions compared to the other carduelines (9–11 compared to 0–5, table 2). It is also aberrant concerning morphological and behavioral traits and has frequently been placed near the base of the carduelines (e.g., Martin and Johnson 1986). The position of A. flammea could not be resolved by the cytochrome b data (fig. 2a, 2e, and 2f). According to Desfayes (1971), A. flammea belongs to a group of genera not included in the present study. In a cladistic analysis based on morphological and behavioral characters (van den Elzen and Nemeschkal 1991), A. flammea appears in a cluster including species of the Serinus and Carduelis groups.

In any tree building analysis, the spectrum of cardueline species formed a cluster separate from all non-carduelines, confirming their monophyly as was derived from other evidence.

In summary, the different approaches supported or complemented each other, yielding no evidence for the general superiority of a particular method of investigation.

Discussion

Phylogenetic analyses of the cardueline cytochrome b sequences led to a branching pattern that was widely independent of the method of analysis, the cardueline taxa involved, and the choice of outgroup. This considerable tree stability stands in marked contrast to the low bootstrap values obtained. The question arises whether this lack of resolution provides some kind of artifact or might reveal some aspects of cardueline speciation reflecting the group’s history. Both possibilities will be discussed.

Lack of Resolution: Artifact . . .

First, artifacts can be due to systematic errors, such as violation of the underlying assumptions of the methods applied. Since the debate on the methods for phylogenetic analysis is still ongoing and since it is not clear which of the (simplified) assumptions realistically match the evolutionary process leading to the observed character distribution (Simmons et al. 1994), a variety of methods and assumptions have been applied in the present study. The resulting tree topologies were widely congruent, no general rearrangements of taxa were observed, and the branching pattern was not affected by different combinations of ingroup and outgroup taxa. Although tree stability does not necessarily mean that the "true" phylogenetic tree has been inferred, it provides a good reassurance against systematic errors. Exceptions are the unstable positions of Serinus mozambicus and Acanthis flammea whose comparably long branches (fig. 2c and 2g) probably reflect unequal rates of evolution and may contribute to systematic errors (Swofford and Olsen 1990). While the position of S. mozambicus was very constrained and could be determined by detailed character and subset analysis (see above), the position of A. flammea can probably be resolved by the addition of a presumably closely related
taxon subdividing the long branch and/or by further sequence data. In contrast to the character-based methods (parsimony and ML), the "true" position of *S. mozambicus* was found with the distance analyses, and also, the position of *A. flammaea* was stable (fig. 2c and 2d), indicating that conflicting character states might be a general problem.

Second, there might be the argument that the low bootstrap support reflects a lack of data. As a minimum, the number of phylogenetically informative characters should exceed the number of species to be resolved (Stewart 1993). In the present data, 97 variable/57 phylogenetically informative characters are used to construct a tree with 12 taxa; for 8 cardueline taxa, 62 variable/30 phylogenetically informative characters are available. Distance methods probably performed better because they use all variable characters for analysis, but the same holds for ML, leading back to the conflicting character states. In bootstrapping analyses, at least three congruent synapomorphies are required for each node to be significant at the 95% level (Kluge and Wolf 1993). Two examples show that caution is needed to apply bootstrapping here in the usual way, i.e., expecting percentages above 95%: (1) The two canary specimens whose relationships are known from history always clustered but never gained statistical significance with the bootstrap. (2) Although carduelines always constituted a monophyletic group, which was in agreement with the general opinion that they are well separated from the other so-called "finch" groups, bootstrapping did not separate outgroup species from carduelines at a statistically significant level. So, there may be either too few data to reach statistical significance with the bootstrap or the characters available are incongruent.

A closer examination of the bootstrap percentages, especially a consideration of less supported branches not appearing in the majority rule consensus trees (table 3) gives an idea of the reliability of certain positions and branching patterns (Swofford and Olsen 1990).

First, the cause of uncertainty differs substantially between the situation of the generally weakly supported branches consistently found with every method employed and the unsuccessful integration of *A. flammaea*, whose position changed according to method of analysis and chosen outgroup. Obviously, a branch occurring at different positions should lower the statistical support of the remaining internodes (table 3). Indeed, exclusion of *A. flammaea* raised the bootstrap values of many nodes by about 10%–20% with every method of analysis (not shown).

Second, the low bootstrap values of the internal branches were not due to numerous rearrangements, which would imply an arbitrary branching pattern, but rather to similarly supported groups, which would cause only minor topology modifications. For instance, placing *Serinus serinus* next to the Canaries instead of *S. mozambicus* hardly affects the general topology. Additionally, both arrangements occurred with every method of analysis (compare fig. 2b, 2d, and 2h and table 3). For example, when the *S. canaria/S. serinus* group was suppressed in the consensus tree by the *S. canaria/S. mozambicus* cluster because of somewhat lower bootstrap percentages, it still gained considerable support, and vice versa. The same held for a *Pyrrhula/Coccothraustes* clade found in many analyses with considerable percentages compared to the other internodes, but suppressed in most cases by the *Carduelis chloris/Pyrrhula pyrrhula* group. Inversely, *Pyrrhula* and *Coccothraustes* generally clustered in analyses including only cardueline taxa (not shown) while the *C. chloris/P. pyrrhula* group always appeared among the less supported groups with similar percentages.

A main cause for the lack of resolution therefore seems to be conflicting data, i.e., part of the nucleotide states supports one group and another part supports a group excluding the first. Furthermore, the contradictory clusters are not arbitrary, judged by morphological and behavioral data, but are indeed the same as found with these independent data sets. Why should any of the mutually excluding groups (table 3) make sense if the low bootstrap support were due to an artifact of the molecular analysis? Although the latter possibility can never be excluded completely, it is not considered to be the most obvious explanation for the cardueline data. Instead, the assumption is made that the conflicting character states observed may indeed reflect an historical reality.

... Or History?

Bootstrapping revealed the existence of similarly supported groups, which excluded each other and which therefore did not appear together in the majority rule consensus trees. None of these groups would cause large rearrangements of the tree topology, but rather inversion of two neighboring branches (e.g., *S. mozambicus* and *C. carduelis*) or clustering of two species or branching off one after another (e.g., *C. chloris* and *P. pyrrhula*). The same contradictory arrangements have been proposed earlier with independent data sets (see above). Additionally, groups not appearing in the molecular analyses have never been suggested to be closely related (e.g., *Serinus* and *Pyrrhula* or *Carduelis* and *Coccothraustes*). Thus, the cytochrome *b* data reasonably confirm earlier assumptions on species relationships and also suffer from the same difficulties to resolve them definitely. In other words, none of the approaches was able to remove its own contradictions.
Two general explanations to conflicting character distributions in molecular data are available: homoplasy or shared ancestral polymorphism (Simon et al. 1994). On the one hand, a high level of homoplasy (convergences, parallelisms, and reversals) could be responsible for the low statistical support of the nodes. However, multiple hits of an order capable of explaining the situation found in this study are unlikely because of the low overall variability and the kinds of substitutions observed (see above). Furthermore, ML as well as the transformation methods turning sequences into distances include models of evolution that correct for such events. Also, using the uncorrected substitution numbers in a distance matrix did not alter the tree topology (not shown). The likelihood of parallel substitutions also increases with increasing distance. When distances are small, parallelisms frequent enough to affect the phylogenetic signal are only to be expected when a strong selection acts upon the sequences. But most of the mutations are silent substitutions. Even those leading to amino acid differences are nearly neutral in the sense that all provide a functional cytochrome b. So, selection on the protein level should not affect the phylogenetic analysis. On the nucleotide level, the strong bias in base composition (table 1) at the different codon positions might reflect some kind of selection eventually concerning tRNAs and the translation process. The biases, probably maintained by selective pressure, narrow the possibilities of "allowed" nucleotides. For example, transversions of A→C at the third codon position are most frequent and reflect the base composition. Also, the possibility of, e.g., the unbiased nucleotide composition of the first codon position also being stabilized by selection cannot be excluded. Therefore, every kind of substitution might be subject to selection (favoring parallel substitutions) as well as reflect species evolution. At least a clustering of species only because of a similar nucleotide distribution is not to be expected, because base composition was highly similar for every taxon involved (table 1). The general congruence of cytochrome b relationships with species relationships derived from other studies investigating independent character complexes suggests the presence of a considerable phylogenetic signal in the molecular data. So, either the homoplasies are not sufficient to obscure the phylogenetic signal or the problem of statistical insignificance is not primarily related to homoplasy.

Contradictory character states also observed repeatedly in other fields of investigation yielding a mosaic-like distribution of characters across the whole range of cardueline species have often led to the assumption of parallel evolution in the different lineages (Nicolai 1959; Gütinger 1978; van den Elzen 1985). A few examples of plumage patterns of cardueline species exceeding those involved in this study shall be given to show that the problem of conflicting character distribution is neither constrained to a few taxa nor to the cytochrome b data:

Light wing tips occur in different cardueline lineages (Gütinger 1978). Wing bars are developed in many different species, and a red pigment present in adult males also occurs in various genera that are not necessarily more closely related than those not sharing this character. A complex head pattern is shown in several cardueline species, which are not supposed to be very closely related (Gütinger 1978). The "Canary-type" characterized by streaked plumage pattern and a short, conical bill was suggested to have evolved several times (Nicolai 1959) and represents rather an ecotype submitted to adaptive constraints.

While parallel evolution might be an adequate explanation for adaptive features, e.g., related to feeding specializations, there is no rationale to postulate numerous parallel gains and losses of features for which no obvious selective advantage can be given, as for many plumage characters. Additionally, recent works have shown common ancestry also to play an important role in the resemblance of closely related species, even in highly adaptive features, e.g., bill morphology (Richman and Price 1992; Björklund and Merilä 1993) so that an interpretation in terms of parallel evolution is not the only reasonable explanation even for traits most likely subjected to adaptive constraints.

Instead of assuming parallel evolution of numerous morphological characters, the patterns of character distribution can also be explained by a mosaic of retained ancestral states spread by early recombination throughout the different lineages. Some observations on complex plumage characteristics give further examples that can be interpreted in terms of shared ancestral polymorphisms: The complex head pattern mentioned above has been considered as an ancestral character complex because it also occurs in the juvenile plumage of several species with otherwise derived morphological characters (van den Elzen 1985). The plumage patterns of many interspecific hybrids resemble the "Canary-type", which can therefore also be interpreted as a re-established ancestral state, i.e., an atavistic pattern (Fehrer 1993). Thus, the cardueline data can be easily fitted into a model assuming ancestral polymorphisms of morphological characters. The same interpretation may hold for the findings on the mitochondrial DNA level because a major contribution of homoplasy is not considered to be the most reasonable explanation for the conflicting nucleotide states (see above). Ancestral polymorphism is suggested to have been present very early in cardueline history, because (1) conflicting character states affect all lineages examined, on the molecular as well as on the
marginal homogeneity of mitochondrial DNA haplotypes has been shown to yield a trend toward monophyly with increasing time after speciation (Neigel and Avise 1986), which can explain why the intraspecific variability of the cytochrome \( b \) can be low despite postulated ancestral polymorphisms. The phylogenetic signal of the cardueline data is therefore thought to reflect mainly the subsequent evolution of the separated lineages.

The problems shared ancestral polymorphisms pose to phylogeny reconstruction are known to be more severe when time between speciation events is short (Pamilo and Nei 1988). Speciation processes taking place in a short period of time increasingly turn out to be a rule rather than an exception (Helm-Bychowski and Cracraft 1993). In cardueline cytochrome \( b \) trees, the short internodes compared to the long tips (fig. 2c and 2g) may trace back to a rapid radiation of the different lineages. Also, the low bootstrap support of the internodes can be interpreted in terms of a so-called "star-phylogeny" (Helm-Bychowski and Cracraft 1993), i.e., a bush-like radiation of lineages.

Taken together, we can hypothesize a scenario of a highly polymorphic gene pool common to all cardueline ancestors. Part of the polymorphism could have been retained in the speciation process, leading to a conflicting, i.e., cladistically incongruent character distribution. Recombination within the assumed highly polymorphic population could have provided an enormous variety of forms serving as a driving force to give rise to a burst of speciation. Possibly, after a period of explosive radiation, proceeding isolation has led to independently evolving lineages with separate histories.

A similar model has recently been proposed (Richman and Price 1992) for the songbird genus Phytoeclops based also on cytochrome \( b \) and morphological data, assuming a rapid speciation occurring very early in the history of the group and the present species persisting relatively unchanged over long periods of time.

Some predictions can be made to test the validity of the outlined interpretation of the cardueline data: As Pamilo and Nei (1988) have pointed out, phylogeny reconstruction from a single gene cannot be improved by increasing the number of alleles sampled at the same locus when the ancestral population was genetically polymorphic and the time of divergence between the species was short. In this case, more sequence data of the cytochrome \( b \) or other mitochondrial genes should not be able to improve the resolution substantially. Instead, further sequences should reveal further conflicting nucleotide distributions. Different weighting schemes should not yield considerable improvement of the tree unless parallel evolution played a role more important than assumed here. Additional information, e.g., on nuclear genes may or may not improve the phylogenetic signal, depending on the extent of polymorphism, which is not constrained to the mitochondrial genome as indicated by the distribution of plumage characters discussed above.

Conclusion

In the mitochondrial cytochrome \( b \) gene, character distributions leading to contradictory species clusters were found in phylogenetic analysis. On the morphological level, a similar situation made it difficult to resolve species relationships convincingly. While no evidence for considerable homoplasia could be demonstrated, the observed character distributions are attributed to shared polymorphisms of the ancestral cardueline population, concerning the mitochondrial DNA as well as phenotypic patterns. The speciation model derived for cardueline finches provides an explanation for the difficulties experienced with their classification (Paynter 1968, p. 207; Voous 1977). It further suggests that at least some of them might be unremovable on principle because shared ancestral polymorphisms and a bush-like radiation of the lineages affect reconstruction of cardueline phylogeny, indicating that the problem may indeed be founded in the concrete history of the group.

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