BMC Evolutionary Biology

BioMed Central

Research article

Inter-familial relationships of the shorebirds (Aves: Charadriiformes) based on nuclear DNA sequence data

Per GP Ericson*[1], Ida Envall[1], Martin Irestedt[1,2] and Janette A Norman[3]

Address: [1]Department of Vertebrate Zoology and Molecular Systematics Laboratory, Swedish Museum of Natural History, P.O. Box 50007, SE-10405 Stockholm, Sweden, [2]Department of Zoology, University of Stockholm, SE-106 91 Stockholm, Sweden and [3]Sciences Department, Museum Victoria, G.P.O. Box 666E, Melbourne, Vic. 3001, Australia

Email: Per GP Ericson* - per.ericson@nrm.se; Ida Envall - ida.envall@nrm.se; Martin Irestedt - martin.irestedt@nrm.se; Janette A Norman - jnorman@museum.vic.gov.au

* Corresponding author

Published: 23 July 2003

BMC Evolutionary Biology 2003, **3**:16

Received: 23 May 2003
Accepted: 23 July 2003

This article is available from: http://www.biomedcentral.com/1471-2148/3/16

Abstract

Background: Phylogenetic hypotheses of higher-level relationships in the order Charadriiformes based on morphological data, partly disagree with those based on DNA-DNA hybridisation data. So far, these relationships have not been tested by analysis of DNA sequence data. Herein we utilize 1692 bp of aligned, nuclear DNA sequences obtained from 23 charadriiform species, representing 15 families. We also test earlier suggestions that bustards and sandgrouses may be nested with the charadriiforms. The data is analysed with methods based on the parsimony and maximum-likelihood criteria.

Results: Several novel phylogenetic relationships were recovered and strongly supported by the data, regardless of which method of analysis was employed. These include placing the gulls and allied groups as a sistergroup to the sandpiper-like birds, and not to the plover-like birds. The auks clearly belong to the clade with the gulls and allies, and are not basal to most other charadriiform birds as suggested in analyses of morphological data. *Pluvialis*, which has been supposed to belong to the plover family (Charadriidae), represents a basal branch that constitutes the sister taxon to a clade with plovers, oystercatchers and avocets. The thick-knees and sheathbills unexpectedly cluster together.

Conclusion: The DNA sequence data contains a strong phylogenetic signal that results in a well-resolved phylogenetic tree with many strongly supported internodes. Taxonomically it is the most inclusive study of shorebird families that relies on nucleotide sequences. The presented phylogenetic hypothesis provides a solid framework for analyses of macroevolution of ecological, morphological and behavioural adaptations observed within the order Charadriiformes.

Background

The order Charadriiformes (shorebirds) comprises 343 species divided into 18 families (*sensu* del Hoyo *et al* [1], whose taxonomic nomenclature is followed here, Table 1). Following Peters [2] the families are placed in three suborders; Alcae, Lari and Charadrii. The Alcae contains a single family, Alcidae (auks, puffins, murrelets and allies), while the Lari is comprised of the Stercorariidae (skuas and jaegers), Laridae (gulls), Sternidae (terns and noddies) and Rynchopidae (skimmers) The largest and most diverse assemblage is the Charadrii comprising the remaining 13 families.

Table 1: Family-names for charadriiform birds The families included in the order Charadriiformes (*sensu* del Hoyo *et al.* 1996). Asterisks mark taxa that are not included in this study.

Family	English name
Alcidae	Auks
Burhinidae	Thick-knees
Charadriidae	Lapwings and plovers
Chionidae	Sheathbills
Dromadidae *)	Crab plover
Glareolidae	Coursers and pratincoles
Haematopodidae	Oystercatchers
Ibidorhynchidae *)	Ibisbill
Jacanidae	Jacanas
Laridae	Gulls
Pedionomidae *)	Plains-wanderer
Recurvirostridae	Avocets and stilts
Rostratulidae	Painted snipes
Rynchopidae	Skimmers
Scolopacidae	Sandpipers, snipes and phalaropes
Stercorariidae	Skuas and jaegers
Sternidae	Terns
Thinocoridae	Seedsnipes

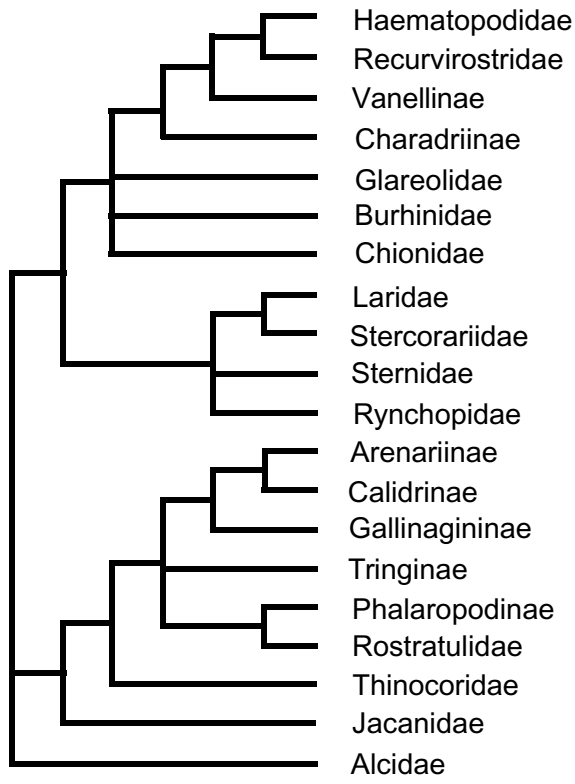

Figure 1
Phylogeny proposed by Strauch (1978) Systematic relationships among major groups of charadriiform birds proposed by Strauch (1978) based on 70 morphological characters analysed by a character compatibility analysis.

Morphological support for monophyly of the Charadriiformes is weak and conflicting hypotheses of charadriiform relationships have been proposed based on analysis of morphological, osteological, and genetic characters. In a detailed osteological study of the order, which included 227 charadriiform taxa, Strauch [3] identified three lineages, Alcae, Scolopaci and Charadrii (Fig. 1). The Scolopaci comprised the Jacanidae (jacanas), Rostratulidae (painted snipe), Scolopacidae (sandpipers, stints, snipe, curlews and allies) and Thinocoridae (seedsnipe). The Charadrii comprised the Dromadidae (Crab Plover), Haematopodidae, Ibidorhynchidae, Recurvirostridae, Burhinidae, Glareolidae, Charadriidae, Chionidae (sheathbills), Stercorariidae, Laridae, Sternidae and Rynchopidae while the Alcae comprised only the Alcidae. Strauch's [3] analysis could not resolve the affinities between the three lineages but reanalyses of the data by Mickevich and Parenti [4], Björklund [5] and Chu [6] identified Alcae as the basal lineage. Additionally, Björklund's [5] reanalysis placed the Charadriidae within the Scolopaci suggesting it formed a monophyletic clade with the Scolopacidae, Jacanidae and Rostratulidae. Consistent with the osteological data, Jehl's [7] analysis of downy young plumage patterns within the Charadrii supported a relationship between the Haematopidae, Ibidorhynchidae, Recurvirostridae, Burhinidae, Glareolidae and Charadriidae.

Sibley and Ahlquist [8] using DNA-DNA hybridisation data for 69 charadriiform identified only two major lineages; the Scolopaci of Strauch [3] together with Pedionomidae (plains-wanderer) and an expanded Charadrii comprising the remaining families (Fig. 2). A protein allozyme study by Christian *et al.* [9] also confirmed that the Burhinidae, Haematopodidae, Recurvirostridae and Charadriidae represented an assemblage distinct from the Scolopacidae. The protein study could not confirm a close association between the Laridae and the Charadriidae assemblage or between the Scolopacidae and Jacanidae, but did suggest a sister relationship between the Lari and the Glareolidae.

While in broad agreement with the studies of Strauch [3] and Chu [6] the DNA-DNA hybridisation data of Sibley and Ahlquist [8] did not identify auks (Alcidae) as a separate lineage instead placing them as the sister taxon to gulls, terns, skimmers and skuas. Moreover, alternative relationships were recovered from the data when different tree-building algorithms were applied. This resulted in the gulls and their allies being placed as the sister group to the

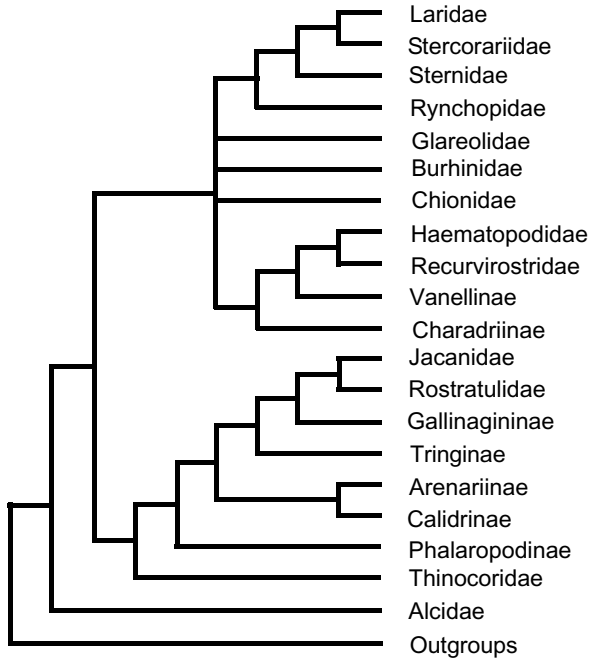

Figure 2
Phylogeny proposed by Chu (1995) Systematic relationships among major groups of charadriiform birds proposed by Chu (1995) based on a parsimony analysis of a morphological data set almost identical to that of Strauch (1978).

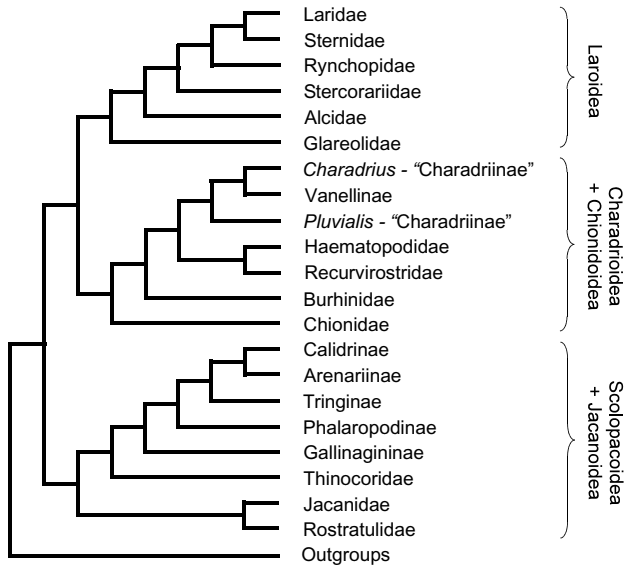

Figure 3
Phylogeny proposed by Sibley and Ahlquist (1990) Systematic relationships among major groups of charadriiform birds proposed by Sibley and Ahlquist (1990) based on an analysis of DNA-DNA hybridisation data.

sandpiper-like birds rather than to the plover-like birds (op. cit. fig. 337). Several other differences occur between the results produced by the various datasets. For example, the Scolopacidae was found to be monophyletic in the analyses of Strauch [3] and Sibley and Ahlquist [8], while Chu [6] and Mickevich and Parenti [4] found the scolopacids to be paraphyletic. Obviously, the higher-level phylogeny of the shorebirds is still ambiguous. In this paper we investigate if DNA sequence data can be used to produce a more robust hypothesis of charadriiform relationships.

The choice of molecular markers becomes critical when old divergences are studied. Although the times of divergences among the extant charadriiform lineages are largely unknown, fossils of extinct groups commonly referred to the order Charadriiformes occur already in deposits dated to the Late Cretaceous [10]. The oldest fossils assigned to modern families of charadriiforms date from the late Tertiary [11,12]. It can thus be safely assumed that some branches in the phylogenetic tree for these taxa date back to the early Tertiary, if not longer. This is in agreement with the assumption, based on biogeography and molecular-clock models, that the evolution and diversification of several orders and families of extant birds have occurred in the late Cretaceous or Early Tertiary, some 100 to 50 mya [13–17]. It should be noted, however, that estimates of divergence times based on the fossil record are generally lower than those based on molecular data [18].

The old age of many bird taxa presents one explanation of why their higher-level systematic relationships have been difficult to resolve using mitochondrial sequence data [16,19,20]. The mitochondrial genes most commonly used in these studies (cytochrome *b* and 12S) are normally assumed to evolve at a rate of ~1% per million years [21]. It has been argued that the cytochrome *b* gene gives reliable information in birds only for divergences younger than 9 million years [22], i.e. evolutionary events considerably younger than the evolution of most modern orders and families. Thus, to use DNA sequence data to resolve higher-level branching patterns among birds requires molecular markers that evolve considerably slower than the mitochondrial genome.

In recent years nuclear genes have been utilized for this purpose. The most commonly used nuclear markers are the protein-encoding c-*myc*, RAG-1, and c-*mos* genes with

the former two evolving at rates 4 to 12 times slower than the mitochondrial cytochrome *b* gene in the same taxa (Ericson pers. obs.). The evolutionary rates of these genes may, however, be too slow for many studies as the number of mutations observed to accumulate between evolutionary branching events can be very low. This results in short internodes with poor statistical support in the phylogenetic tree, which in turn makes the phylogenetic hypotheses weak. To enhance studies of medium-old (ca 20–60 my) divergences in birds it is desirable to find molecular markers with slightly faster evolutionary rates than the protein-encoding nuclear genes used so far, and introns in nuclear genes may provide such markers.

Herein we use an intron positioned in the myoglobin gene, which has proven especially useful to resolve relationships among passerine birds [23–25], in addition to the protein-encoding RAG-1 gene. By using two independent molecular markers we can also compare the phylogenetic signal produced by each genic region. While a tree based on a single gene or region may be misleading purely because of stochastic factors [26,27], observed congruence between individual gene trees increases the probability that they represent the true evolutionary history of the group.

Results

Molecular variation and base compositions

A segment of 930 homologous base pairs from RAG-1 was obtained from all taxa with no indels detected. Sequences for the myoglobin intron II ranged from 693 base pairs (*Alca torda*, *Larus fuscus*) to 734 base pairs (*Afrotis atra*). The intron exhibited several indels, of which two were phylogenetically informative (one between positions 38 and 48, and the other between positions 532 and 544).

Despite sequencing in both directions with several primers, certain nucleotide positions could not be determined unambiguously. This relates to 24 positions in the RAG-1 sequences (0.1%), and 48 positions in the myoglobin sequences (0.3%). The observed polymorphisms may be due to true heterozygosity in the individual, but also to PCR or sequencing artefacts.

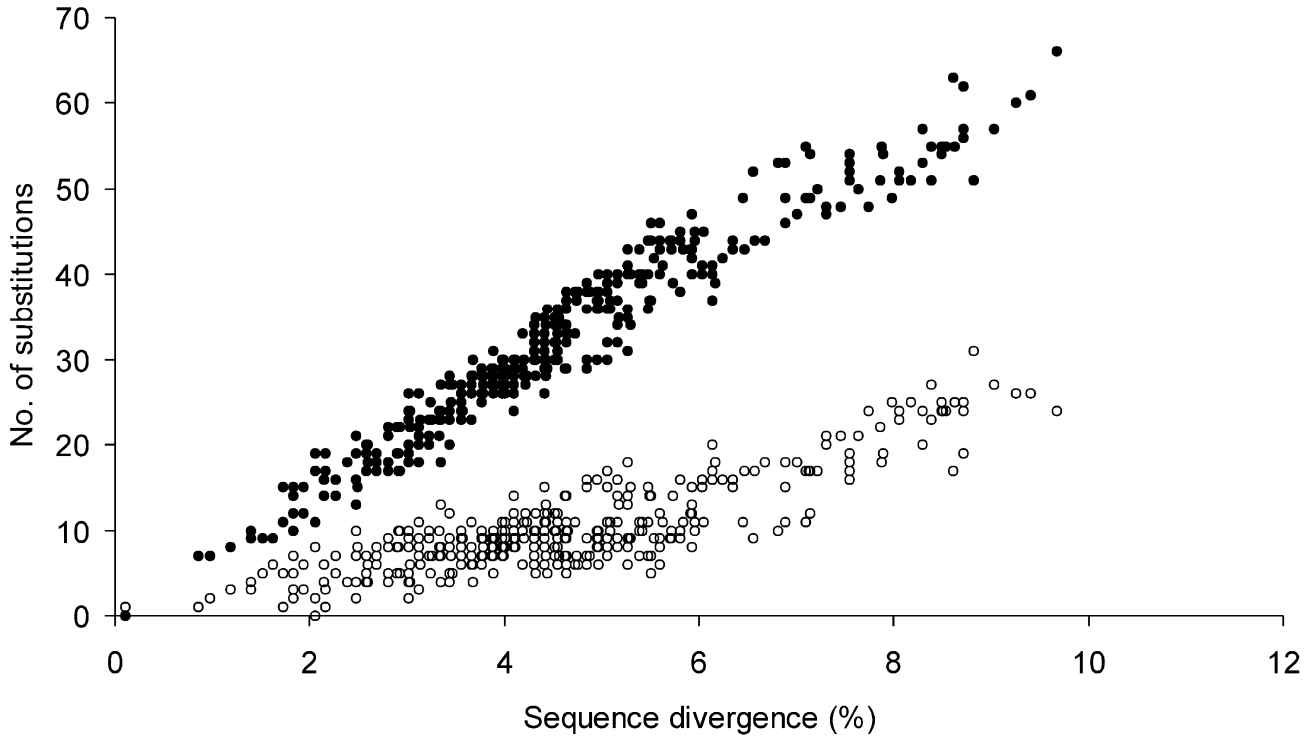

Figure 4
Saturation plots for RAG-1 Transitions (open circles) and transversions (closed circles) at all codon positions plotted against the observed pairwise sequence divergences (p-distance).

After alignment the data matrix consisted of 1692 base pairs, of which 930 base pairs derive from RAG-1 and 762 base pairs from myoglobin intron II. In RAG-1, 644 positions (69%) were constant, 164 (18%) were variable but not phylogenetically informative, while 122 (13%) were potentially informative. In myoglobin intron II 395 positions (52%) were constant, 207 (27%) variable but not phylogenetically informative, and 160 (21%) were potentially informative.

Sequence distances and patterns of substitutions

In RAG-1 the uncorrected sequence divergences between charadriiform taxa ranged from 0.1% (*Catharacta* vs. *Stercorarius*) to 7.1% (*Chionis* vs.*Thinocorus*) (Table 2, see Additional file 1). Divergences between charadriiform taxa and the columbiforms (dove and sandgrouse) and gruiforms (bustard and crane), ranged from 2.4% to 7.1%. Generally, larger distances were observed in comparisons involving the outgroup megapod and screamer (5.5% to 9.7%). The corresponding ranges in sequence divergences for myoglobin intron II were from less than 0.1% (*Catharacta* vs. *Stercorarius*) to 10.3% (*Cursorius* vs.*Jacana*) within the charadriiforms, from 6.1% to 14.6% between the charadriiforms and the columbiforms and gruiforms, and from 9.5% to 17.1% in comparisons involving the outgroups (Table 2, see Additional file 1). The pairwise sequence divergences were higher, on average, in myoglobin intron II than in RAG-1.

Neither RAG-1, nor myoglobin intron II, showed any sign of saturation with both transitions and transversions linearly related to the observed pairwise sequence distances, with no obvious tendency to level off (Figs. 4,5). A plot of the pairwise, uncorrected sequence distances observed in RAG-1 and myoglobin intron II, respectively, showed an almost linear relationship between the two genic regions (Fig. 6). The slope of the regression line estimated to fit these data indicates that the myoglobin intron II evolves at a rate that is about 60% faster than that in RAG-1.

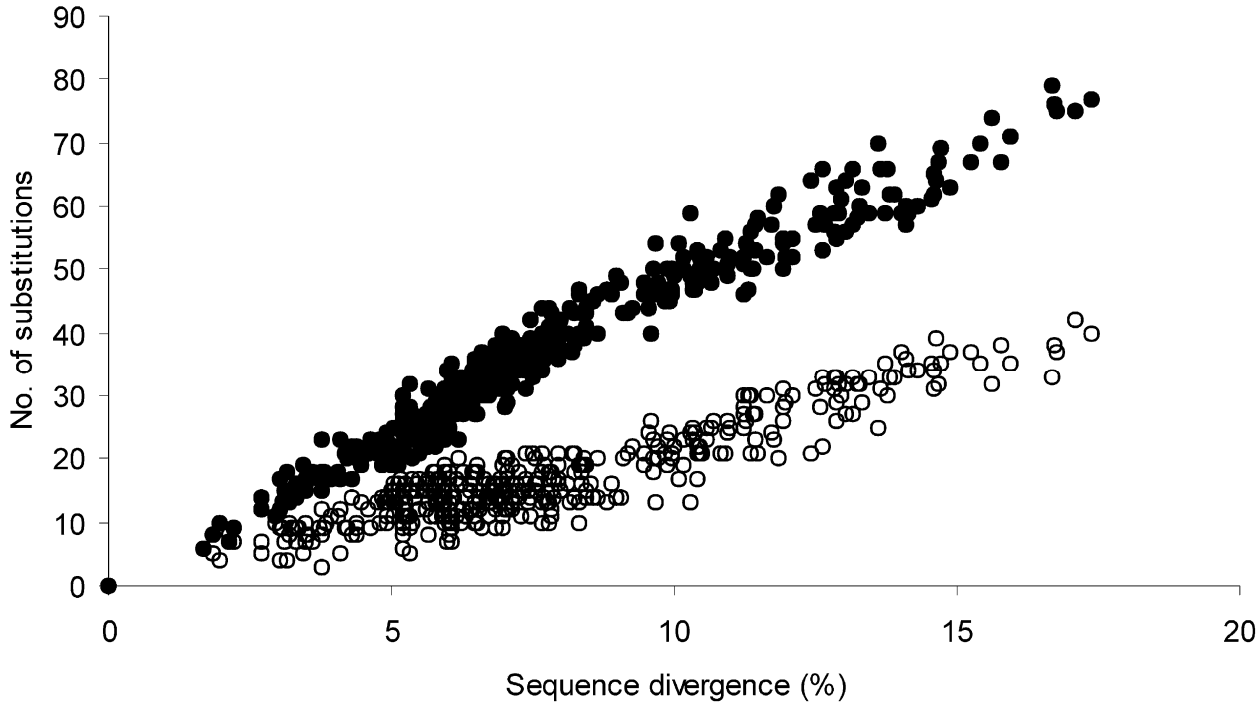

Figure 5
Saturation plots for myoglobin intron II Transitions (open circles) and transversions (closed circles) at all positions plotted against the observed pairwise sequence divergences (p-distance).

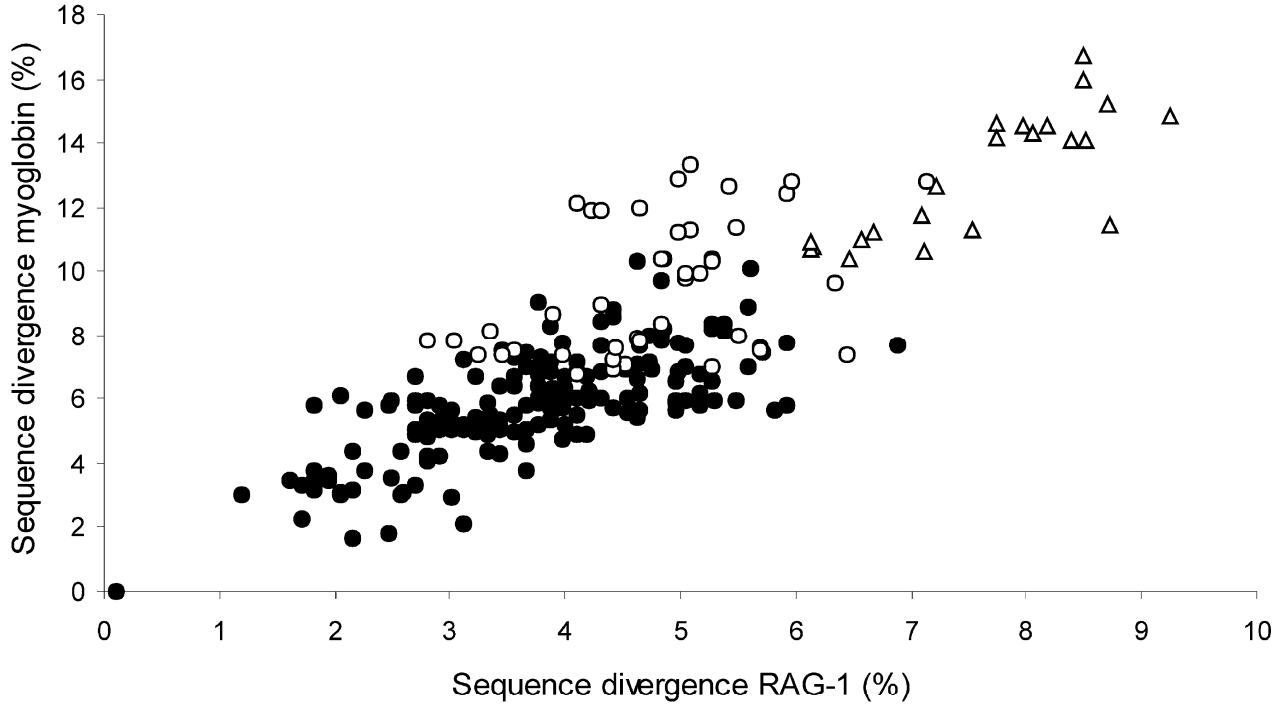

Figure 6
Comparison between pairwise sequence divergences in RAG-1 and myoglobin intron II Solid circles indicate observed pairwise distances observed within the charadriiforms; open circles indicate distances between the charadriiform taxa and the bustard, crane, pigeon and sandgrouse; and triangles indicate distances between charadriiforms and the anseriform and galliform outgroup.

Phylogenetic relationships

Parsimony and maximum-likelihood analyses of the combined sequence data yielded virtually identical tree topologies, as did the parsimony analyses of the individual genic regions (Figs. 7,8,9). The parsimony analyses yielded four most parsimonious trees (length 1128 steps, consistency index [CI] 0.71, retention index [RI] 0.60) for the combined data set, 72 most parsimonious trees (length 483, CI 0.70, RI 0.60) for RAG-1, and 76 most parsimonious trees (length 639, CI 0.72, RI 0.61) for myoglobin intron II. Bootstrap values and Bayesian support values are indicated in the trees.

Monophyly of the Charadriiformes (*sensu* [1]) is strongly supported by the combined data set, with a bootstrap value of 94% in the maximum parsimony (MP) tree (Fig. 8), and a 100% Bayesian support in the maximum-likelihood (ML) tree (Fig. 7). Neither the sandgrouse (Pteroclididae) nor bustard (Otididae) clustered within the Charadriiformes, nor does either taxon unambiguously form the sister-group.

There is strong bootstrap support in both the parsimony and maximum-likelihood analyses for a division of the order into two major clades. The first consists of sandpiper-like birds (representing the families Scolopacidae, Jacanidae, Rostratulidae, Thinocoridae) along with gulls and their allies (Laridae, Sternidae, Rynchopidae, Stercorariidae), auks (Alcidae) and the coursers (Glareolidae). The second consists of plover-like birds (representing Charadriidae, Haematopodidae, Recurvirostridae) along with the sheathbill (Chionidae) and thick-knee (Burhinidae).

Within the first clade, two subclades are evident. One, comprising Alcidae, Stercorariidae, Laridae, Sternidae, Rynchopidae, and Glareolidae is strongly supported (MP: 100%, Bayesian: 100%) and defined by a synapomorphic indel between positions 532 and 544 in the myoglobin intron II. Relationships within this clade cannot be determined with certainty, but there is support (MP: 61%, Bayesian: 100%) for a dichotomy between Glareolidae and the rest. This dichotomy is also supported by a

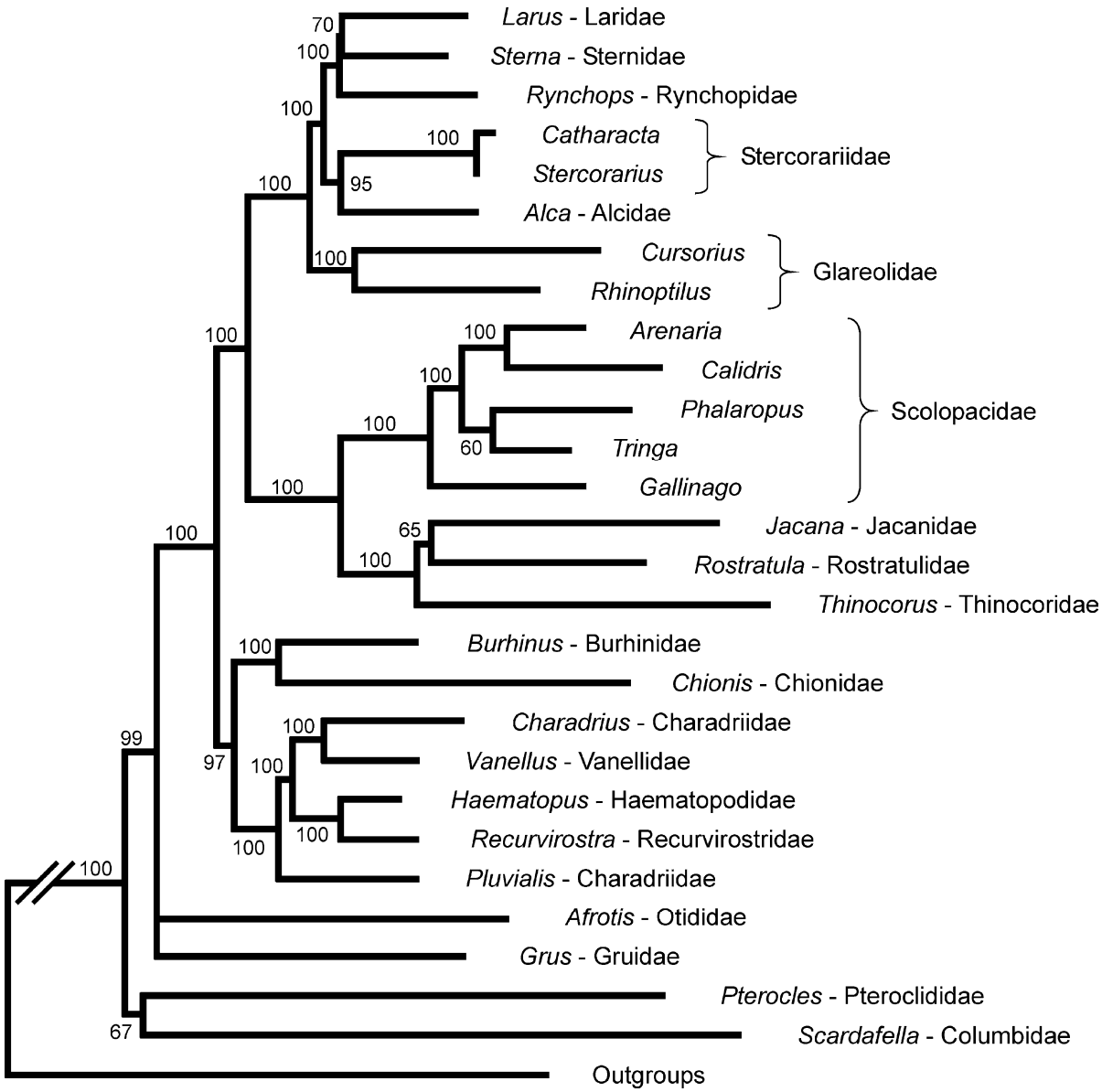

Figure 7

Maximum-likelihood analysis Best-fit maximum likelihood tree calculated under the GTR + Γ + G time-reversible model for nucleotide substitutions. The two outgroups (*Alectura* and *Chauna*) are combined in the tree and the branch leading to them is not drawn proportional to the number of substitutions. The Bayesian inference analysis resulted in the same tree topology. The numbers indicated on branches refer to nodal support values estimated in the Bayesian analysis.

synapomorphic indel, between positions 38 and 48 of the myoglobin intron II. The second subclade comprises the family Scolopacidae (*Arenaria*, *Calidris*, *Tringa*, *Phalaropus*, *Gallinago*) that is sistergroup to a clade consisting of Jacanidae, Thinocoridae and Rostratulidae. The relationships within Scolopacidae are more difficult to determine although *Gallinago* is identified as the sister to all the others, in both trees. The relationships between Jacanidae, Thinocoridae and Rostratulidae cannot be established, although they appear to constitute a monophyletic group.

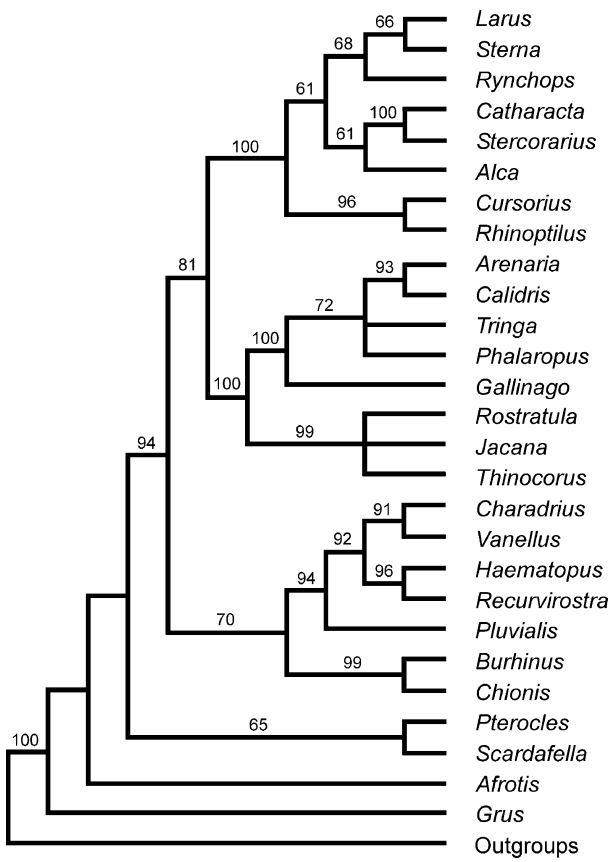

Figure 8
Maximum parsimony analysis Strict consensus tree calculated from the four most parsimonious trees (length 1116 steps, CI 0.71, RI 0.60) based on the combined data set.

Within the second major clade a relationship between Burhinidae and Chionidae was strongly supported (MP: 99%, Bayesian: 100%), and a lineage including Charadriidae, Vanellidae, Recurvirostridae and Haematopodidae (MP: 94%, Bayesian: 100%) constitutes their sistergroup. The family Charadriidae is not recovered as monophyletic. Instead, *Charadrius* (Charadriidae) and *Vanellus* (Vanellidae) group together, with *Recurvirostra* (Recurvirostridae) and *Haematopus* (Haematopodidae) as their sister. *Pluvialis* (considered as belonging to Charadriidae) falls outside these four taxa.

There are two contradictions between the trees based on RAG-1 and myoglobin intron II, respectively (Fig. 9). According to the myoglobin intron II tree, Jacanidae and Thinocoridae constitute one group, and this group is the sister to Rostratulidae. In the RAG-1 tree, Jacanidae and Rostratulidae group together, with Thinocoridae as their sister. The low nodal support values indicate that the relationships between these three taxa are effectively unresolved in the present analyses. Furthermore, Alcidae is the sister to Glareolidae in the myoglobin tree, and to Stercorariidae in the RAG-1 tree, but neither suggestion receives bootstrap support.

Discussion

Utility of the RAG-1 and myoglobin intron II sequences

Both nuclear DNA regions examined here were suitable for phylogenetic analyses at this taxonomic level as neither showed any sign of saturation in the present data set. This was expected for RAG-1 based on previous results obtained for analyses of avian data sets [24,28–30] but was unexpected for the non-coding myoglobin intron II. Slade *et al*. [31] and Lessa [32] have previously advocated the use of nuclear introns as markers for resolving patterns of intra-specific differentiation in vertebrates and this approach has been adopted in population genetic studies of birds [33,34]. More recently, nuclear introns have been used to reconstruct species-level phylogenies [35–41]. Their application at this taxonomic level is supported by comparative studies, which suggest that beta-fibrinogen intron 7 contains similar phylogenetic signal to the mitochondrial cytochrome *b* gene in birds [36]. Based on these limited findings, saturation of nuclear intron sequences and poor phylogenetic resolution might logically be expected to occur at higher taxonomic levels. This was not observed for myoglobin intron II in the present data set, which examined intra-ordinal relationships. Despite its non-coding function myoglobin intron II appeared to evolve at a rate only about 60% faster than that of the RAG-1 gene.

Phylogenetic relationships

The analysis of the concatenated nuclear DNA sequences strongly supports monophyly of the charadriiform order, albeit that the two genes provide markedly less support when analysed individually. Contrary to certain previous suggestions [11,42,43] there is no support for the placement of the sandgrouse or bustard within the charadriiforms. Neither do the data provide strong support for a link between the Columbiformes and Charadriiformes with the two columbiform outgroups (*Pterocles* and *Scardafella*) occurring basal in the maximum-likelihood tree and as part of an unsupported basal polytomy in the maximum-parsimony analysis.

The charadriiform families clearly divide into three clades consistent with DNA-DNA hybridisation data although the relationships among these clades differ from those reported by [8]. One of the best-supported groupings of charadriiform families in the present analysis consists of the Lari (gulls, terns, skimmers and jaegers) along with the

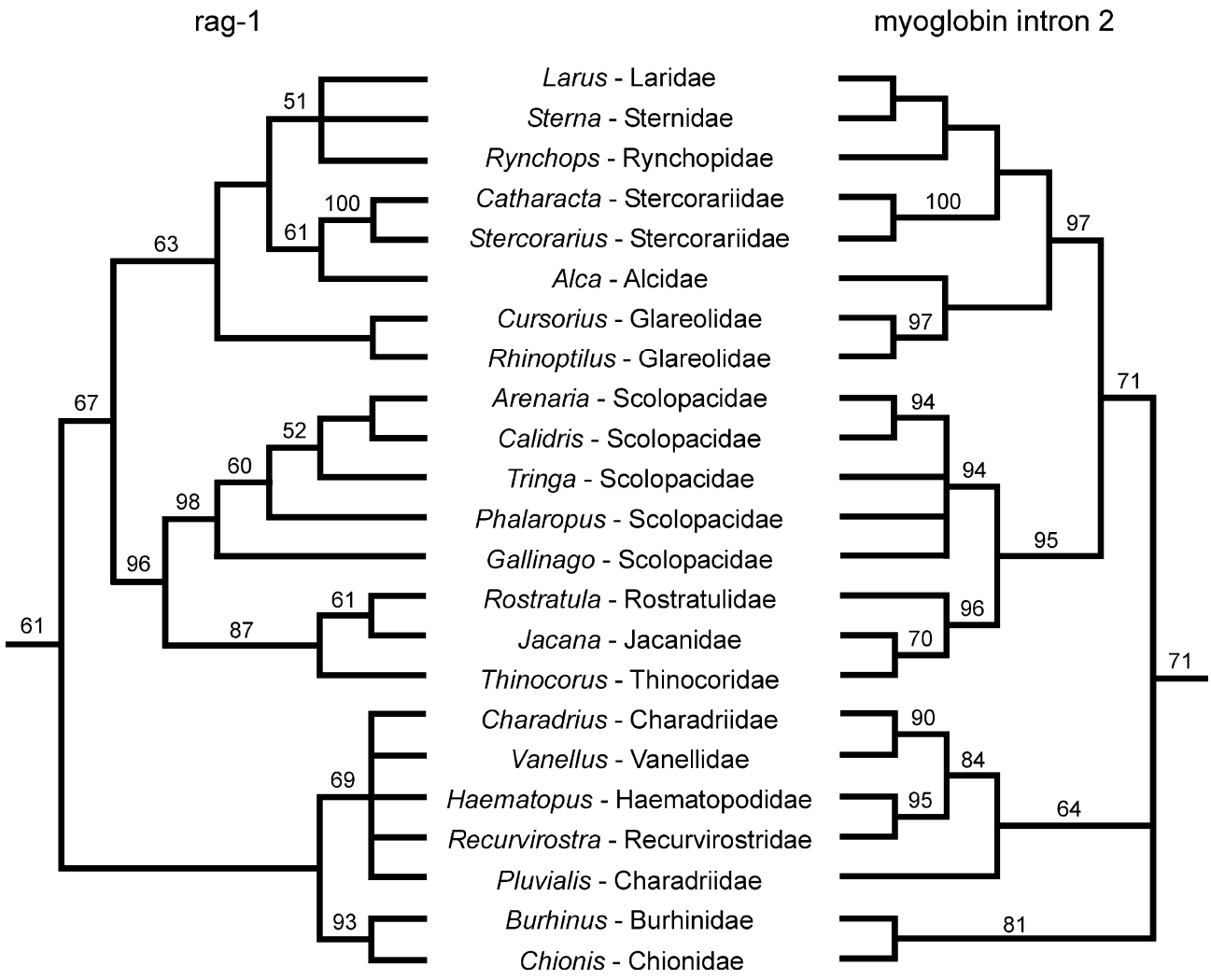

Figure 9
Comparison between the two gene trees Strict consensus trees from parsimony analyses of the RAG-1 and the myoglobin intron II data sets, respectively. The RAG-1 tree is calculated from the 72 most parsimonious trees (length 483 steps, CI 0.70, RI 0.60), and the myoglobin tree is calculated from the 30 most parsimonious trees (length 627 steps, CI 0.73, RI 0.62).

Alcae (auks, puffins, murrelets and allies) and glareolids (coursers and pratincoles). This group constitutes the Laroidea of [8] and has never been suggested as forming a monophyletic group on the basis of morphology. The auks and their allies, considered basal in morphological analyses [4–6], are clearly nested within the Lari and form the sister group to the Stercorariidae (skuas and jaegers) in most analyses. The Glareolidae are basal in this group. Although the taxonomic boundaries of the Glareolidae have been disputed it is most often considered to include six genera *Glareola*, *Cursorius*, *Rhinoptilus*, *Smutsornis*, *Stiltia* and *Pluvianus*, of which only two were studied herein. Given the uncertainty about the monophyly of Glareolidae, it is worth noting that the DNA-DNA hybridization study included two other glareolid genera (*Glareola* and *Stiltia*) than those used herein (*Cursorius* and *Rhinoptilus*).

Relationships within the Lari are not firmly resolved by the present data, although the relationships suggested, with the skimmers (Rynchopidae) as sister to gulls and terns (Laridae and Sternidae) and skuas (Stercorariidae)

basal are identical to the results based on DNA-DNA hybridization and consistent with the morphological analyses of Schnell [44,45]. In comparing 51 measurements from skeletal elements among 93 species of jaegers, skuas, gulls, terns, and skimmers, Schnell concluded that skimmers are most similar to terns, and that the differences between jaegers and gulls are greater than those between gulls and terns.

The remaining Charadrii families included in this study divide into two clades, one comprising the sandpiper-like birds (Scolopacidae, Jacanidae and Thinocoridae), and the other the plover-like birds (Charadiidae, Recurvirostridae, Haemtopodidae) along with sheathbills (Chionidae) and thick-knees (Burhinidae). The sandpiper-like birds unambiguously form the sistergroup to the Lari-Alcae-glareolid clade with strong bootstrap support in both methods of analysis (MP: 81%, Bayesian: 100%). This systematic arrangement is rather unusual. Based on morphology, the gulls and allies have traditionally been placed together with the plover-like birds [3,6], a relationship also suggested by UPGMA analysis of DNA-DNA hybridization data [8]. However, the application of an alternative clustering algorithm (FITCH) to the DNA-DNA hybridization data groups the gull and allies with the sandpiper-like birds [[8]: fig. 337], in accordance with the results of the present study.

The clade containing the sandpiper-like birds includes a well-supported, monophyletic group of scolopacid taxa, and a likewise well-supported group consisting of the jacana, painted snipe and seedsnipe. Relationships among the latter three taxa are unresolved although maximum-likelihood analysis, skeletal morphology [3] and the down patterns of the young [7] suggest a closer relationship between jacanas and painted-snipes. Their sister clade includes all scolopacid representatives included in the study with the snipe (*Gallinago*) as the basal taxon and a sister-relationship between the turnstone (*Arenaria*) and sandpiper (*Calidris*) recovered in all analyses. These scolopacid relationships agree well with the results based on DNA-DNA hybridization data. Neither the low (60%) support for this clade in the maximum-likelihood analysis, nor the likewise low (0.3) delta-T_{50} value in the study of the DNA-DNA hybridization data, allow strong hypotheses about the evolutionary relationships between phalaropes, shanks, and other scolopacids to be formulated.

The remaining clade containing the plover-like birds (plover, lapwing, avocet and oystercatcher), sheathbill and thick-knee is basal in the phylogeny. The intra-relationships of this clade match closely those suggested by DNA-DNA hybridization data, and only the position of the plover *Pluvialis* differs. In the DNA-DNA hybridization study *Pluvialis* groups adjacent to a clade containing the plover (*Charadrius*) and lapwing, while the present data places it basal to all other plover-like birds including the oystercatcher and avocet. The distinctiveness of *Pluvialis* and its basal position relative to other plover-like birds is also supported by the allozyme data of [9]. It is highly unexpected that the oystercatchers and avocets should be nested within the Charadriidae, as suggested by the present data, and this issue needs further investigation.

The analysis of the sequence data places a clade with the thick-knee and sheathbill as the sistergroup to the plover-lapwing-oystercatcher-avocet group, but the branch leading to this larger clade is short and receives no statistical support. The relationship between the thick-knee (*Burhinus*) and sheathbill (*Chionis*) is well supported, however. As with many other systematic relationships supported by the DNA sequence data analysed herein, the grouping of the thick-knee with the sheathbill is unexpected when morphology and general behaviour are taken into consideration. However, the same relationship is strongly suggested by Paton *et al.* [46] who analyzed DNA sequences obtained from the complete RAG-1 gene.

Conclusions

The DNA sequence data obtained from two segments of the nuclear genome provides independent estimates of the phylogenetic relationships of the charadriiform birds. The great similarity between the two resulting phylogenies suggests that the concatenated data set can be used to infer the true evolutionary history of the group. Parsimony and maximum-likelihood analyses of the combined sequences resulted in a well-resolved phylogenetic tree with many strongly supported internodes. The DNA sequence data suggests that the order Charadriiformes is monophyletic, and that the sandgrouses and bustards are not part of it. It is also suggested that the order be divided into two major groups. In the first, a clade of gulls, terns, skimmers, auks and jaegers forms the sistergroup of the coursers and pratincoles, while another clade, consisting of scolopacids (sandpipers and allied groups), jacanas, painted-snipes and seedsnipes, constitutes the sistergroup of them. The second major group of charadriiform birds consists of, on one hand, the plovers, lapwings, oystercatchers and avocets, and the thick-knees and sheathbills on the other. The presented phylogenetic hypothesis provides a solid framework for analyses of macroevolution of ecological and behavioural adaptations observed within the order Charadriiformes.

Materials and Methods

Taxon selection

23 individuals representing 15 of the 18 families of the order Charadriiformes were selected for study (Table 3). As monophyly of Charadriiformes has not been unequiv-

Table 3: Samples used in the study Acronyms: FMNH: Field Museum of Natural History; LSUZM: Museum of Natural Science, Lousiana State University; NMWM: National Museum of Namibia; NRM: Swedish Museum of Natural History; PFIAO: Percy FitzPatrick Institute of African Ornithology, University of Cape Town; TJP: Thomas J. Parsons.

Species	Vernacular name	Family	Sample no.	Origin
Larus fuscus	Lesser Black-backed Gull	Laridae	NRM 946583	Russia
Sterna hirundo	Common Tern	Sternidae	NRM 20016389	Sweden
Rynchops niger	Black Skimmer	Rynchopidae	LSUMZ B-2457	USA
Catharacta maccormicki	South Polar Skua	Stercorariidae	NRM 896303	Antarctica
Stercorarius pomarinus	Pomarine Jaeger	Stercorariidae	NRM 946710	Russia
Alca torda	Razorbill	Alcidae	NRM 986504	Sweden
Cursorius temminckii	Temminck's Courser	Glareolidae	NMWM 796F	Namibia
Rhinoptilus africanus	Double-banded Courser	Glareolidae	NMWM 2127F	Namibia
Arenaria interpres	Ruddy Turnstone	Scolopacidae	NRM 946593	Russia
Calidris melanotos	Pectoral Sandpiper	Scolopacidae	NRM 937394	Paraguay
Phalaropus lobatus	Red-necked Phalarope	Scolopacidae	NRM 976541	Sweden
Tringa totanus	Common Redshank	Scolopacidae	NRM 946526	Sweden
Gallinago gallinago	Common Snipe	Scolopacidae	NRM 20016235	Sweden
Jacana jacana	Wattled Jacana	Jacanidae	NRM 937364	Paraguay
Rostratula benghalensis	Greater Painted-snipe	Rostratulidae	FMNH 358238	Philippines
Thinocorus orbignyanus	Grey-breasted Seedsnipe	Thinocoridae	LSUMZ B-1205	Bolivia
Charadrius collaris	Collared Plover	Charadriidae	TJP	unknown
Pluvialis dominica	American Golden-Plover	Charadriidae	NRM 947050	Paraguay
Vanellus vanellus	Northern Lapwing	Charadriidae	NRM 996200	Sweden
Haematopus ater	Eurasian Oystercatcher	Haematopodidae	TJP	unknown
Recurvirostra avosetta	Pied Avocet	Recurvirostridae	NRM 966075	Sweden
Burhinus bistriatus	Double-striped Thick-knee	Burhinidae	LSUMZ B-19210	Captive
Chionis alba	Snowy Sheathbill	Chionidae	LSUMZ B-9907	Antarctica
Afrotis atra	Black Bustard	Otididae	LSUMZ B-8672	Captive
Grus canadensis	Sandhill Crane	Gruidae	TJP	unknown
Pterocles gutturalis	Yellow-throated Sandgrouse	Pteroclididae	PFIAO 37YtS	South Africa
Scardafella squammata	Scaled Dove	Columbidae	NRM 956728	Paraguay
Chauna torquata	Southern Screamer	Anhimidae	TJP	unknown
Alectura lathami	Australian Brush-turkey	Megapodiidae	LSUMZ B-20851	Captive

ocally demonstrated by morphology, we also included representatives of two taxa that have been proposed to be close to the shorebirds, or nested within them [cf. [11,42,43]]. These are a sandgrouse (Pterocles gutturalis, Pteroclididae) and a bustard (Afrotis atra, Otididae). As the alternative systematic positions of these two taxa are with the pigeons and the gruiform birds, respectively, two representatives of these were also included (Scardafella squammata, Columbidae, and Grus canadensis, Gruidae). As outgroups, we include a screamer (Chauna torquata, Anhimidae) and a megapod (Alectura lathami, Megapodiidae) which are members of the Galloanserae, the presumed sistergroup to Neoaves (sensu [47]) to which the shorebirds belong.

Extraction, amplification, and sequencing

DNA was extracted from tissue or blood specimens using the QIAamp® DNA Mini Kit (QIAGEN®) following the manufacturer's recommendations, or by standard techniques of Proteinase K/SDS digestion followed by phenol chloroform extraction and ethanol precipitation, as described in [48]. Polymerase chain reaction (PCR) amplifications were carried out with Ready-To-Go® PCR Beads (Amersham Pharmacia Biotech, Uppsala, Sweden), with 1 μl of each primer, 22 μl distilled water, and 1 μl template. Different combinations of four primers: R17, R22, R50, and R51 were used for the amplification of RAG-1 [28,29] using a step-down PCR protocol. This involved an initial soak at 94°C for 5 min, followed by four cycles of 94°C for 40 s, 63°C for 60 s, and 72°C for 60 s, followed by four cycles at 60°C annealing and 32 cycles at an annealing temperature of 55°C with the same temperatures and intervals. There was a final soak; 72°C for 5 min. Myoglobin intron II was amplified using the primer Myo2 in combination with Myo3F or Myo3 [31,49]. For some taxa nested PCR was used, whereby the Myo2/Myo3 amplicon was re-amplified with Myo2/Myo3F. Thermocycling procedures for the myoglobin intron began with a soak at 94°C for 5 min, followed by

40 cycles of 94°C for 40 s, 59°C for 40 s and 72°C for 5 min and completed with a final soak at 72°C for 5 min.

The products from the PCR were purified using QIAquick™ PCR purification Kit (QIAGEN®), and then sequenced with Perkin Elmer Applied BioSystems 377 automated fluorescent sequencing instruments, and Perkin Elmer Applied BioSystems PRISM terminator cycle sequencing kits, with AmpliTaq FS polymerase with BigDye terminators. Both strands were sequenced. Sequencing of myoglobin intron II was performed using the primers Myo2, Myo3F, Myoint.c and Myoint.nc [25,31,49]. The segment corresponds to the region between positions 303 (exon 2) and 400 (exon 3) in humans (GenBank XM009949) and includes the complete intron II, as well as 13 and 10 base pairs of the flanking exons 2 and 3, respectively [49]. RAG-1 was sequenced using the primers R50, R51, R52, and R53 [29]. The fragment obtained corresponds to positions 1054–1983 in the published chicken sequence [50].

Sequences for *Chauna torquata* and *Alectura lathami* have been published previously [28,30,51]. New sequences were deposited in GenBank (AY339073-AY339126).

Data analysis

The multiple segments obtained by sequencing with different primers were assembled to complete sequences using SEQMAN II™ (DNASTAR®). When nucleotides could not be unambiguously determined the IUB coding system [52] was used. The combined sequences were then aligned by eye with MEGALIGN™ (DNASTAR®). The sequences are deposited in GenBank (accession numbers AY339073-AY339126).

MEGA 2.0 [53] was used to determine nucleotide variation and divergence values. To investigate the extent of saturation in RAG-1 and the myoglobin intron the number of transitions and transversions were plotted against uncorrected sequence divergence (p-distance). This was done to determine whether RAG-1 should be partitioned in order to down-weight saturated positions and to assess the phylogenetic usefulness of the non-coding myoglobin intron at these taxonomic levels. In addition, the pairwise uncorrected sequence divergences for the two genic regions were plotted against each other to compare their evolutionary properties.

Parsimony and maximum-likelihood analyses were performed using PAUP* 4.0b10 [54], designating *Chauna torquata* and *Alectura lathami* as outgroups. Parsimony analyses were performed for the two genic regions separately, and for both genic regions combined. Searches for most parsimonious trees were done under the heuristic search option, with random additions of taxa and tree bisection-reconnection (TBR) branch-swapping. Ten random additions were performed to reduce the risk of finding local optima only. Data were unweighted and coded as unordered. Gaps were treated as missing values. Strict consensus trees were generated and nodal supports estimated with 1000 bootstrap replicates with a 50% majority rule applied.

MODELTEST 3.06 [55] was used to choose the model of substitutions for the maximum-likelihood analysis of the combined data set. This program determines the simplest model of evolution that cannot be rejected in favour of a more complex one that gives a significantly better tree. The model chosen for the analysis was the GTR + Γ + I time-reversible model for nucleotide substitutions, with six types of substitutions and the proportions of invariable sites and shape parameter alpha estimated (I = 0.212 and α = 1.105). These estimates were used in a heuristic search with TBR branch-swapping. Ten random additions of taxa were performed. A Bayesian inference analysis was performed using MRBAYES 2.01 [56], with the Markov chain Monte Carlo method. 400,000 generations were run and every hundredth tree after stabilisation was saved. The remaining 3000 trees were imported into PAUP* 4.0b10 [54], and posterior probabilities were obtained from the 50% majority-rule consensus tree calculated from these.

Added in proofs

An analysis of DNA sequences of the entire RAG-1 gene (c. 2.9 kb) obtained from representatives of a similar set of taxa as studied herein will shortly be published by Paton *et al.* [46]. The two studies are entirely independent but arrive at very similar conclusions about phylogenetic relationships within the Charadriiformes. The studies complement each other despite having analyzed partly overlapping DNA sequences obtained from the RAG-1 gene. Although we studied a shorter stretch (930 bp) of the RAG-1 gene than did Paton *et al.*, we also included information from a second nuclear marker (myoglobin intron II). The low degree of conflicts observed between the RAG-1 and myoglobin gene trees, along with the general agreement between the studies of Paton *et al.* and ourselves, lend confidence to the hypothesis that the observed phylogenetic patterns accurately reflect the evolutionary history of the shorebirds.

Authors' contributions

Author 1 (PE) designed and conceived the study, participated in the phylogenetic analyses and drafted the manuscript. Author 2 (IP) carried out the labwork, sequence alignments and initial phylogenetic analyses in partial fulfilment of a master's degree in zoological systematics at the University of Stockholm. Author 3 (MI) assisted in the design of the study and supervised the labwork and

sequence alignments. Author 4 (JN) developed laboratory protocols to enable the use of the myoglobin intron in analyses of higher-level relationships in birds and assisted with drafting, revisions and editing of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1
Table 2 – Observed pairwise sequence distancesFile name: Table 2.pdf
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2148-3-16-S1.pdf]

Acknowledgements

We thank the Field Museum of Natural History, Chicago; Museum of Natural Science, Louisiana State University; National Museum of Namibia, Windhoek; Percy FitzPatrick Institute of African Ornithology, University of Cape Town; Swedish Museum of Natural History; and Thomas J. Parsons for providing tissue. We are also thankful to Ulf S. Johansson, Pia Eldenäs and Mari Källersjö for help and advice in the laboratory. Thanks also to Les Christidis, Christer Erseus, Niklas Janz, Ulf S. Johansson and two anonymous reviewers for commenting on the manuscript. This work has been funded by the Swedish Research Council (grant no. 621-2001-2773 to P.E.) and the Swedish Museum of Natural History.

References

1. del Hoyo J, Elliott A, Sargatal J and Eds: *Handbook of the Birds of the World, volume 3. Hoatzin to Auks Barcelona: Lynx editions*; 1996.
2. Peters JL: *Check-list of Birds of the World Volume 2. Cambridge: Harvard University Press*; 1934.
3. Strauch JG Jr: **The phylogeny of the Charadriiformes (Aves): A new estimate using the method of character compatibility analysis.** *Transactions of the Zoological Society of Lond* 1978, **34:**263-345.
4. Mickevich MF and Parenti LR: **Review of, "The phylogeny of the Charadriiformes (Aves): a new estimate using the method of character compatibility analysis.".** *Systematic Zoology* 1980, **29:**108-113.
5. Björklund M: **Phylogenetic relationships among Charadriformes: reanalysis of previous data.** *Auk* 1994, **111:**825-832.
6. Chu PC: **Phylogenetic reanalysis of Strauch's osteological data set for the Charadriiformes.** *Condor* 1995, **97:**174-196.
7. Jehl JR Jr: **Relationships in the Charadrii (shorebirds): a taxonomic study based on color patterns of the downy young.** *San Diego Soc Nat Hist Memoir* 1968, **3:**1-54.
8. Sibley CG and Ahlquist JE: *Phylogeny and classification of Birds. A study in molecular evolution New Haven: Yale University Press*; 1990.
9. Christian PD, Christidis L and Schodde R: **Biochemical systematics of the Charadriiformes (shorebirds): relationships between the Charadrii, Scolopaci and Lari.** *Australian Journal of Zoology* 1992, **40:**291-302.
10. Hope S: **The Mesozoic radiation of Neornithes.** In *Mesozoic Birds: Above the Heads of Dinosaurs* Edited by: *Chiappe LM, Witmer L. Berkeley: University of California Press*; 2002:339-388.
11. Olson SL: **The fossil record of birds.** In *Avian Biology 8* Edited by: *Farner DS, King JR, Parkes KC. New York: Academic Press*; 1985:79-238.
12. Hou L and Ericson PGP: **A Middle Eocene shorebird from China.** *Condor* 2002, **104:**896-899.
13. Cooper A and Penny D: **Mass survival of birds across the Cretaceous-Tertiary boundary: molecular evidence.** *Science* 1997, **275:**1109-1113.
14. van Tuinen M and Hedges SB: **Calibration of avian molecular clocks.** *Molecular Biology and Evolution* 2001, **18:**206-213.
15. Cracraft J: **Avian evolution, Gondwana biogeography and the Cretaceous-Tertiary mass extinction event.** *Proceedings of the Royal Society of London Ser B* 2001, **268:**459-469.
16. Ericson PGP, Christidis L, Cooper A, Irestedt M, Jackson J, Johansson US and Norman JA: **A Gondwanan origin of passerine birds supported by DNA sequences of the endemic New Zealand wrens.** *Proceedings of the Royal Society of London Ser B* 2002, **269:**235-241.
17. Paton T, Haddrath O and Baker AJ: **Complete mitochondrial DNA genome sequences show that modern are not descended from transitional shorebirds.** *Proceedings of the Royal Society of London Ser B* 2002, **269:**839-846.
18. Feduccia A: *The Origin and Evolution of Birds New Haven: Yale University Press*; 1996.
19. van Tuinen M, Sibley CG and Hedges SB: **The early history of modern birds inferred from DNA sequences of nuclear and mitochondrial ribosomal genes.** *Molecular Biology and Evolution* 2000, **17:**451-457.
20. García-Moreno J and Mindell DP: **Rooting a phylogeny with homologous genes on opposite sex chromosomes (gametologs): a case study using avian CHD.** *Molecular Biology and Evolution* 2000, **17:**1826-1832.
21. Fleischer RC, McIntosh CE and Tarr CE: **Evolution in a volcanic conveyor belt: using phylogeographic reconstructions and KAr-based ages of the Hawaiian Islands to estimate molecular evolutionary rates.** *Molecular Ecology* 1998, **7:**533-545.
22. Moore WJ and DeFillippis VR: **The window of taxonomic resolution for phylogenies based on mitochondrial cytochrome *b*.** In *Avian Molecular Evolution and Systematics* Edited by: *Mindell DP. San Diego: Academic Press*; 1997:84-119.
23. Hunt JS, Bermingham E and Ricklefs RE: **The molecular systematics and biogeography of antillean thrashers, tremblers, and mockingbirds (Aves: Mimidae).** *Auk* 2001, **118:**35-55.
24. Ericson PGP, Christidis L, Irestedt M and Norman JA: **Systematic affinities of the lyrebirds (Passeriformes: *Menura*), with a novel classification of the major groups of passerine birds.** *Molecular Phylogenetics and Evolution* 2002, **25:**53-62.
25. Irestedt M, Fjeldså J, Johansson US and Ericson PGP: **Systematic relationships and biogeography of the tracheophone suboscines (Aves: Passeriformes).** *Molecular Phylogenetics and Evolution* 2002, **23:**499-512.
26. Pamilo P and Nei M: **Relationships between gene trees and species trees.** *Molecular Biology and Evolution* 1988, **5:**568-583.
27. Avise JC: **Gene trees and organismal histories: a phylogenetic approach to population biology.** *Evolution* 1989, **43:**1192-1208.
28. Groth JG and Barrowclough GF: **Basal divergences in birds and the phylogenetic utility of the nuclear RAG-1 gene.** *Molecular Phylogenetics and Evolution* 1999, **12:**115-123.
29. Irestedt M, Johansson US, Parsons TJ and Ericson PGP: **Phylogeny of major lineages of suboscines (Passeriformes) analysed by nuclear DNA sequence data.** *Journal of Avian Biology* 2001, **32:**15-25.
30. Johansson US, Parsons TJ, Irestedt M and Ericson PGP: **Clades within "higher land birds", evaluated by nuclear DNA sequences.** *Journal of Zoological Systematics and Evolutionary Research* 2001, **39:**37-51.
31. Slade RW, Moritz C, Heideman A and Hale PT: **Rapid assessment of single-copy nuclear DNA variation in diverse species.** *Molecular Ecol* 1993, **2:**359-373.
32. Lessa EP: **Rapid surveying of DNA sequence variation in natural populations.** *Molecular Biology and Evolution* 1992, **9:**323-330.
33. Friesen VL, Congdon BC, Walsh HE and Birt TP: **Intron variation in marbled murrelets detected using analyses of single-stranded conformational polymorphisms.** *Molecular Ecology* 1997, **6:**1047-1058.
34. Congdon BC: **Mechanisms of population differentiation in marbled murrelets: historical versus contemporary processes.** *Evolution* 2000, **54:**974-986.
35. Johnson KP and Clayton DH: **A molecular phylogeny of the dove genus *Zenaida*: Mitochondrial and nuclear DNA sequences.** *Condor* 2000, **102:**864-870.
36. Johnson KP and Clayton DH: **Nuclear and mitochondrial genes contain similar phylogenetic signal for pigeons and doves (Aves: Columbiformes).** *Molecular Phylogenetics and Evolution* 2000, **14:**141-151.

37. Prychitko TM and Moore WS: **Comparative evolution of the mitochondrial cytochrome b gene and nuclear beta-fibrinogen intron 7 in woodpeckers.** *Molecular Biology and Evolution* 2000, **17:**1101-1111.
38. Johnson KP, de Kort S, Dinwoodey K, Mateman AC, ten Cate C, Lessells CM and Clayton DH: **A molecular phylogeny of the dove genera *Streptopelia* and *Columba*.** *Auk* 2001, **118:**874-887.
39. Nicholls JA: **Molecular systematics of the thornbills, *Acanthiza*.** *Emu* 2001, **101:**33-37.
40. Weibel AC and Moore WS: **A test of a mitochondrial gene-based phylogeny of woodpeckers (genus *Picoides*) using an independent nuclear gene, beta-fibrinogen intron 7.** *Molecular Phylogenetics and Evolution* 2002, **22:**247-257.
41. Saetre GP, Borge T, Lindroos K, Haavie J, Sheldon BC, Primmer CR and Syvanen AC: **Sex chromosome evolution and speciation in *Ficedula* flycatchers.** *Proceedings of the Royal Society of London. Ser. B.* 2003, **270:**53-59.
42. Maclean GL: **Die systematische Stellung der Flughühner (Pteroclididae).** *Journal für Ornithologie* 1967, **108:**203-217.
43. Hendrickson HT: **A comparative study of egg white proteins of some species of the avian order Gruiformes.** *Ibis* 1969, **111:**80-91.
44. Schnell GD: **A phenetic study of the suborder Lari (Aves) I: Methods and results of principal component analyses.** *Systematic Zoology* 1970, **19:**35-57.
45. Schnell GD: **A phenetic study of the suborder Lari (Aves) II. Phenograms, discussion, and conclusions.** *Systematic Zoology* 1970, **19:**264-302.
46. Paton TA, Baker AJ, Groth JG and Barrowclough GF: **RAG-1 sequences resolve phylogenetic relationships within Charadriiform birds.** *Molecular Phylogenetics and Evolution* .
47. Sibley CG and Monroe BL Jr: *Distribution and taxonomy of birds of the world New Haven: Yale University Press*; 1990.
48. Norman JA, Christidis L, Westerman M and Hill AR: **Molecular analysis confirms the species status of the Christmas Island Hawk-Owl (*Ninox natalis*).** *Emu* 1998, **98:**197-208.
49. Heslewood MM, Elphinstne MS, Tidemann SC and Baverstock PR: **Myoglobin intron variation in the Gouldian Finch *Erythura gouldiae* assessed by temperature gradient gel electrophoresis.** *Electrophoresis* 1998, **19:**142-151.
50. Carlson LM, Oettinger MA, Schatz DG, Masteller EL, Hurley EA, McCormack WT, Baltimore D and Thompson CB: **Selective expression of RAG-2 in chicken b cells undergoing immunoglobulin gene conversion.** *Cell* 1991, **64:**201-208.
51. Johansson US and Ericson PGP: **Molecular support for a sister group relationship between Pici and Galbulae (Piciformes sensu Wetmore 1960).** *Journal of Avian Biology* 2003, **34:**185-197.
52. IUB Nomenclature Committee: *European Journal of Biochemistry* 1985, **150:**1-5.
53. Kumar S, Tamura K, Jakobsen IB and Nei M: **MEGA2: molecular evolutionary genetics analysis software.** *Bioinformatics* 2001, **17:**1244-1245.
54. Swofford DL: *PAUP*: Phylogenetic analysis using parsimony (* and other methods), version 4.0 Sunderland: Sinauer*; 1998.
55. Posada D and Crandall KA: **MODELTEST: testing the model of DNA substitution.** *Bioinformatics* 1998, **14:**817-818.
56. Huelsenbeck JP, Ronquist F and Hall B: **MrBayes: Bayesian inference of phylogeny.** *Bioinformatics* 2001, **17:**754-55.