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Beyond phylogeny: pelecaniform and ciconiiform birds, and long-term niche stability

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ABSTRACT

Phylogenetic trees are a starting point for the study of further evolutionary and ecological questions. We show that for avian evolutionary relationships, improved taxon sampling, longer sequences and additional data sets are giving stability to the prediction of the grouping of pelecaniforms and ciconiiforms, thus allowing inferences to be made about long-term niche occupancy. Here we report the phylogeny of the pelecaniform birds and their water-carnivore allies using complete mitochondrial genomes, and show that the basic groupings agree with nuclear sequence phylogenies, even though many short branches are not yet fully resolved. In detail, we show that the Pelecaniformes (minus the tropicbird) and the Ciconiiformes (storks, herons and ibises) form a natural group within a seabird water-carnivore clade. We find pelicans are the closest relatives of the shoebill (in a clade with the hammerkop), and we confirm that tropicbirds are not pelecaniforms. In general, the group appears to be an adaptive radiation into an 'aquatic carnivore' niche that it has occupied for 60–70 million years. From an ecological and life history perspective, the combined pelecaniform–ciconiform group is more informative than focusing on differences in morphology. These findings allow a start to integrating molecular evolution and macroecology.

1. Introduction

What do we do when we have solved the tree of life? While a phylogeny is informative in itself, the interpretations that can be drawn from a stable phylogeny are even more interesting, and should help focus our goals for future work – beyond phylogeny. When phylogenetic agreement has been reached by multiple datasets, it is possible to use this framework to ask broader questions about macroecology and long-term ecological niche stability (Poole et al., 2003). Here we use mitochondrial genomes to investigate the relationships within the 'water bird' clade proposed by Hackett et al. (2008), particularly focusing on the traditional pelecaniform and ciconiiform taxa. We ask whether some basic phylogenetic agreement has been reached between mitochondrial and nuclear data, and use this to investigate whether the group has occupied an essentially consistent niche space, with species turnover within it, for a very long time (many tens of millions of years).

In this context, we are more concerned with general niche stability that can operate at several nested levels, rather than a more specific species-level niche occupancy. If, as we hypothesize (and it is consistent with nuclear data, Hackett et al., 2008), the Pelecaniformes and Ciconiiformes form a natural group within a larger framework of water-carnivores, what can be said about the long-term life history of the whole group? Looking to the future, our longer-term goal is the integration of evolutionary trees with a range of life history traits (see Kennedy et al., 1996; Paterson et al., 1995; Slikas, 1998).

1.1. Consilience of induction

To have confidence in our findings it is important in general to have agreement among multiple datasets. Datasets are more likely to agree as taxon sampling and information content increases. For example Paton and Baker, 2006 showed that relationships within the Charadriiformes were resolvable with long mitochondrial sequences whereas shorter mitochondrial sequences had been unable to resolve the groupings. Additionally, their results agreed with independent findings from nuclear data (Paton et al., 2003). At deeper phylogenetic levels we have also found good agreement between mitochondrial and nuclear datasets (Hackett et al., 2008; McCormack et al., 2013; Pacheco et al., 2011; Pratt et al., 2009; Slack et al., 2007). We predict this will be the case for the water-carnivores and their allies as well.

The problem of deep avian phylogeny resolution is especially compounded because many internodes in the deeper neoavian tree

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branches are relatively short (Hackett et al., 2008; Pratt et al., 2009). Nevertheless, if there is an adaptive radiation (see Gavrilets and Losos, 2009; Gibb et al., 2007) we may find that a group as a whole may be stable, even if the group has short internal branches within it that are not yet stably resolvable. In such cases, progress can be made by focusing on the adaptive radiation, rather than just on the details of the phylogeny.

Recent nuclear-based studies have increased taxon sampling and sequence length for the water-carnivores (Ericson et al., 2006; Hackett et al., 2008). However, further investigation is required, as a high proportion of current nuclear data comes from introns, which could be problematic to align at deeper divergences (Morgan-Richards et al., 2008; Saurabh et al., 2012; Wong et al., 2008). Nevertheless, introns are very useful for resolving within-family level phylogenies (e.g. de Kloet and de Kloet, 2005). Importantly, the findings of these nuclear-based studies provide phylogenetic predictions we can test with whole mitochondrial genomes, and vice versa.

Traditionally, the Pelecaniformes were considered a monophyletic group comprising the frigatebirds (Fregatidae), tropicbirds (Phaethontidae), pelicans (Pelecanidae), darters (Anhingidae), cormorants/shags (Phalacrocoracidae), and gannets/boobies (Sulidae). Despite its 'divergency of structure' (Beddard, 1898), this group was apparently united by morphological characters including totipalmate feet (all four toes being connected by a web), gular pouch, lack of a brood patch, and the salt gland being completely enclosed within the orbit (rather than being in a cavity on top of the skull) (Cracraft, 1985; Hedges and Sibley, 1994; Nelson, 2005; summarized in Table 1). The discovery that this group may not be monophyletic lead Sibley and Ahlquist (1990) to suggest that the Pelecaniformes 'may present the most complex and controversial questions in the avian phylogeny'.

Table 1
Taxa used in these analyses.

Scientific name	Common name	GenBank ID	Genome size	Duplicated region?
Accipiter gentilis	Northern goshawk	AP010797	18,266	Remnant CR2
Aegotheles cristatus cristatus	Australian owlet-nightjar	EU344979	18,607	No
Alectura lathami	Australian brush-turkey	AY346091	16,698	No
Anhinga rufa	African darter	GU071055[a]	19,385	1/2 cytb-CR
Anseranas semipalmata	Magpie-goose	AY309455	16,870	No
Apus apus	Common swift	AM237310	17,037	No
Archilochus colubris	Ruby-throated hummingbird	EF532935	16,356	No
Ardea novaehollandiae	White-faced heron	DQ780878	17,511	1/2 cytb-CR
Arenaria interpres	Ruddy turnstone	AY074885	16,725	No
Aythya americana	Redhead duck	AF090337	16,616	No
Balaeniceps rex	Shoebill	GU071053[a]	15,752[b]	Unknown
Buteo buteo	Common buzzard	AF380305	18,674	Remnant CR2
Ciconia boyciana	Oriental white stork	AB026193	17,622	No
Ciconia ciconia	White stork	AB026818	17,347	No
Thalassarche (Diomedea) melanophris	Black browed albatross	AY158677	18,976	tThr-CR
Diomedea chrysostoma	Grey-headed albatross	AP009193	14,877[b]	Unknown
Egretta eulophotes	Chinese egret	EU072995	17,579	No[c]
Eudyptes chrysocome	Rockhopper penguin	AP009189	16,930[b]	No
Eudyptula minor	Little blue penguin	AF362763	17,611	No
Fregata sp.	Frigatebird	AP009192	14,790[b]	Unknown
Gallus gallus	Chicken	AP003317	16,788	No
Gavia pacifica	Pacific loon	AP009190	15,574[b]	Unknown
Gavia stellata	Red-throated loon	AY293618	17,573	No
Haematopus ater	Blackish oystercatcher	AY074886	16,791	No
Ixobrychus cinnamomeus	Cinnamon bittern	HQ690247	17,180	No[c]
Larus dominicanus	Southern black-backed gull	AY293619	16,701	No
Morus serrator	Australasian gannet	GU071056[a]	19,285	1/2 cytb-CR
Mycteria americana	Wood stork	AY274076, DQ433030, AF082066, DQ485797	7497[b]	Unknown
Nipponia nippon	Crested ibis	AB104902	16,732	No[c]
Nisaetus alboniger	Blythe's hawk eagle	AP008239	17,977	Remnant CR2
Nisaetus nipalensis	Mountain hawk eagle	AP008238	17,667	Remnant CR2
Nycticorax nycticorax	Black-crowned night-heron	JN018412	17,829	No[c]
Pandion haliaetus	Osprey	DQ780884	17,864	CR
Pelagodroma marina	White-faced storm petrel	KC875856[a]	17,360[b]	Probably 1/2 cytb-CR
Pelecanus conspicillatus	Australian pelican	DQ780883	16,846[b]	1/2 cytb-CR
Phaethon rubricauda	Red-tailed tropicbird	AP009043	17,777	No
Phalacrocorax chalconotus	Stewart Island shag	GU071054[a]	19,073	1/2 cytb-CR
Phoenicopterus ruber roseus	Greater flamingo	EF532932	17,446	Remnant CR
Platalea leucorodia	Eurasian spoonbill	GQ199608	16,715	No[c]
Platalea minor	Black-faced spoonbill	EF455490	16,918	1/2 cytb-CR[d]
Podiceps cristatus	Great crested grebe	AP009194	16,134[b]	No
Procellaria cinerea	Grey petrel	AP009191	14,818[b]	Unknown
Pterodroma brevirostris	Kerguelen petrel	AY158678	16,414	No
Pygoscelis adeliae	Adélie penguin	KC875855[a]	17,486	No
Scopus umbretta	Hammerkop	AF339360, EU372682, U08936	6,324[b]	Unknown
Spilornis cheela	Crested serpent eagle	JN191388	18,291	CR
Sula dactylatra	Masked booby	KC875857[a]	13,205	1/2 cytb-CR
Synthliboramphus antiquus	Ancient murrelet	AP009042	16,730	No
Tachybaptus novaehollandiae	Australasian little grebe	EF532936	18,002	No
Threskiornis aethiopicus	Sacred ibis	GQ358927	16,960	No[c]
Tigrisoma fasciatum	Fasciated tiger-heron	EU167034, EU166937, EU166980	4220[b]	Unknown

[a] New mitochondrial genomes reported in this publication.
[b] Genome is not full length.
[c] Suggest duplication exists, but was not detected.
[d] Genbank accession has no duplication, but Cho et al. (2009) report the duplication.

From recent studies (morphological, mitochondrial and nuclear) it appears that the main pelecaniform families are closely associated with ciconiiform families (broadly speaking, storks, herons and ibises), within a larger adaptive radiation of aquatic and semi-aquatic carnivores (Cracraft, 2001; Hackett et al., 2008; Pratt et al., 2009; van Tuinen et al., 2001). This wider seabird water-carnivore clade probably also includes the tubenoses, loons and penguins, but probably not the shorebirds nor flamingoes and grebes. Deeper nodes in this clade are often poorly resolved (e.g. Brown et al., 2008; Ericson et al., 2006). We expect that longer sequences and improved taxon sampling will improve this resolution, but nevertheless, the group as a whole is strongly supported and that is what we test here.

The monophyly of the 'core pelecaniforms' (this includes the cormorants, darters and sulids, though ironically not the pelicans) has never really been in question (Cracraft, 1985; Kennedy and Spencer, 2004; Nelson, 2005). It is now also clear the frigatebirds are the deepest branch within this group. Recently authors have suggested both Suliformes and Phalacrocoraciformes as names to describe this group of non-pelican pelecaniforms (Christidis and Boles, 2008; Gill and Donsker, 2012). Until the naming settles down, we refer to this group as the core pelecaniforms. While molecular studies have in the past had varying degrees of coverage with taxon sampling and resolution, a molecular consensus is forming regarding the pelicans being closely allied with the ciconiiform shoebill and hammerkop, and the tropicbirds being very distantly related to other pelecaniform birds (e.g. Hackett et al., 2008 with nuclear data).

In this study, we use complete mitochondrial DNA sequences to test hypotheses about the phylogenetic relationships within the water-carnivores, and particularly the Pelecaniformes and Ciconiiformes. We report seven new mitochondrial genomes, and analyze the most comprehensive water-carnivore molecular dataset to date. With these analyses, we demonstrate the extent to which the mitochondrial, nuclear and morphological results agree or disagree for water-carnivore phylogeny, and the implications the results have for interpreting the morphology and life history of this seabird water-carnivore group. We find that the group appears to have occupied a 'water-carnivore' role for many tens of millions of years, diverging from the shorebird water-carnivores about 70 million years ago, before the K/Pg impact that marks the end of the Cretaceous.

2. Material and methods

2.1. Taxon sampling, DNA extraction, PCR and sequencing

Species sampled were *Morus serrator* (Australasian gannet), *Anhinga rufa* (African darter), *Phalacrocorax chalconotus* (Stewart Island shag,), *Sula dactylatra* (masked booby), *Balaeniceps rex* (shoebill), *Pelagodroma marina* (white-faced storm petrel), and *Pygoscelis adeliae* (adélie penguin). Total genomic DNA was extracted from the samples using standard phenol/chloroform extraction and ethanol precipitation. Mitochondrial genomes were amplified in 2–3 overlapping long-range fragments, which were subsequently amplified in shorter 0.5–3 Kb fragments using primers from our database. Doing long-range PCR first reduces the possibility of sequencing nuclear copies of mtDNA (numts). Primer details can be found in Table S1. Within each genome, sequences were aligned and manually checked using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI).

2.2. Species for analysis

In addition to the seven new sequences, mitochondrial genomes from all water-carnivore taxa available on GenBank were included in this study. This resulted in a dataset of 27 seabird genomes. Shorter mitochondrial DNA sequences are available for three key additional taxa (hammerkop, tiger-heron and woodstork), and these were included in some analyses. These sequences mostly overlap among species, and total 3.5–5.7 Kb for each species. Sequences included are from 12S to COI, Atp6/8 and cyt*b*.

As well as water-carnivores, our dataset contains representatives from groups that have been suggested to be their close relatives, and that could potentially disrupt the group. There are indications that the tropicbird may not group with any of these families, therefore shorebirds (Charadriiformes), flamingo and grebe (Mirandorniths), hummingbird, swift and owlet nightjar (Apodiformes and Caprimulgiformes) were also added as potential relatives for the tropicbird (see Hackett et al., 2008). In previous work (Gibb et al., 2007), we suggested that some raptors (here eagles, hawks and osprey, Accipitriformes) may group with the water-carnivores, and this is also tested here. Four Galloanseres species were also used as a known outgroup to all these taxa (Hackett et al., 2008; Slack et al., 2007). The complete dataset comprises 48 taxa, and a full list of species and accession numbers is provided in Table 1.

2.3. Alignment

Sequences were aligned by gene and checked using Geneious 5.5.7 (Drummond et al., 2011) at the amino acid level for protein coding genes and based on stem loop secondary structure for rRNA genes (Gutell et al., 1993; Springer and Douzery, 1996) and tRNA cloverleaf pattern. A conservative alignment procedure was used where gaps, ambiguous alignments next to gaps, NADH6 (light strand encoded), non-coding regions and stop codons are excluded from the final alignment. Conserved amino acid and stem columns were used to define the boundaries of ambiguous regions next to gaps. The full dataset is 13,544 nucleotides long. Third codon positions in protein coding regions are coded as RY (as explained in Gibb et al., 2007; Phillips et al., 2010). The alignments and trees are available from TreeBASE (TB2:S14121).

2.4. Phylogenetic analyses and tree building

Maximum Likelihood with rapid bootstrapping was implemented in RAxML using a general time reversible model with gamma distribution (GTR + G) (Stamatakis et al., 2008). Bayesian posterior probabilities (bpp) were estimated in MrBayes (Huelsenbeck and Ronquist, 2001) using 5,000,000 generations and a burn in of 10%. For RAxML and MrBayes analyses the dataset was partitioned into codon positions (protein) and stems and loops (RNA) (Harrison et al., 2004; Powell et al., 2013). Analyses were performed using the CIPRES Science Gateway (Miller et al., 2010). Trees were viewed using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/ [accessed September 2012]) and SplitsTree 4.8 (Huson and Bryant, 2006).

2.5. Molecular dating

Dated analyses were performed using BEAST v1.6.2 (Drummond and Rambaut, 2007) with the 48-taxon dataset partitioned as for Bayesian analyses. The XML file was generated using BEAUti v1.6.2 (Drummond and Rambaut, 2007) and with subsequent modification by hand. An uncorrelated relaxed clock model was used with rates among branches distributed according to a lognormal distribution (Drummond et al., 2006). Nucleotide partitions used an estimated GTR + I + G model; for the RY partition the at-gc scale operators and delta exchange were removed. The following dates and calibration priors were used following the recommendations of Ho and Phillips (2009). The root prior had a normal distribution of 66–121 Ma (98% range). Galloanserae had normal distribution of

66–86 Ma (95% range), with the minimum based on *Vegavis* (Clarke et al., 2005). The sphenisciform stem prior had a normal distribution of 61.5–65.5 Ma (95% range), based on the Waimanu penguin fossils (Slack et al., 2006). Runs totaling 30,000,000 MCMC generations ensured ESS values >200 (as estimated in Tracer v 1.5 Rambaut and Drummond, 2003). Chains were sampled every 5000th generation after removing a burnin of at least 10% (3,000,000 generations). Trees were viewed using Figtree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/ [accessed September 2012]).

Ancestral state reconstruction was performed in the Mesquite package (Maddison and Maddison, 2011) using the default Markov k-state 1 (Mk1) parameter model was for Maximum Likelihood character state reconstructions with equal probability for any particular character state change. General foraging modes were sourced from Schreiber and Burger (2002). The tree used for the reconstruction is the BEAST chronogram, pruned so only seabird water-carnivore taxa remain.

3. Results and discussion

3.1. New mitochondrial genomes and gene orders

We report seven new mitochondrial genomes; genome size, gene order and accession numbers can be found in Table 1. The core pelecaniforms have a duplicated mitochondrial gene order (see Gibb et al., 2007; Mindell et al., 1998), with the duplicated region spanning half of cyt*b*, and all of tRNA Thr, tRNA Pro, NADH 6, tRNA Glu and the control region (Fig. 1a). The booby mitochondrial genome is incomplete, as the DNA was too degraded to sequence through the control regions. However Morris-Pocock et al. (2010) have shown the same gene duplication is also present in boobies. It is unknown if this gene duplication is also present in the frigatebird, because the frigatebird mitochondrial genome retrieved from GenBank (AP009192) does not have the full control region.

3.2. Implications of core pelecaniform gene duplications for phylogenetic studies

Discovery of gene duplications around the control region of avian mitochondrial genomes is now becoming so common it could almost be considered the default state (Table 1, Gibb et al., 2007; Pacheco et al., 2011; Sammler et al., 2011; Singh et al., 2008). The three complete pelecaniform genomes are all over 19 Kb in length, and are among the longest avian mitochondrial genomes reported to date. All three genomes show evidence of concerted evolution, in that the duplicated coding regions are nearly identical within each individual, but very different between the three genera (shown in Fig. 1b). These mitochondrial gene duplications will need to be taken into consideration for pelecaniform population level studies, which often use cyt*b* and the control region in their analyses. It is possible that structure within a population could be inferred incorrectly because of differential amplification success between the two control regions (see also Morris-Pocock et al., 2010).

There is a high probability that other sequenced taxa in the pelecaniform-ciconiiform group also have an undetected gene duplication around the control region. The pelican and the heron (sequenced previously by our group) showed indications of a potential duplication (Gibb et al., 2007) but numts (nuclear copies of mtDNA) could not be completely excluded, and the quality and quantity of the DNA available precluded extensive testing. The shoebill did not show evidence of duplication but again DNA quality was poor. Other groups have sequenced all remaining mitochondrial genomes in the Pelecaniformes and Ciconiiformes, and we do not know if a gene duplication was tested for. In some cases only the coding regions of the mitochondrial genome have been published (e.g. Watanabe et al., 2006). The nature of the duplication means that if it is not deliberately excluded by testing 'otherwise incompatible' PCR primers pairs (i.e., pairs that bind in sites that would only amplify products if a duplication is present), an apparently complete mitochondrial genome can be assembled, and a duplication may go undetected (Gibb et al., 2007). This appears to be the case for the spoonbills, where the published genomes do not have a duplication, but more recent work shows duplications are clearly present (Cho et al., 2009).

In the wider water-carnivore clade, duplication has not been detected in penguins and loons, but a similar duplication is present in albatross (with only 50 bp of cytb duplicated, Abbott et al., 2005), it may be present in petrels (Lawrence et al., 2008) and we found evidence for a duplication in storm petrels, although the control region was not completely mapped. Alternative dupli-

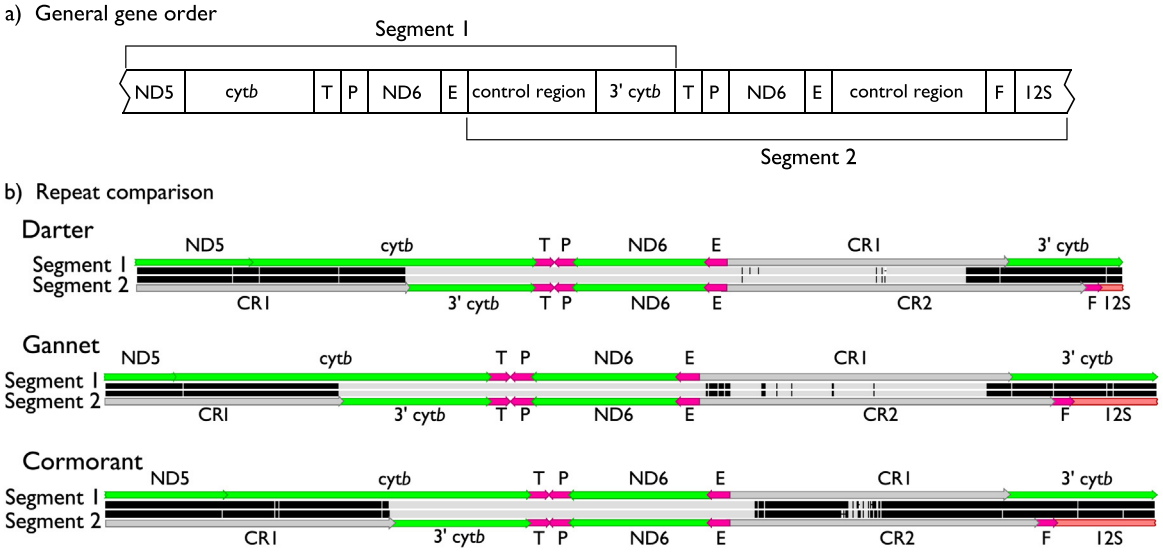

Fig. 1. (a) Mitochondrial gene order from ND5 to 12S showing the duplicated gene regions. (b) Comparison of the duplicated regions within each species by aligning segments 1 and 2. Pale grey regions indicate identical sequences, black regions indicate a mis-match.

cation/rearrangement patterns have also been detected in a number of other more distant avian lineages (Gibb et al., 2007; Mindell et al., 1998). To discover how, how often, and when, these duplications have evolved thus requires further explicit testing.

3.3. Phylogenetic analyses

We begin by analyzing the full-length mitochondrial dataset (48 taxa, Fig. 2). As expected, all ordinal level relationships are highly supported except those within the Pelecaniformes and Ciconiiformes (explored further below). Above the ordinal level, the previously identified flamingo-grebe clade, and the hummingbird, nightjar, swift clade are also well supported. In agreement with nuclear studies, we find good support separating the water-carnivores from other clades in similar niches, namely the flamingo-grebes, shorebirds, accipiters and the tropicbird (87% bootstrap, 1.0 bpp).

As a cautionary note, we have not sampled widely enough outside the water-carnivores to comment on the precise relationships between our outgroups, and can only say the tropicbird clearly does not group with the other pelecaniforms. Because the tropic bird is a single long branch, the current weak grouping of tropicbird and accipiters is possibly an artifact of 'long branch attraction' (Kennedy et al., 2005; Slack et al., 2007), and has not been seen by nuclear or morphological studies.

We then looked more closely at the relationships within the two uncertain orders, Pelecaniformes and Ciconiiformes. In addition to the 17 complete genomes within this clade, we also included three key species with shorter DNA sequences (3.5–5.7 Kb) in a reduced 20 taxon dataset. While adding taxa with shorter sequences increases the amount of missing data in the dataset, this is balanced against the advantage of breaking long branches within the radiation. Most of the branches have very good support, and are in agreement with nuclear and morphological findings, however there are a few key regions where mitochondrial DNA sequences show local rearrangement/conflict (Fig. 3). Firstly, within the core pelecaniforms there is some signal for darters as sister group to gannets and boobies (30% bootstrap support), although the majority links darters with cormorants (66% bootstrap support), this latter topology is also found with nuclear data (Hackett et al., 2008). These conflicting signals were also found with shorter mitochondrial sequences, where long-branch attraction made it difficult to resolve the short internal branch that separates the three families (Kennedy et al., 2005). Secondly, there is a fairly even split (58% and 40% bootstrap support) in signal between the pelican joining either the shoebill or the hammerkop. This is contra to the nuclear signal for shoebill and hammerkop (68%, Hackett et al., 2008). We comment further on this relationship shortly.

The third region of uncertainty involves the very short internodes where the five well-supported groups join together. Given the short length of these internodes it is little wonder that these branches have low support, and that placing a root in this network is difficult. These short internodes mean a small signal in the data

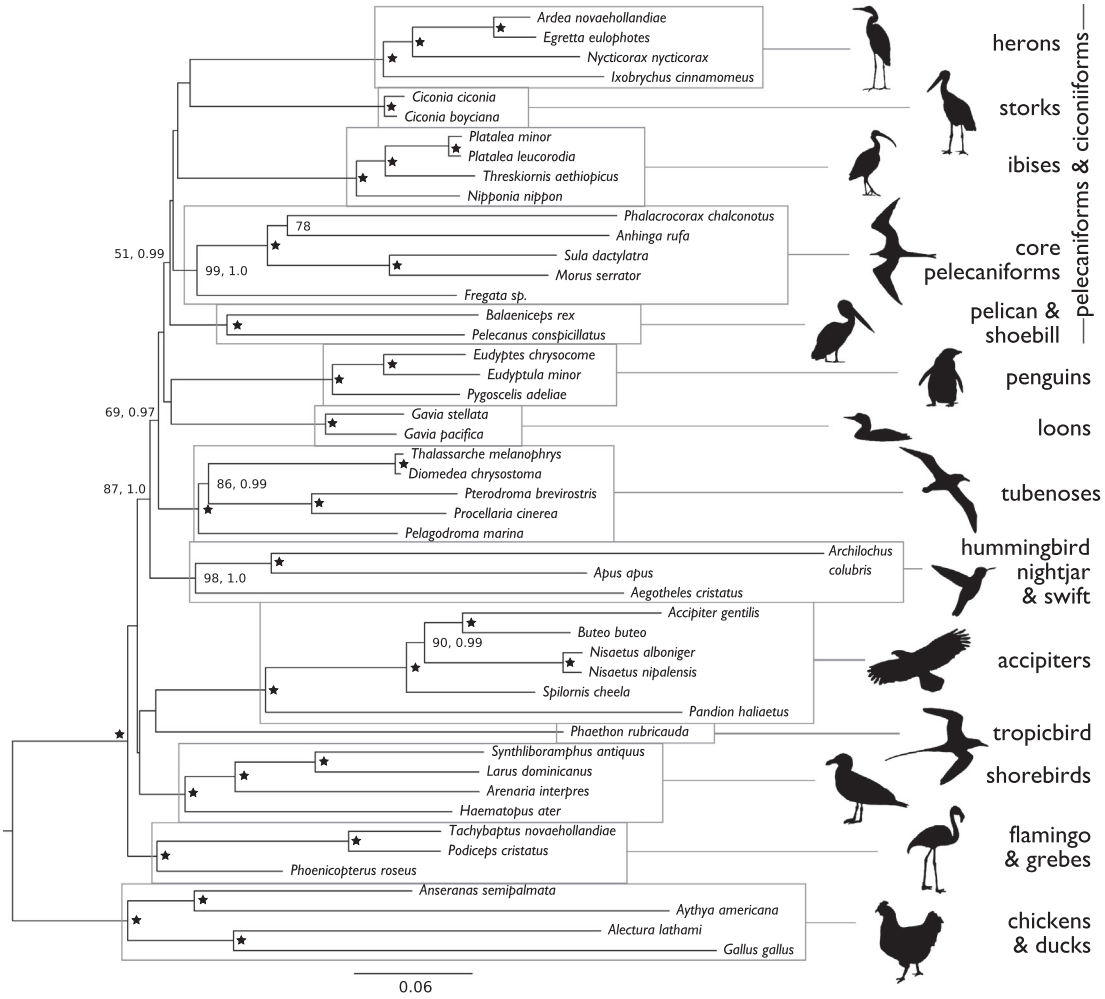

Fig. 2. Maximum likelihood reconstruction of complete mitochondrial genome analysis, showing relationships among the water-carnivores and their relatives. RAxML rapid bootstrap support over 50%, and Bayesian posterior probabilities over 0.8 are indicated. Support of 100/1.0 is indicted with a star.

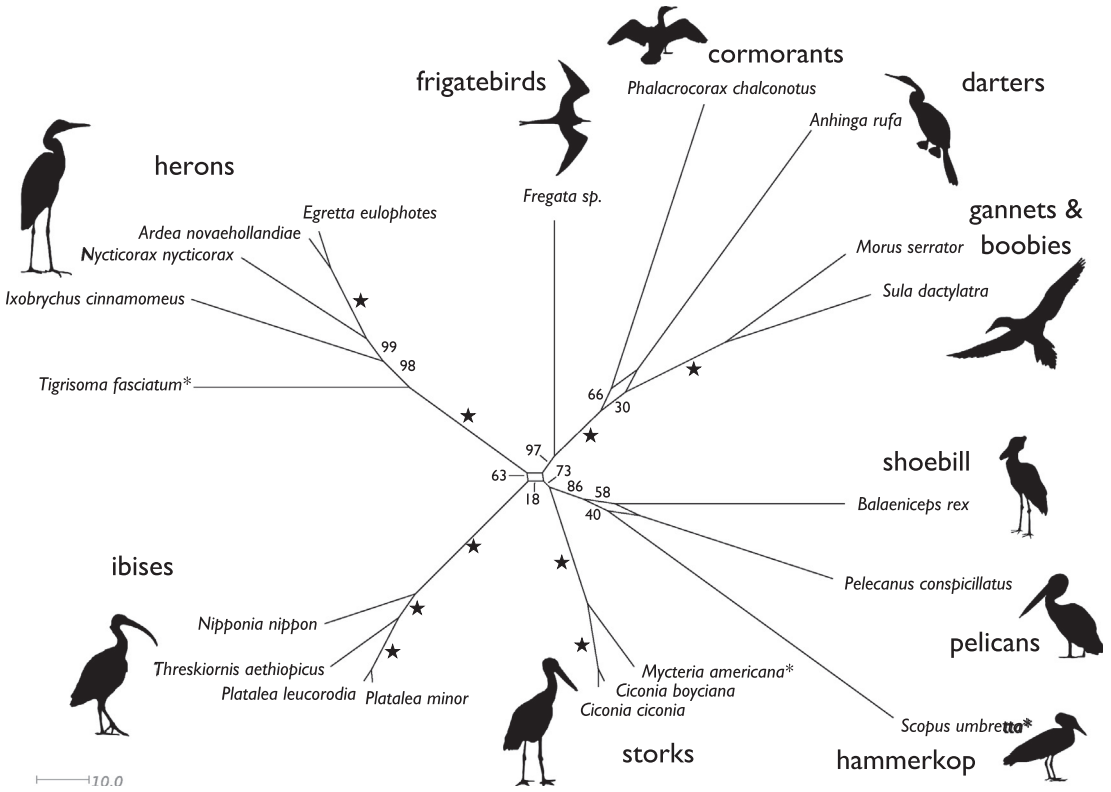

Fig. 3. Unrooted splits-network of relationships among the Ciconiiform and Pelecaniform taxa, showing the consensus of splits greater than 15% from RAxML rapid bootstrapping. 100% support is indicated with a star. Asterisked species have partial mitochondrial genomes.

can easily be confused by noise, leading to the uncertainty and low support for branches at the base of this group (Figs. 2 and 3). These short internal branches are consistent with an adaptive radiation in this group, and this finding is much more interesting than focusing on precisely resolving the branching order (as, if they did radiate almost contemporaneously, they may not be able to be accurately resolved).

3.4. The pelican is not a pelecaniform

There is now an agreement from nuclear and mitochondrial studies that the pelican, shoebill and hammerkop form a monophyletic group and are not sister to the core Pelecaniformes (Fig. 4). The exact relationship between the three taxa is not yet clear, and both nuclear and mitochondrial studies have low support for alternative arrangements (as discussed above). With the mitochondrial data it is possible the grouping is influenced by the hammerkop missing substantial amounts of data, and full mitochondrial sequences will be needed to investigate this. Nevertheless, the three taxa are 'locally stable' (Cooper and Penny, 1997): the trees are simply the alternative arrangements around a single internal branch, and this variation does not affect our discussion.

There have been several morphological studies investigating the possibility that the shoebill is closely related to the pelicans (e.g. Cottam, 1957; Cracraft, 1985; Saiff, 1978). However, in doing so, most suggested that the shoebill is an aberrant pelecaniform, rather than pelicans being 'aberrant long-legged waders' (van Tuinen et al., 2001). The morphological characters linking pelicans to cormorants, darters and sulids (primarily totipalmate feet, but see Table S2), appeared to preclude any alternative placements of pelicans, thereby bringing the shoebill into Pelecaniformes. However, when Cracraft (1985) examined the characters linking the pelicans

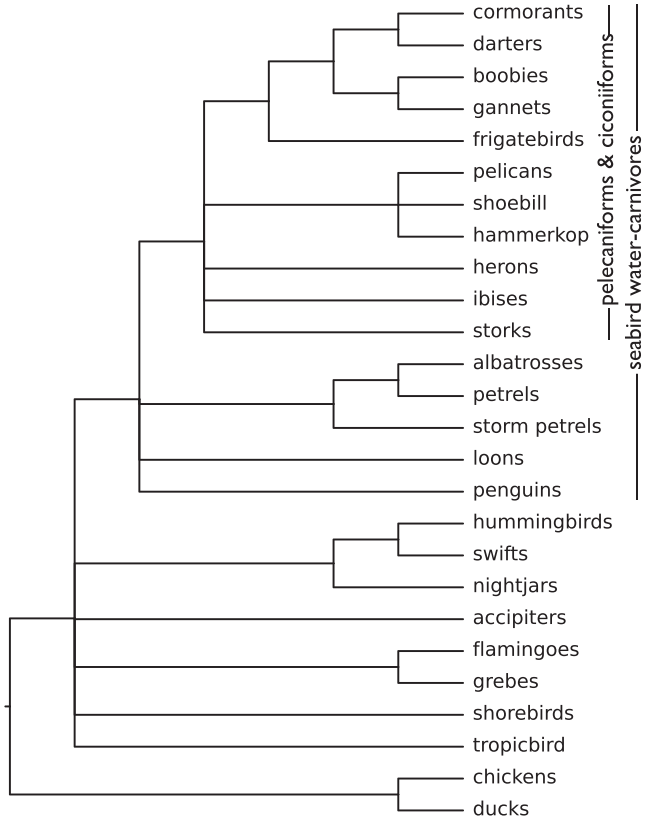

Fig. 4. Consensus diagram showing agreement in avian relationships between this complete mitochondrial genome analysis and the nuclear analyses of Hackett et al. (2008).

and shoebills he concluded they were convergences that arose as mechanical responses to similarities in feeding behavior. In the light of the modern DNA-based consensus, we can now say that morphological characters linking pelicans and shoebills represent a shared history, and those tying pelicans to the core pelecaniforms may be either convergent responses to the mechanical stresses of feeding behavior, or else are ancestral to the whole pelecaniform and ciconiiform group.

The morphological findings of Livezey and Zusi (2007), however, still placed the tropicbirds and pelicans within Pelecaniformes, with the shoebill as a monotypic sister order (but see discussion in Mayr, 2008). This result is apparently supported by four diagnostic apomorphies grouping the shoebill as the sister order to the Pelecaniformes, four diagnostic apomorphies grouping the Pelecaniformes, and seven and nine supportive apomorphies for these groupings respectively (see Livezey and Zusi's Table 2). The phylogenetic signal in morphological characters can, however, be obfuscated by homoplasy (e.g. Holland et al., 2010). We suggest that it is more informative and interesting to use the genetic evidence to establish the phylogeny, and then to map the ecological life history/morphological characters onto this tree. From this, we hope to understand the important life history and biological changes that have occurred.

The totipalmate foot, for example, would usually be argued to have evolved in the common ancestor of the pelecaniforms (perhaps as an adaptation for foraging in water). However, given the relationships of the pelecaniform and ciconiiform group it appears that the totipalmate foot is either convergent, or has been lost multiple times in the ciconiiforms. Possible explanations for the evolution of the totipalmate foot in the supposedpelecaniformcommon ancestor include foraging mode, but these modes have evolved to differ substantially in the extant taxa. Tropicbirds, gannets and boobies are plunge divers, pelicans are mostly surface filterers (though brown pelicans also plunge dive), frigatebirds are entirely aerial feeders that specialize in dipping, and cormorants in foot propelled pursuit diving (Schreiber and Burger, 2002). We return to foraging mode later.

Thus, the totipalmate foot condition should be discounted as a unifying morphological character for phylogeny. Siegel-Causey (1997) argued that the foot webbing of the tropicbird *and* frigatebird was not the same as the other Pelecaniformes (including the pelican), which is, in part, supported by the molecular results placing the tropicbirds apart from the other pelecaniforms. We do not find evidence for the tropicbird grouping with the Caprimulgiformes, nor with the flamingo-grebe clade, as was proposed (albeit with low support) by Hackett et al. (2008). Because we are not confident yet of where the tropicbird is placed, all we can currently conclude is that it is a deeply diverging Neoavian lineage, definitely outside the water-carnivore group, and an independent lineage of water-carnivores. The similarity of the foot webbing of the tropicbird and the other pelecaniforms may, thus, be a by product of it also being a plunge diver, or some other life history characteristic it shares with the others.

3.5. Molecular dating

Evolution of water-carnivory appears to have happened relatively quickly and early in Neoaves (approximately 60–70 Ma, Fig. 5). By 60 Ma, already six extant lineages within the seabird water-carnivores were present. Previous studies have suggested a decline in pterosaur diversity (potential non-avian competitors),

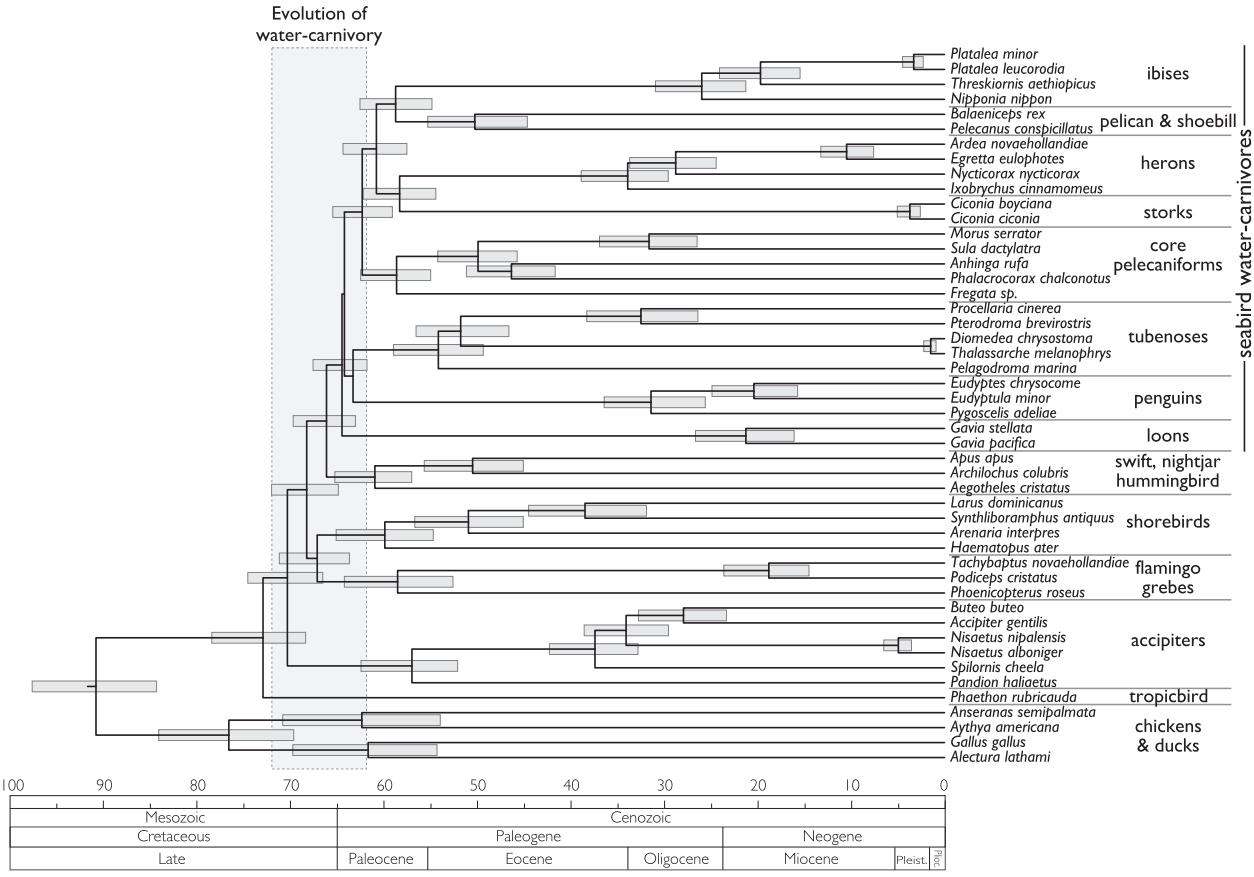

Fig. 5. Maximum clade credibility chronogram inferred using BEAST. Error bars represent 95% posterior credibility intervals and are displayed for nodes present in more than 50% of trees sampled. The time scale is in millions of years ago (Ma).

and an increase in 'web-footed birds' during the Campanian/Maastrichtian (end of the Cretaceous, Kim et al., 2003; Slack et al., 2006). Our findings would suggest water-carnivory in neoavian birds was also evolving around this time. Whilst we cannot yet draw direct links between these observations, it is interesting to see 'consilience of induction', in this case congruent results from molecular trees and fossil footprint data.

The core pelecaniform birds (including the frigatebird) began diversifying a short time later, around 58 Ma. This is consistent with the oldest known fossil frigatebirds, found in the Eocene Green River Formation in Wyoming (~49 Ma, Olson and Matsuoka, 2005). Within the core pelecaniforms, Friesen and Anderson (1997) estimated the boobies (*Sula*) are a fairly deep relative (>20 million years ago) of the gannets (*Morus*) based on cyt*b* sequences and an estimated transversion rate of 0.2 base pairs per 100 Million years (Patterson et al., 2011, found a similar date, ~17 Ma, for this split with their relaxed clock analysis). Our findings indicate an even deeper split between *Sula* and *Morus*, around 30 Ma (Fig. 5). The date of our estimate of the cormorant/darter split (>40 millions years ago) is consistent with Oligocene cormorant and darter fossils from Australia (Worthy, 2011; 2012). Within the wider water-carnivores, our dated analysis is also consistent with the oldest fossil pelican attributed to *Pelecanus* (~30 Ma, Louchart et al., 2011).

3.6. Water-carnivory in Neoaves, adaptive radiations and niche stability

The core pelecaniforms, the grouping of pelecaniforms and ciconiiforms, and the seabird water-carnivore group are supported by both nuclear and mitochondrial data (summarized in Fig. 4). However, within the seabird water-carnivore group, the deep branching is not certain, with very short internodes supporting an early adaptive radiation of this group as a whole. Our results show these lineages began diverging from each other around 65 Ma, over a period of about five million years. While it should be possible to resolve branching over a five million year period from the recent past (say, within the Miocene), it stands to reason that resolution becomes more challenging the longer ago this radiation occurred. While it will be hard to resolve exactly short branching patterns over a five million year period 65 Million years ago, an early adaptive radiation into this general niche is probably a more important biological finding than determining the exact order in which these lineages diversified. With marine pterosaur diversity declining at about this time (Slack et al., 2006), ancestral birds were diversifying into the niches pterosaurs had dominated.

What type of bird may have been most ready to exploit these niches? From the foraging mode reconstruction (Fig. 6), it appears a wading lifestyle is likely to have been the ancestral state of the seabird water-carnivores, although foot-propelled pursuit diving is also a significant possibility. These findings are consistent with Paleogene fossil evidence (Mayr, 2009). The adaptations of the pelecaniform birds into a more specialized foraging niche (plunge diving, surface diving and surface feeding) have evolved more recently. A wading lifestyle is also seen in a number of other more distantly related groups, for example, rails, cranes, and flamingoes. Perhaps wading is an ancestral foraging mode for all Neoaves? This is certainly something to explore further. In relation to the long-legged wading lifestyle, the more general 'ground-foraging' appears to be the ancestral state in modern birds. This state occurs

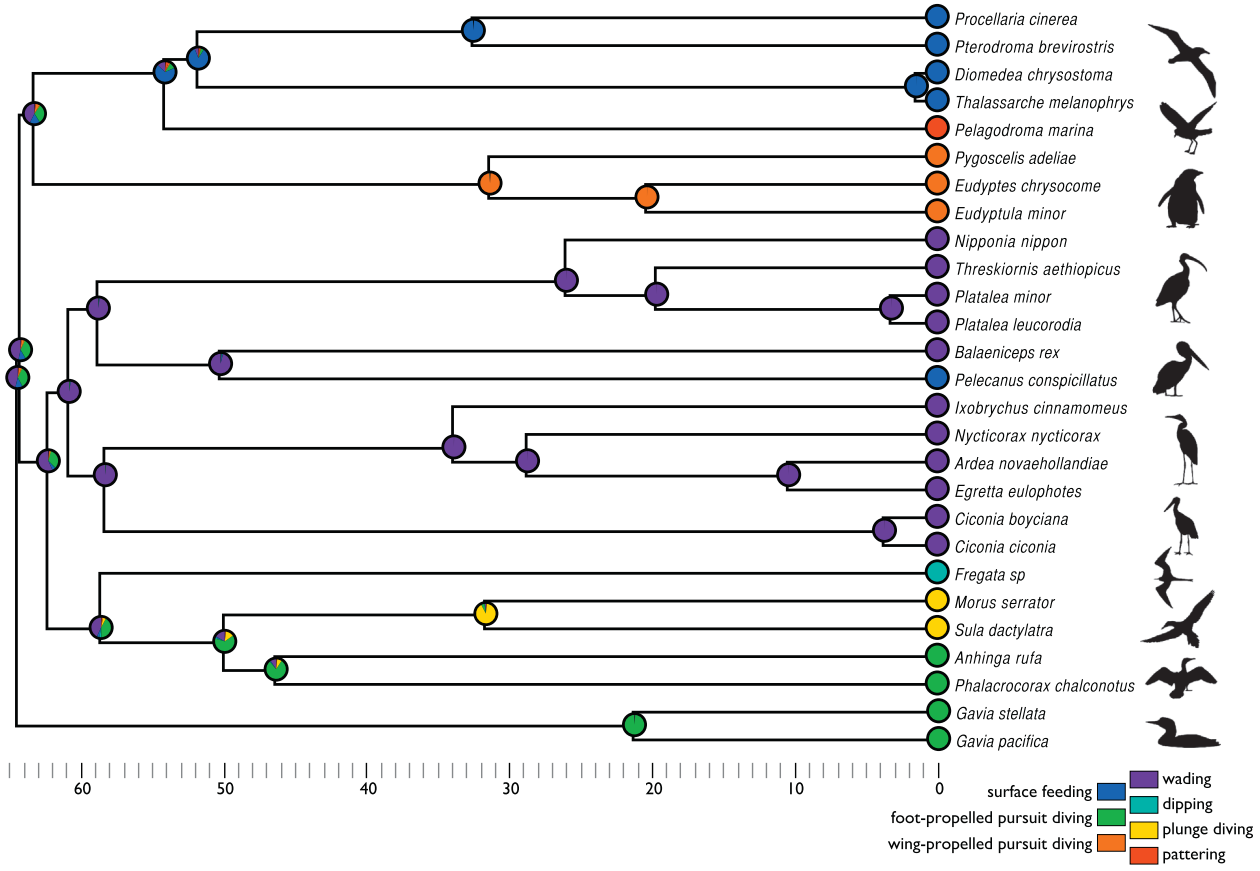

Fig. 6. Maximum likelihood reconstruction of foraging mode ancestral state for the seabird water-carnivores. Branch lengths are in millions of years, and are from the BEAST dated analysis.

in the Palaeognathae and the Galloanserae, and in several deeply diverging Neoaves lineages.

The Pelecaniformes and Ciconiiformes are part of the major water-carnivore radiation, which also includes penguins, loons and tubenoses (loosely, seabirds, ~320 species). Distinct from this clade, it appears the other major water-carnivore group (shorebirds – Charadriiformes, ~350 species) evolved separately, and perhaps began radiating slightly later (Fig. 5). Among other Neoaves, a few individual species or clades have also adopted some kind of water-carnivory (for example, tropicbird, osprey, fish owl, sea eagles, cranes and kingfishers) but no group is as successful in terms of number of species, or breadth of water-carnivory niches as either the seabird or shorebird groups. Additionally, having evolved water-carnivory, there has been a low probability within the seabird water-carnivores of transition out of this niche, even though a number of groups have moved between sea and freshwater (e.g. ciconiiform families, darters, some cormorants).

4. Conclusions

Increasingly, we see it is essential to integrate the results of phylogenetic research with life history, developmental and behavioral information (or more generally, the biology of organisms). As a start to this longer-term aim we have discussed our findings regarding the Pelecaniformes and their allies. Our findings suggest that there has been long-term niche stability within the water-carnivores, the depth of which is further emphasized by a ~30 million year old fossil pelican that has an anatomically modern bill (Louchart et al., 2011). It is now clear from nuclear and mitochondrial data that we have a consensus on the main features of pelecaniformphylogeny, as well as an established grouping of pelecaniform and ciconiiform birds within a larger seabird water-carnivore clade (Fig. 4).

We see that within the long term stability of the seabird water-carnivore niche there have been local changes in lifestyle and ecology (Fig. 6), but no water-carnivores have had ecologically diverse radiations out of this niche, for example becoming 'higher land birds' (sensu Olson, 1985), or vice versa. Niche conservation at the levels of genus, family and order may be buffered against local variation such as environmental changes, and may be more affected by intrinsic life history traits (Hadly et al., 2009). Evolutionary-stable niche-discontinuity (ESND) is a theoretical framework for exploring this concept (Poole et al., 2003). Generally, any group diversifying into a new niche must compete not only with those already specialized for that niche, but also with its relatives in the ancestral niche. This competition can lead to long-term stability of general niche boundaries, such as seen here in the water-carnivores.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.03.021.

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