When morphology is not reflected by molecular phylogeny: the case of three 'orange-billed terns' Thalasseus maximus, T. bergii and T. bengalensis (Charadriiformes: Laridae).

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When morphology is not reflected by molecular phylogeny: the case of three 'orange-billed terns' *Thalasseus maximus*, *T. bergii* **and** *T. bengalensis* **(Charadriiformes: Laridae).**

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Running title: Species status of West African Royal Tern *T. m. albididorsali*s

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ABSTRACT

In order to elucidate genetic structure within the Royal Tern *Thalasseus maximus*, genetic analyses and phylogenetic reconstructions were performed on Royal Terns *T. m. albididorsalis* from the West African breeding population and compared with sequences from American populations *T. m. maximus*. The analysis shows that Royal Tern as currently defined is a paraphyletic species: West African Royal Tern is genetically distinct from American breeding populations of the nominate subspecies and forms part of a genetic cluster with Lesser Crested Terns (of all subspecies) and Greater Crested Terns *T. bergii*. This represents the first published analysis of the genetic relationship between the two subspecies of Royal Terns, suggests that the West African population should be treated as a distinct species, and provides support to previous studies suggesting that morphological and genetic similarities are poorly correlated in the genus *Thalasseus*. Conservation and taxonomic implications are discussed.

Keywords

mtDNA – molecular phylogeny – *Sterna* - Sterninae – The Gambia.

INTRODUCTION

Phylogenetics can be used both to differentiate between morphologically close congeneric taxa and also to interrogate the distribution and breeding patterns of species (Bridge *et al*. 2005; Pons *et al*. 2005; Whittier *et al*. 2006; Efe *et al*. 2009; Taylor and Friesen, 2012). The Royal Tern *Thalasseus maximus* (Boddaert, 1783) (formerly *Sterna maxima*) has two geographically separated subspecies: nominate *T. m. maximus* breeding in coastal USA, the West Indies and coastal South America to Patagonia; and *T. m. albididorsalis* (Hartert, 1921), breeding in coastal West Africa. Both are large, crested, orange-billed terns and the recognised differences between the subspecies are mean bill morphology (redder and deeper in nominate birds) biometric variations in mass (*albididorsalis* is on average smaller) and wing : bill-length ratio; hence the validity of the subspecies has been questioned (del Hoyo *et al*., 1996). The taxonomy of yellow/orange-billed *Thalasseus* terns remains under review: Buckley and Buckley (2002) suggested that South American populations of nominate Royal Tern shared similarities with the West African population and may represent an undescribed taxon. Mayr and Short (1970) suggested that Royal Tern forms a superspecies with the superficially similar yellow-billed Greater Crested Tern *T. bergii* which breeds in south-west and eastern Africa, the Arabian Peninsula, tropical Indian Ocean isands through to the Oriental Region and Australia. Recent genetic analyses have clarified the systematic relationships within terns (Sternidae) (Bridge *et al*., 2005; Efe *et al*., 2009) and have confirmed, for example, that the nominate Royal Tern resides within the genus *Thalasseus*, closely related to the Greater Crested Tern and the smaller, yellow-billed Lesser Crested Tern *T. bengalensis* which breeds primarily in Libya, North African Mediterranean coast (*T. b. emigratus*), the Red Sea and northwest Indian Ocean (*T. b. bengalensis*), New Guinea and northern Australia (*T. b. torresii*). However, to date there has been no phylogenetic analysis of Royal Terns from West Africa. Given the large genetic difference between the morphologically similar Palearctic Sandwich Terns *T. sandvicensis* (Latham, 1787) and Nearctic Cabot's Tern *T. acuflavidus* (S. Cabot, 1847), which underlay their recent split (Efe *et al*., 2009; Sangster *et al*., 2011), a comparable analysis of *albididorsalis* Royal Terns will provide important biogeographic information to determine whether they have experienced a long period of isolation from nominate Royal Terns of the Americas. Such information is fundamental for setting conservation priorities.

In this study, we have performed phylogenetic analysis using mitochondrial DNA (mtDNA) and nuclear DNA gene sequences of Royal Terns from Mauritania and the internationally important West African breeding population at Tanji Bird Reserve and Bijol Islands, The Gambia (Cosgrove *et al*., 2013) recently damaged by storms and erosion such that only a much smaller breeding colony persists. We also include Lesser Crested Terns from breeding colonies in the Mediterranean, Red Sea

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and Arabian Peninsula to complement the previously published sequence from this species taken from Australian individuals. It is concluded that Royal Tern as currently defined is paraphyletic and shows that the 'orange-billed terns' are another example of avian taxa in which morphological divergence is not a good indicator of evolutionary relationship.

METHODS

Taxonomy used in this paper follows the *Howard and Moore Complete Checklist of the Birds of the World*, 4th edition (Dickinsen and Remsen, 2013), and hence places these 'crested' terns in genus *Thalasseus*, based substantially on the work of Bridge *et al*. (2005) which shows that they form a strongly supported monophyletic criteria that is sister to other terns in genus *Sterna* s.s. However we recognise that the argument for this arrangement is subjective (compared to retaining crested terns in genus *Sterna*) because retention in genus *Sterna* does not lead to paraphyly (Sangster *et al.,* 2005).

Royal Terns from the breeding populations in West Africa, were collected as follows. Two birds were found freshly dead at the Tanji Bird Reserve and Bijol Islands, The Gambia, coordinates N 13° 23' 07.8'' W 16° 48' 49.3'', on 11 March 2014 ('MAX01') and 8 June 2012 ('MAX02'). A wing ('S1') was collected from Cap Blanc peninsula, Mauritania, as described in Dufour *et al*. (2016). Skeletal and wing preparations were retained as vouchers. Genomic DNA was extracted from feathers using the Qiagen Blood and Tissue Extraction Kit or QIAamp DNA Micro Kit (Qiagen, UK) according to the manufacturer's instructions, with the addition of dithiothreitol to 0.1 M concentration in the proteinase K digestion mix and elution in 80 µl of Qiagen buffer AE. PCR, DNA gel extraction and sequencing was performed using protocols as described in Shannon *et al*. (2014). Primers used were: for *cytb*, L14993 and H16065 described in Helbig *et al.,* 1995); for *COI*, BirdF1 and Bird R1 (Hebert *et al*., 2004); for *ND2*, L5216 and H6313 as described in Shannon *et al*. (2014). Negative (extraction blank and water) controls were used to eliminate the possibility of contamination. Sequencing was performed by Source BioScience (Cambridge, UK) and Eurofins Genomics (Ebersberg, Germany). Sequence homology was analysed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). DNA was also extracted from Lesser Crested Tern feathers and muscles at six locations: 1) on the Ashrafi archipelago, Egyptian Red Sea, 27°46'N 33°41'E (*T. b. bengalensis*); 2) Al Jarrim island, Persian Gulf, Bahrain 26°28' N 50°30'E (*T. b. bengalensis*) and; 3) Libya (*T. b. emigratus*) at Gara Island, Ajdabiyah 30°47'N 19°54E, Jeliana Islet, Benghazi, 32°05'N 20°03'E and Elba Island, Derna, 32°14'N 23°17'E. ND2 was amplified and sequenced as above. A 306 bp fragment of *cytb* was amplified using primers

L15008 (5'-AACTTCGGATCTCTACTAGG-3') and H15326 (5'-GAATAAGTTGGTGATGACTG-3') with

annealing temperature 58°C. Existing database *cytb*, *COI* and/or *ND2* sequences were downloaded

from GenBank for multiple individuals of nominate Royal Terns, Greater and Lesser Crested Terns,

Sandwich, Cabot's and Elegant Terns *T. elegans*.

Seven nuclear loci - CRMIL(14), RGS4(3), 3682, ACL(16), BFIB7, FGB, and TGF - were also amplified

and sequenced from multiple individuals of all relevant taxa (Dufour *et al*., 2016) using protocols and

primers as described in Dufour *et al.* (2016). Sequences were concatenated to produce a

phylogenetic reconstruction based on 4597 bp from all seven loci.

For mitochondrial and analyses, sequences were aligned with MEGA7.0 (Tamura

et al. 2013) with further adjustment by eye. The appropriate models of evolution were selected

using MEGA7.0 and Bayesian analysis was performed using the program BEASTv1.8.3 (Drummond *et*

al., 2012) available from (http://beast.bio.ed.ac.uk).The MCMC chains were run for 10,000,000

generations with trees sampled every 1000 generations using the lognormal uncorrelated relaxed

clock model. The priors of all remaining parameters, such as the base frequencies, gamma shape

parameter, and root height of the tree, were kept at default values. Estimated sample size was

calculated using Tracer (Rambaut *et al*, 2014) and was confirmed to be >1000 in all cases. The

phylogenetic tree estimate was carried out in TreeAnnotatorv1.8.3 using the maximum tree clade

credibility target tree and median node height.

For Maximum Likelihood Analyses, alignments were performed with MEGA6 or CLC Sequence

Viewer (http://www.clcbio.com/products/clc-sequence-viewer/), and phylogenetic reconstructions

with PhyML (Dereeper *et al*., 2008) and TreeDyn (Chevenet *et al*., 2006) online using the South of

France Bioinfomatics Platform (http://www.atgc-montpellier.fr/index.php?type=pg) and 1000

Bootstrap replicates to indicate statistical support for nodes. For the nuclear gene analysis, Mega 6

was used to select the best model of sequence evolution (no partition). The best model was T92 + G

(Tamura 3 parameter + gamma distribution of rates among sites). Mega 6 was used to build a

Maximum Likelihood tree with 1000 bootstrap repetitions using the default parameters in Mega and

the "use all sites" option for gap and missing data.

Novel sequences have been uploaded to the European Nucleotide Database with Accession

numbers: tba.

RESULTS AND DISCUSSION

In the first instance, two West Africa Royal Terns from The Gambia (see Methods) were 'barcoded' by obtaining 656 bp of sequence of the mitochondrial *COI* gene. The two African Royal Tern (MAX01 and MAX02) sequences were only 1 bp different, but were distinct (1.1% uncorrected genetic divergence) from multiple nominate Royal Terns from both North and South America, suggestive of a long period of geographic isolation of the two subspecies. An indicative ML phylogenetic tree based on these sequences compared with database sequences of multiple individuals of related tern species (**Figure 1**) resolved strongly supported clades clustering nominate Royal Terns *T. m. maximus*, West Africa Royal Terns *T. m. albididorsalis* and Lesser Crested Terns *T. bengalensis* together, a clade grouping the Greater Crested Tern samples together, and a clade containing Sandwich Tern *T. sandvicensis*, both subspecies of Cabot's Tern *T. acuflavidus acuflavidus* and *T. a. eurygnathus* and Elegant Tern *T. elegans*. The relationships between these clades were not clearly resolved. Nominate Royal Terns from the North American breeding population clustered with birds from the South American population with strong statistical support, to the exclusion of all other taxa.

Both available archived *COI* sequences of *T. bengalensis* were from Australian birds *T. b. torresii*. To determine the relationships of Mediterranean breeding birds *T. b. emigratus* and nominate birds from the Red Sea and Persian Gulf, further *ND2* sequences (1041 bp) were obtained from multiple individuals sampled by Hamza (2014). A Bayesian gene tree based on *ND2* from these birds, the West African Royal Terns and archived database sequences from the other taxa confirmed that Lesser Crested Tern *T. bengalensis* (of all subspecies) forms a single clade with strong statistical support (posterior probability = 1) (**Figure 2**). The Australian bird *T. b. torresii* was sister to a clade containing all Mediterranean *T. b. emigratus* and Red Sea/Persian Gulf *T. b. bengalensis* individuals and there was no evidence from this analysis of genetic differentiation between these latter two subspecies. Nominate and *albididorsalis* Royal Terns showed 1.5% uncorrected divergence at the *ND2* locus and a node with strong statistical support showed that they were not sister taxa. Nevertheless the relationships between Lesser Crested Terns, Greater Crested Terns, and American and West African populations of Royal Tern were not robustly resolved by ND2 alone.

The analyses were re-run to perform a Bayesian reconstruction based on a concatenated 2484 bp sequence of *cytb*, *COI* and *ND2* genes obtained from both West African Royal Terns, and representative examples of nominate Royal Tern, Lesser Crested Tern, Greater Crested Tern and Sandwich Tern (**Figure 3**). This firmly resolved the mitochondrial phylogeny of the tern taxa: West African Royal Tern *T. m. albididorsalis* is a sister taxon to Lesser Crested Tern *T. bengalensis* (posterior probability 0.99), together these two taxa are sister to nominate Royal Terns *T. m.*

maximus (posterior probability 0.99). Greater Crested tern *T. bergii* is sister to the clade containing the Royal and Lesser Crested Terns (posterior probability 0.93) with Sandwich Tern *T. sandvicensis* as the outgroup consistent with previous studies. Hence in spite of the strong morphological similarity between *albidorsalis* and nominate Royal Terns and the multiple biometric and plumage differences from Greater and Lesser Crested Tern, *albididorsalis* and nominate Royal Terns were suggested not to be each other's closest relatives. A further analysis including Mediterranean Lesser Crested Tern confirmed this conclusion (**Supplementary Figure 1**).

A 4549 bp alignment of concatenated sequences from 7 nuclear genes was assembled from individuals of all relevant taxa as described in Materials and Methods and subject to a separate phylogenetic analysis. Congruent with the mitochondrial data, the nuclear tree resolved West African Royal Tern *T. m. albididorsalis* and Lesser Crested Terns *T. bengalensis* as sister taxa (99/100 bootstrap support), sister to nominate Royal Terns *T. m. maximus* (83/100 bootstrap support) (**Figure 4)**. The position of Greater Crested Tern *T. bergii* was not clearly resolved by the nuclear tree alone, but the paraphyly of Royal Tern s.l. was confirmed.

The level of genetic divergence between nominate and *albididorsalis* Royal Terns described here (1.1% *COI*, 1.5% *ND2*, 1.2% cytb) suggests at least 400,000 - 500,000 years of genetic isolation between these geographically isolated taxa (Weir and Schluter, 2008). The level of uncorrected divergence is comparable to that which separates the biological sister species, Cabot's Tern *T. acuflavidus* and Elegant Tern *T. elegans* (approx. 1.9% *COI*, 0.5% *ND2*, 1.8% cytb) and less than that which separates the morphologically similar Nearctic Cabot's Tern and Palearctic Sandwich Tern *T. sandvicensis* (3.9% *COI*, 2.6% *ND2*, 3.2% cytb), but this latter pairing are not sister species. The relatively low level of divergence between nominate and *albididorsalis* Royal Terns does not by itself allow any conclusion with respect to their specific status, however the paraphyly of Royal Tern demonstrated in this study, by nuclear and mtDNA would preclude their retention as a single species. The fact that mtDNA and a concatenated dataset of 7 nuclear loci supports exactly the same relationships between *maximus*, *bengalensis* and *albididorsalis* excludes that this result is due to introgression rather than reflects the true evolutionary relationships of these taxa. Nuclear gene flow between *bengalensis* and *albididorsalis* in Africa would move the position of *albididorsalis* closer to *bengalensis* and could explain their position in the nuclear tree even if *albididorsalis* shared a more recent common ancestor with *maximus* than with *bengalensis* but would not affect the mtDNA tree. In addition multiple diagnostic positions in several nuclear loci separate *albididorsalis* from *bengalensis*, demonstrating reduced, if any, nuclear gene flow. We are thus confident that our

result establish that African Royal Tern is truly more closely related to Lesser Crested Tern than to American Royal Tern.

Whereas splitting of Royal Tern s.l. into morphologically near-identical Nearctic and Palearctic species is counterintuitive, it would reflect the split of the near-identical Palearctic Sandwich Tern and Nearctic Cabot's Tern based primarily on genetic data showing that 'Sandwich Tern' s.l. was paraphyletic, with Cabot's Tern being genetically closer to the morphologically divergent Elegant Tern *T. elegans* (Gambel, 1849) (Efe *et al.*, 2009; this study). Vocalisations have been shown to provide phylogenetic information in terns (Massey, 1976) and an analysis of vocalisations would be useful. In contrast, the available mtDNA data suggested that subspecies of Lesser Crested Tern form a monophyletic clade with no evidence of cryptic speciation. ND2 alleles from nominate *T. b. bengalensis* and Mediterranean *T. b. emigratus* were found to be genetically clustered without clear differentiation.

The conservation status of Royal Tern (both subspecies combined) is currently regarded as good, on the basis of large population size and breeding range (BirdLife International, 2016). However, massive erosion and catastrophic loss of eggs recently observed at breeding colonies in Senegal and The Gambia suggests that are at least local threats to *albididorsalis.*

Our genetic data suggest that there has been no persistent gene flow between the two taxa of Royal Tern for perhaps half a million years. Sampling of more individuals would be required to confirm this and would be informative to measurement of the amount of genetic diversity in Royal Tern populations. A bird identified as Royal Tern and apparently carrying an American ring was recorded in the UK in 1979 (Moon, 1983), but Royal Tern remains an extreme rarity in Europe, suggesting little transatlantic interchange of migrant individuals. The data presented in this paper demonstrate the two Royal Tern subspecies should better be treated as separate species.

We would presume that the two taxa of Royal Tern share a retained ancestral phenotype from which the smaller, yellow-billed Lesser Crested Tern *T. bengalensis*, and possibly Greater Crested Tern *T. bergii*, have diverged. The problem of retained ancestral features and/or convergent evolution of distantly related organisms may lead to misleading phylogenetic reconstructions based on morphological, vocal and ecological criteria alone.   Genetic analyses based on markers that are neutral to morphology provide a more objective and potentially more accurate line of evidence for reconstructing evolutionary relationships. For this reason, taxonomic workflows that explicitly ignore genetic criteria, such as that formulated by Tobias *et al.* (2010), fail to recognize those valid biological species that differ very little in phenotype (cryptic species) and systematic lists based on

them (e.g. del Hoyo *et al.*, 2014) will inherently miss a significant component of biodiversity, with potentially serious consequences for the conservation of these taxa. We recommend inclusion of genetic criteria along other sources of information in all taxonomic frameworks.

Acknowledgements

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FIGURE LEGENDS

Figure 1: Genetic status of West African Royal Terns. *COI* gene tree (Maximum Likelihood) showing the relative levels of genetic divergence between nominate *maximus*, *albididorsalis* ('MAX01' and 'MAX02'), and other tern species. Accession numbers of sequences downloaded from NCBI are listed. Bootstrap support (1000 repetitions) is indicated at the nodes. Scale bar represents 0.5% uncorrected sequence divergence. Nominate Royal Terns are represented by both North American individuals (DQ434154, N. Carolina, September 2001; DQ433211, Florida, April 2001; DQ433212 Florida, April 2001, DQ433213 Florida, April 2001) and South American breeding individuals (FJ028398 and FJ028399 both Argentina, December 2004; FJ356198, Sao Paulo, Brazil, date not recorded).

Figure 2: Genetic status of West African Royal Terns. Phylogenetic representation (Bayesian inference) based on 977 bp of *ND2* sequence, showing roughly equivalent genetic distance between *Th. bengalensis* (of all subspecies) and both subspecies of Royal Tern.   The sampling locations of the Lesser Crested Terns being published here for the first time are: *T. b. bengalensis*; Egyptian Red Sea (EGP7 and EGP2), Arabian (Persian) Gulf, Bahrain (P4 and P37); *T. b. emigratus*; Gara (Libya, G_BN and G_T2); Jeliana (Libya, J_BB and J_BC); and Elba (Libya, E_KS and E_AB).Posterior probabilities are indicated at nodes. Scale bar represents 0.2% uncorrected sequence divergence.

Figure 3: Genetic status of West African Royal Terns. Phylogenetic reconstruction (Bayesian inference) of representative examples of *Thalasseus* terns based on 2448 bp of *cytb*, *COI* and *ND2* mtDNA sequence. Statistical support for nodes is illustrated. The phylogeny confirms that 'Royal Tern' is most likely a paraphyletic species.

Figure 4: Nuclear DNA sequence tree of *Thalasseus* terns.

Maximum likelihood phylogeny based on 4549 bp from 7 concatenated nuclear genes as desrbied in main text. Bootstrap support (1000 repetitions) for nodes is indicated. No shared derived indels were found between nominate and *albididorsalis* Royal Terns

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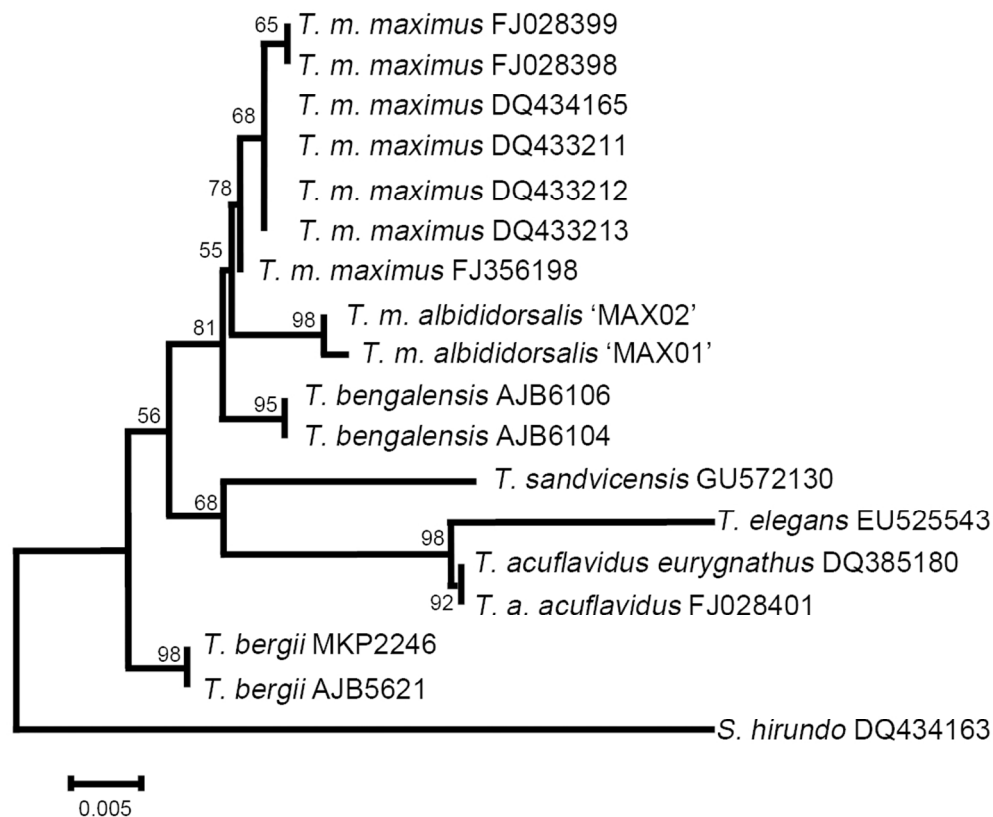17
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Figure 1: Genetic status of West African Royal Terns. COI gene tree (Maximum Likelihood) showing the relative levels of genetic divergence between nominate maximus, albididorsalis ('MAX01' and 'MAX02'), and other tern species. Accession numbers of sequences downloaded from NCBI are listed. Bootstrap support (1000 repetitions) is indicated at the nodes. Scale bar represents 0.5% uncorrected sequence divergence. Nominate Royal Terns are represented by both North American individuals (DQ434154, N. Carolina, September 2001; DQ433211, Florida, April 2001; DQ433212 Florida, April 2001, DQ433213 Florida, April 2001) and South American breeding individuals (FJ028398 and FJ028399 both Argentina, December 2004; FJ356198, Sao Paulo, Brazil, date not recorded).

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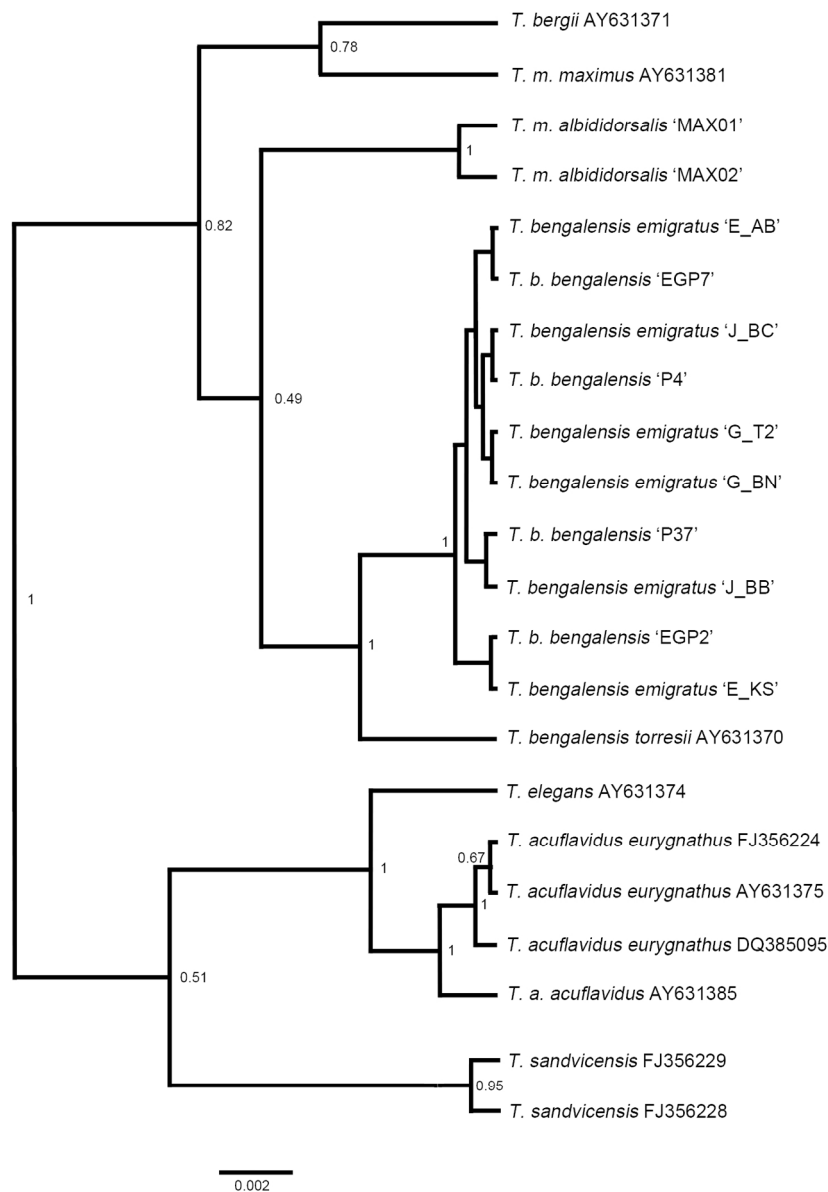48
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Figure 2: Genetic status of West African Royal Terns. Phylogenetic representation (Bayesian inference) based on 977 bp of ND2 sequence, showing roughly equivalent genetic distance between Th. bengalensis (of all subspecies) and both subspecies of Royal Tern.   The sampling locations of the Lesser Crested Terns being published here for the first time are: T. b. bengalensis; Egyptian Red Sea (EGP7 and EGP2), Arabian (Persian) Gulf, Bahrain (P4 and P37); T. b. emigratus; Gara (Libya, G_BN and G_T2); Jeliana (Libya, J_BB and J_BC); and Elba (Libya, E_KS and E_AB).Posterior probabilities are indicated at nodes. Scale bar represents 0.2% uncorrected sequence divergence.
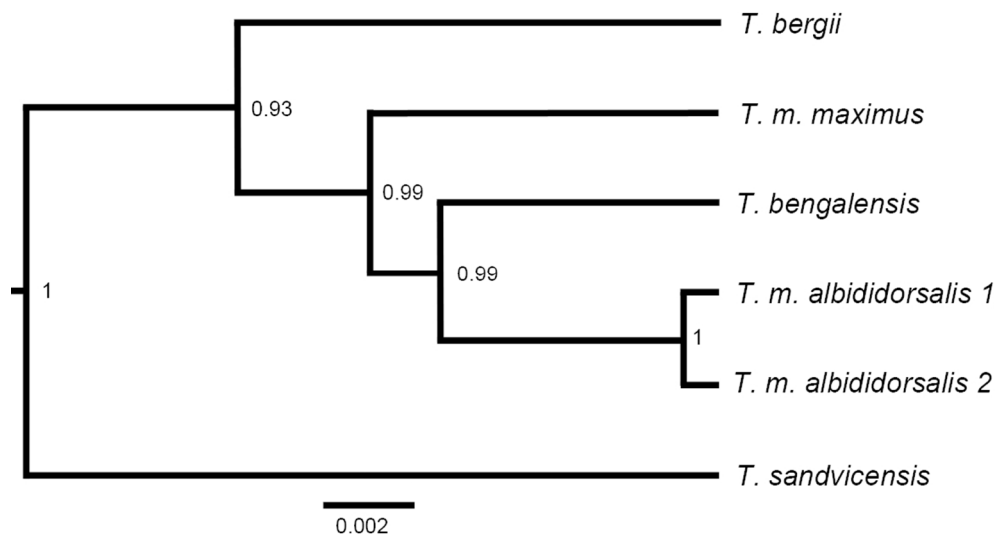
124x179mm (300 x 300 DPI)

Figure 3: Genetic status of West African Royal Terns. Phylogenetic reconstruction (Bayesian inference) of representative examples of Thalasseus terns based on 2448 bp of cytb, COI and ND2 mtDNA sequence. Statistical support for nodes is illustrated. The phylogeny confirms that 'Royal Tern' is most likely a paraphyletic species
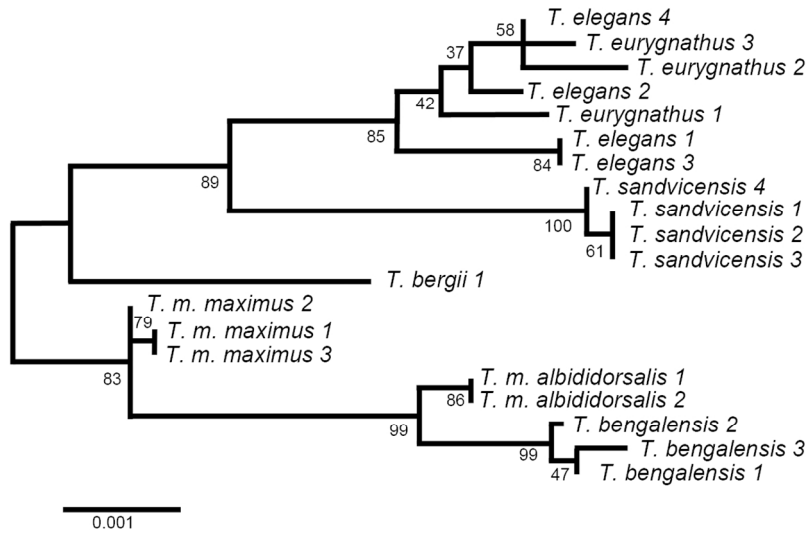
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Figure 4: Nuclear DNA sequence tree of Thalasseus terns.
Maximum likelihood phylogeny based on 4549 bp from 7 concatenated nuclear genes. Bootstrap support
(1000 repetitions) for nodes is indicated.

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When morphology is not reflected by molecular phylogeny: the case of three 'orange-billed terns' *Thalasseus maximus*, *T. bergii* **and** *T. bengalensis* **(Charadriiformes: Laridae).**

J. Martin Collinson[1]*, Paul Dufour[2], Abdulmaula A. Hamza[3,4], Yvonne Lawrie[1], Michael Elliott[4] Clive Barlow[5] and Pierre-André Crochet[2]

SUPPLEMENTARY MATERIAL

Supplementary Figure S1

Maximum likelihood phylogeny of representative examples of *Thalasseus* terns based on concatenated 1272 bp of COI and ND2 mtDNA sequence. Statistical support for nodes is illustrated. 'Royal Tern' is most likely a paraphyletic species.
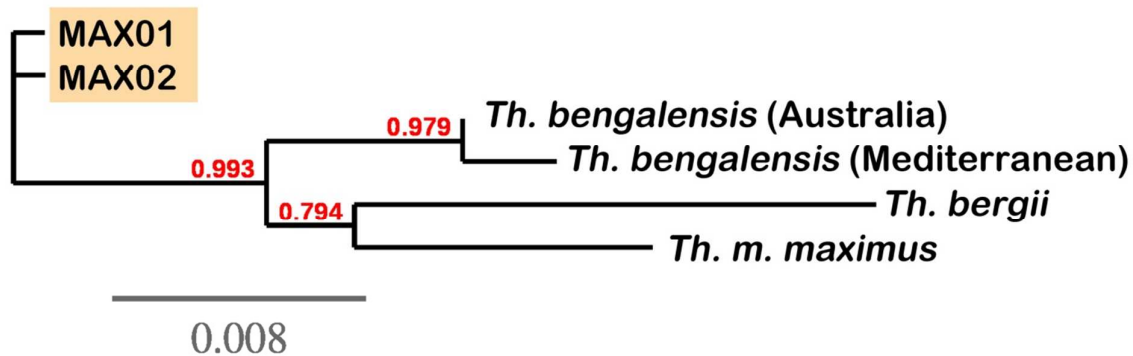
Supplementary Figure S2

Maximum likelihood phylogeny of *Thalasseus* terns based on concatenated 3756 bp of concatenated mtDNA and nuclear sequence.
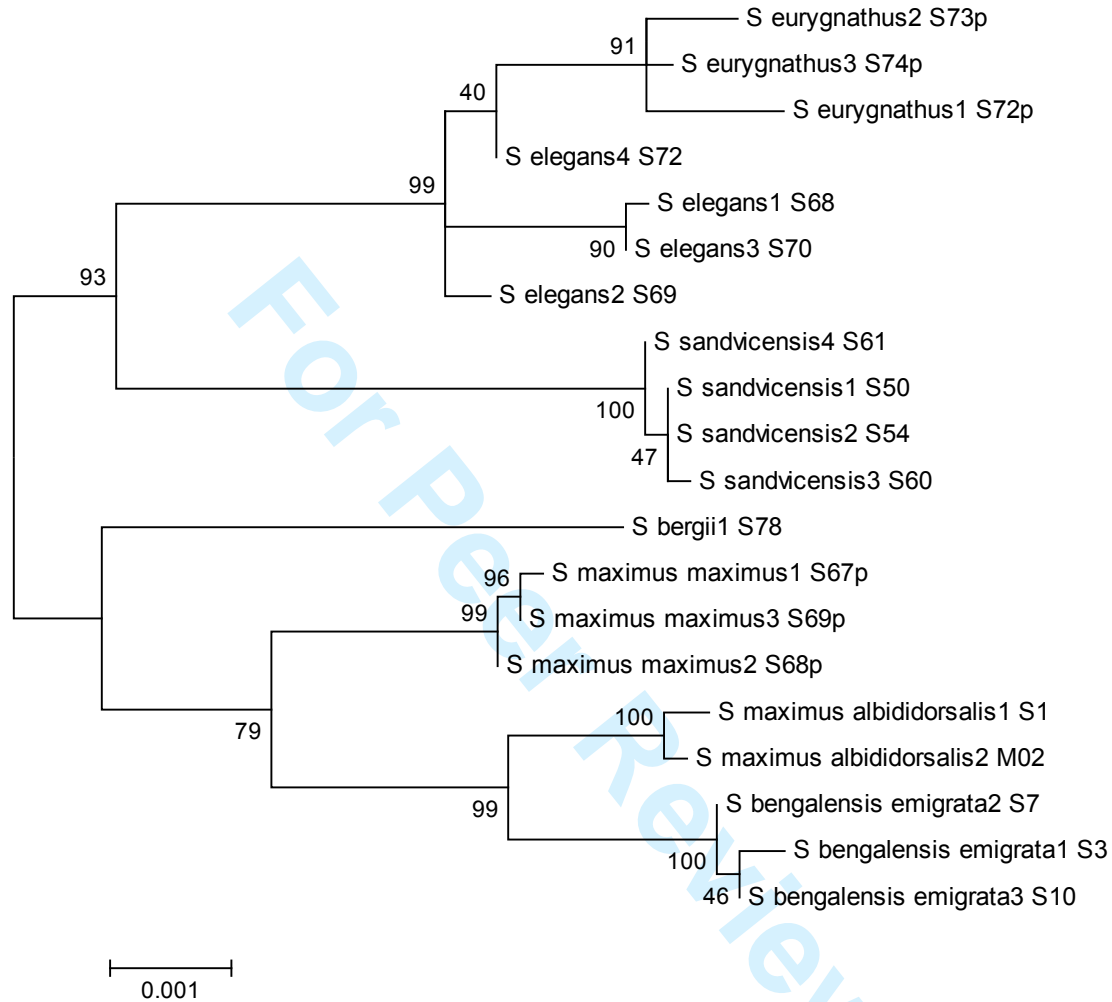
Maximum likelihood, 100 bootstrap, model Tamura 3-parameters with gamma, no partition, performed using Mega. Selection of best model of evolution with Mega using BIC as criterion.