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Short Communication

Multigene phylogeny and DNA barcoding indicate that the Sandwich tern complex (*Thalasseus sandvicensis*, Laridae, Sternini) comprises two species

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1. Introduction

The crested terns are a group of six species of seabirds with a world-wide distribution closely allied to but larger than typical *Sterna* species. They are black-capped with elongated crest feathers and most have a bright yellow or orange to orange–red bill (Gochfeld and Burger, 1996). The taxonomic status of this group as a separate genus, *Thalasseus*, is gaining increasing acceptance following the publication of a molecular phylogeny demonstrating they form a strongly supported monophyletic clade (Bridge et al., 2005).

One of the remaining taxonomic uncertainties in the Sternini is in the classification of the species complex of the Sandwich tern (*Thalasseus sandvicensis*), a taxon whose definition and limits have been controversial over the last century. Within this complex, there are three forms that have been classified either as subspecies or species. The most frequent treatment is to consider them as three subspecies: the Sandwich tern (*T. s. sandvicensis*) that breeds on the Atlantic and Mediterranean coasts of Europe, Cabot's tern (*T. s. acuflavidus*) that breeds on the Atlantic coasts of North America and the Caribbean, and Cayenne tern (*T. s. eurygnathus*) that breeds on the Atlantic coast of South America from Argentina north to the Caribbean. The three races of Sandwich tern were originally described as distinct species (Baird et al., 1884), and due to their morphological and behavioral similarities were later suggested to be part of the same species complex (Baird et al., 1884; Junge and Voous, 1955), an issue that is still controversial (Gochfeld and Burger, 1996; Hayes, 2004).

These taxa are morphologically very similar, with a few distinctions: the Sandwich tern is slightly larger with wider white margin on outer primaries, shorter bill and, paler upperparts (Olsen and Larsson, 1995). Cabot's and Cayenne terns are virtually identical in plumage, although the Cayenne terns possess, on average, a slightly longer, shaggier nuchal crest and slightly darker gray upperparts (Shealer, 1999). The chief distinction between these taxa is in bill coloration. In Sandwich and Cabot's terns the bill is always black with a yellow tip; in the Cayenne tern it is much more variable, typically pale yellow but often with black markings that may be extensive, and rarely orange or even reddish (Hayes, 2004). Cabot's and Cayenne terns often hybridize in Caribbean region (Hayes, 2004).

Breeding habitats of these terns also differ. Sandwich terns nest in open areas with little or no vegetation: bare sand or sand–shell substrates, sandflats, dredge spoil islands and coral cayes (Shealer, 1999). In Europe, they breed in the Ebro Delta in open and sandy beaches and dikes in salinas (Oro et al., 2004). In the Caribbean, Cayenne and Cabot's terns breed in flat islands situated in extensive saline lagoons or on patches of coral debris and sand and a few elevated rocks locally covered with thorny scrub and opuntias (Junge and Voous, 1955). In South America, Cayenne terns nest on islands in Brazil covered by low shrub vegetation, cactus and grasses (Efe et al., 2000), and on coasts characterized by extensive cliffs 30–100 m high and gravel beaches in Argentina (Quintana and Yorio, 1997).

A recent thorough mtDNA analysis of the Sternini species (Bridge et al., 2005) has helped to clarify the phylogenetic relationships of most of the species. However, the relationships of taxa in the *T. sandvicensis* complex was not resolved, as few representatives of Cabot's and Cayenne terns were included and Sandwich terns of the Old World were not examined. Therefore the aim of this study is to clarify the relationships among the Sandwich, Cayenne, and Cabot's terns based on nuclear and mtDNA sequences.

2. Materials and methods

Material was collected for this study by the authors and collaborators, from a wide range of geographic locations, as follows: the

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Sandwich tern, *T. s. sandvicensis*, on Ebro Delta, Spain, 40° 37′N/00° 35′E, (Code ESP, 2004, $n = 3$); the Cabot's tern, *T. s. acuflavidus*, in North Carolina, USA, 35° 32′N/75° 59′W (Code USA, 2005, $n = 2$); the Cayenne tern *T. s. eurygnathus*, in Escalvada Is., Brazil, 20° 41′S/40° 24′W (Code ES, 2002, $n = 3$) and Punta León, Argentina, 43° 03′S/64° 27′W (Code ARG, 2002, $n = 2$). We have also obtained samples from another European population of Sandwich tern (Griend Is., Wadden Sea, The Netherlands) that unfortunately could not be fully sequenced due to sample conservation problems, but we managed to obtain partial sequences from some genes (MyO, COI, cyt *b*, see below) in a few individuals and all resulted in sequences that were indistinguishable from those from Spain (results not shown). Blood samples of breeding birds (adults and nestlings) were taken in the field from the brachial or jugular vein. Samples were preserved in EDTA/Tris-buffer (Dutton, 1995). One additional sample of the Royal tern (*T. maximus*) was from São Paulo, Brazil (provided by P.J. Faria) and another of Trudeau's Tern (*S. trudeaui*) was from Lagoa do Peixe, RS, Brazil. All other samples were described in Bridge et al. (2005).

Total DNA was extracted from the blood samples by a standard phenol/chloroform extraction (Sambrook et al., 1989). DNA was precipitated with cold isopropanol, centrifuged, washed, dried and resuspended in TE buffer. Polymerase chain reaction (PCR) amplifications of the mitochondrial genes cytochrome *b* (cyt *b*), NADH 2 (ND2), and cytochrome oxidase I (COI), and the nuclear genes β-fibrinogen intron 7 (FIB) and Myoglobin intron 2 (MyO) were in 20 μL reactions containing 1 μl DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, 1U *Taq* DNA polymerase (Invitrogen) and 1X buffer (Invitrogen). Primers and PCR conditions for cyt *b* and ND2 were as described in Sorenson et al. (1999), for COI as in Hebert et al. (2003), for FIB as in Prychitko and Moore (1997), and for MyO as in Heslewood et al. (1998). Sequencing of *T. sandvicensis* genes was performed as described in Grazziotin et al. (2006) and of nuclear genes from additional Sternini was performed as described by Bridge et al. (2005). Sequences were deposited in GenBank (Genbank Accession Nos. FJ356177–FJ356229). Other mitochondrial sequences used in this study were obtained from GenBank (Accession Nos. AY631284–AY631390, Bridge et al., 2005).

Traces and sequences were checked manually for ambiguities and aligned using the ClustalW algorithm of MEGA 4.0 (Tamura et al., 2007), with further adjustment by eye. Phylogenies were estimated by maximum parsimony (MP) and maximum likelihood (ML) using PAUP* (Swofford, 1997), and by Bayesian Inference (BI) using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Tree topologies were rooted with the Inca tern (*Larosterna inca*) as an outgroup. The Akaike Information Criterion (AIC) was used in Modeltest v3.7 (Posada and Crandall, 1998) and MrModeltest (Nylander, 2004) to select the best-fit substitution model for use with PAUP* and MrBayes, respectively. MP and ML heuristic searches for optimal trees were conducted using Tree Bisection Reconnection branch-swapping with 100 random addition replicates. Non-parametric bootstrapping was used to assess support for nodes in the MP (1000 replicates) and ML (200 replicates). Random starting trees were used in BI, and four Markov chains were run for one million generations with rate variation among sites modeled as a gamma distribution. Phylogenetic analyses were performed for three datasets: the mtDNA segments combined, the nuclear introns combined, and for all segments concatenated. All analyses were conducted with a partitioned approach (one partition per gene), where the model parameters were estimated independently for each partition.

DNA barcode comparisons using COI sequences of the *T. sandvicensis* complex were performed in a subclade of the main phylogeny, including all the species of *Thalasseus* with additional sequences of *T. s. acuflavidus* ($n = 10$), *T. s. eurygnathus* ($n = 2$), and *T. elegans* (5) detailed in previous papers (DQ433214–DQ433218, DQ434157–DQ434171, Kerr et al., 2007; and EU525544–EU525547, Tavares and Baker, 2008). The best-fit model (HKY with gamma) was selected by AIC in Modeltest v3.7 (Posada and Crandall, 1998). To check for monophyletic sequence clusters a Neighbor-Joining (NJ) tree with the best-fit model parameters was constructed in PAUP*. BI analysis of the barcodes were performed in MrBayes v3.1.2 with four Markov chains (average standard deviation of split frequencies = 0.005633) of 2 million generations, with one cold and four heated chains each, sampling once every 1000 trees and with the burnin time determined after the convergence of likelihood scores (burnin = 200). COI phylogenetically informative characters were mapped on the BI tree in MacClade v4.08 (Maddison and Maddison, 2005), and a test of the chance occurrence of reciprocal monophyly were performed using the coalescent method in Rosenberg (2007) with level of significance $\alpha = 1\%$.

Divergence times were estimated using Markov chain Monte Carlo (MCMC) sampling and a relaxed molecular clock as implemented in Beast v1.4.7 (Drummond and Rambaut, 2007). A root age of 24.4 million years before the present (MYBP) from Paton et al. (2003) and adopted in Bridge et al. (2005) was used. The main parameters and priors used were the uncorrelated log-normal relaxed molecular clock, Yule model of speciation and HKY substitution model with gamma distribution of rates among sites. Samples were drawn every 1000 MCMC steps from a total of 10,000,000 steps, following a discarded burn-in of 1,000,000 steps. Pairwise distances were estimated with MEGA 4.0 using Kimura's two-parameter correction for multiple hits.

3. Results

The size of the alignments for each segment were 420 bp of cyt *b*, 1015 bp of ND2, 684 bp of COI, 730 bp of MyO, and 983 bp of FIB. The number and percentage of variable sites were: cyt *b* (86/20.5%), ND2 (292/28.8%), COI (160/23.4%), MyO (26/3.6%), and FIB (42/4.3%).

The mean Kimura two-parameter (K2P) distance between the *T. s. eurygnathus* and *T. s. acuflavidus* was very small, 0.25% for mtDNA and 0.09% for nuclear genes. However, between *T. s. sandvicensis* and the *T. s. eurygnathus*/*acuflavidus* sequence divergence was similar to among-species distances observed within genera (Table 1). The K2P distances between *T. elegans* and *T. s. eurygnathus*/*acuflavidus* were only 1.08% and 0.2% for mtDNA and nuclear genes, respectively.

Phylogenetic relationships estimated by the different methods and sequence partitions (mtDNA, nuclear, and mtDNA + nuclear) were similar (Fig. 1), with just a few differences due to the low number of informative sites and thus many poorly supported nodes in the nuclear gene tree. However, in all trees individuals of *T. s. sandvicensis* and *T. s. eurygnathus*/*acuflavidus* grouped into distinct monophyletic clades that branched basally in the *Thalasseus* clade. *T. sandvicensis* as currently recognized was paraphyletic, with *T. s. eurygnathus*/*acuflavidus* forming the sister group to the Elegant Tern (*T. elegans*) both from the Americas, rather than to European *T. s. sandvicensis*. *Thalasseus* form a well-supported monophyletic clade separated from other terns by a long branch. Although, the *Thalasseus* clade is sister to two species of *Sterna* (*S. forsteri* and *S. Trudeaui*), thus making *Sterna* paraphyletic, support values at this node are very weak, and further work is required to test the monophyly of this genus.

Trees recovered with Neighbor-Joining and BI analysis of COI barcodes were congruent: the three individuals of *T. sandvicensis* were monophyletic and were sister to a clade including *T. elegans*, *T. s. eurygnathus*, and *T. s. acuflavidus*, with *T. elegans* as a monophyletic group with all clades supported by posterior probability of 1

Table 1
Mean K2P pairwise percent distances among species of terns. MtDNA distances are in the lower triangle and nuclear DNA distances are in the upper triangle.

	1	2	3	4	5	6	7	8	9
1. *T. s. eurygnathus*	–	0.09	0.52	0.27	0.73	0.17	0.27	0.67	0.87
2. *T. s. acuflavidus*	0.25	–	0.55	0.30	0.77	0.23	0.30	0.70	0.90
3. *T. s. sandvicensis*	2.72	2.72	–	0.50	0.96	0.39	0.50	0.81	1.01
4. *T. maximus*	2.87	2.80	2.94	–	0.59	0.21	0.13	0.65	0.85
5. *T. bengalensis*	2.64	2.73	2.67	1.16	–	0.73	0.59	0.72	0.92
6. *T. elegans*	1.06	1.09	2.94	3.01	2.99	–	0.21	0.55	0.83
7. *T. bergii*	2.71	2.63	3.03	1.75	1.83	3.13	–	0.65	0.85
8. *S. sumatrana*	7.58	7.95	7.66	7.72	7.53	7.92	7.39	–	0.59
9. *S. hirundinacea*	8.10	8.26	7.82	7.63	7.27	8.35	7.18	5.69	–
10. *S. albostriatus*	10.05	10.04	9.39	9.59	9.52	10.07	9.54	8.57	9.35
11. *S. dougallii*	8.21	8.36	8.17	7.98	7.67	8.29	7.99	4.38	5.51
12. *S. hirundo*	7.71	7.82	7.43	7.17	7.08	7.93	7.08	5.06	4.90
13. *S. striata*	8.29	8.48	8.31	7.83	7.59	8.33	7.44	4.34	5.12
14. *S. vittata*	8.20	8.38	8.04	7.85	7.60	8.35	7.58	6.21	1.25
15. *S. forsteri*	7.41	7.93	7.61	7.49	7.49	7.34	7.16	7.43	7.51
16. *S. trudeaui*	10.51	10.84	10.60	10.16	10.14	10.28	9.87	9.71	9.99
17. *C. hybridus*	8.71	8.74	8.73	8.63	8.88	8.68	8.95	9.12	9.55
18. *L. inca*	9.56	10.21	9.83	9.26	9.59	9.23	9.37	9.94	9.60
	10	11	12	13	14	15	16	17	18
1. *T. s. eurygnathus*	1.00	0.73	0.54	0.73	0.86	0.86	0.86	0.54	1.33
2. *T. s. acuflavidus*	1.03	0.76	0.57	0.76	0.89	0.89	0.90	0.57	1.36
3. *T. s. sandvicensis*	1.14	0.87	0.67	0.87	1.00	1.09	1.09	0.68	1.47
4. *T. maximus*	0.98	0.72	0.52	0.72	0.85	0.85	0.85	0.52	1.25
5. *T. bengalensis*	1.18	0.79	0.59	0.79	0.92	0.92	0.92	0.79	1.58
6. *T. elegans*	0.90	0.62	0.48	0.62	0.76	0.83	0.83	0.48	1.25
7. *T. bergii*	0.98	0.72	0.52	0.72	0.85	0.85	0.85	0.52	1.31
8. *S. sumatrana*	0.85	0.20	0.26	0.20	0.46	0.85	0.85	0.39	1.38
9. *S. hirundinacea*	0.92	0.52	0.46	0.52	0.33	1.05	1.05	0.52	1.51
10. *C. albostriatus*	–	0.92	0.72	0.92	1.05	1.25	1.18	0.52	1.58
11. *S. dougallii*	9.56	–	0.33	0.13	0.39	0.92	0.92	0.46	1.44
12. *S. hirundo*	8.88	5.39	–	0.33	0.46	0.72	0.72	0.26	1.25
13. *S. striata*	9.45	4.16	5.27	–	0.39	0.92	0.92	0.46	1.44
14. *S. vittata*	9.25	5.97	5.48	5.80	–	1.05	1.05	0.59	1.58
15. *S. forsteri*	10.18	7.92	7.55	7.71	7.49	–	0.39	0.79	1.58
16. *S. trudeaui*	11.62	10.57	10.22	10.44	9.87	6.59	–	0.79	1.58
17. *C. hybridus*	6.12	9.76	9.55	9.89	9.63	9.84	11.90	–	0.98
18. *L. inca*	11.02	10.61	10.26	10.30	9.68	9.95	11.68	10.37	–

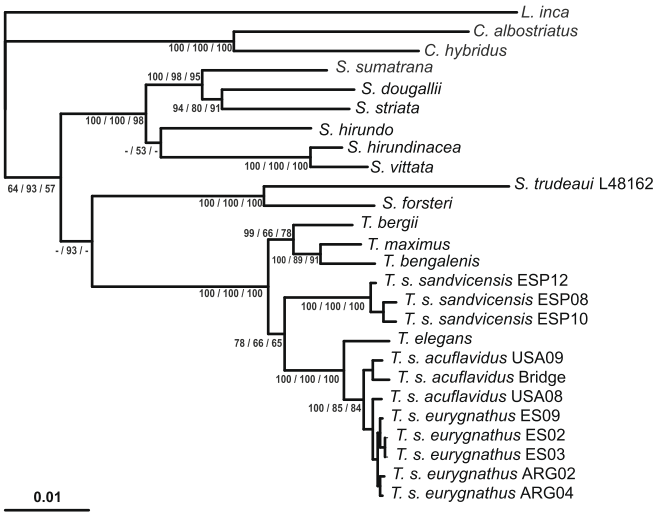

Fig. 1. Phylogenetic tree inferred from Bayesian analysis of 3832 bp from mtDNA + nuclear sequences. Support values are indicated at nodes (Bayesian posterior probabilities, ML and MP bootstrap values, respectively). (-) indicates values lower than 50%, (*) indicates branches where different methods yielded different topologies.

(Fig. 2). Individuals of *T. s. acuflavidus* and *T. s. eurygnathus* were not reciprocally monophyletic. The European terns differed from Cabot's, Cayenne and Elegant terns by 4.2% (HKY + gamma distance) and 3.2% (K2P distance). There are 15 characters that distinguish these clades, 7 on the branch to *T. s. sandvicensis* and 8 on the branch to the other subspecies of the Sandwich tern and the Elegant tern. Chance occurrence of reciprocal monophyly of these two clades was rejected ($p = 2.26 \times 10^{-5}$, $\alpha = 1\%$).

The divergence times estimated with the MCMC Bayesian approach using only mtDNA sequences agree with dates presented by Bridge et al. (2005), e.g. the separation between the *T. sandvicensis/maximus/bengalensis/bergii* and the *T. s. eurygnatus/acuflavidus/T. elegans* clades were about 2.7 MYBP, however using the nuclear + mtDNA dataset the separation between the *T. sandvicensis* and the *T. s. eurygnatus/acuflavidus/T. elegans* clades were older, dated around 3.6 MYBP.

4. Discussion

Our analysis indicates that the Old World (*T. s. sandvicensis*) and the New World (*T. s. acuflavidus/eurygnathus*) tern populations are genetically as divergent as different species in the genus, and do not form a monophyletic group. Instead, the latter are sister to the Elegant tern (*T. elegans*). These results strongly suggest that the current taxonomic treatment of the *T. s. sandvicensis/acuflavidus/eurygnathus* complex as subspecies within a single species or as a northern hemisphere (*T. s. sandvicensis*) and a southern hemisphere species (*T. s. eurygnathus*) are phylogenetically inappropriate. The new arrangement should be one in which the Old World (Sandwich) tern *T. s. sandvicensis* and the New World (Cayenne and Cabot's) terns *T. s. acuflavidus/eurygnathus* are considered two different species. COI barcodes of a larger sample of individuals of the Sandwich tern complex also supported the multigene

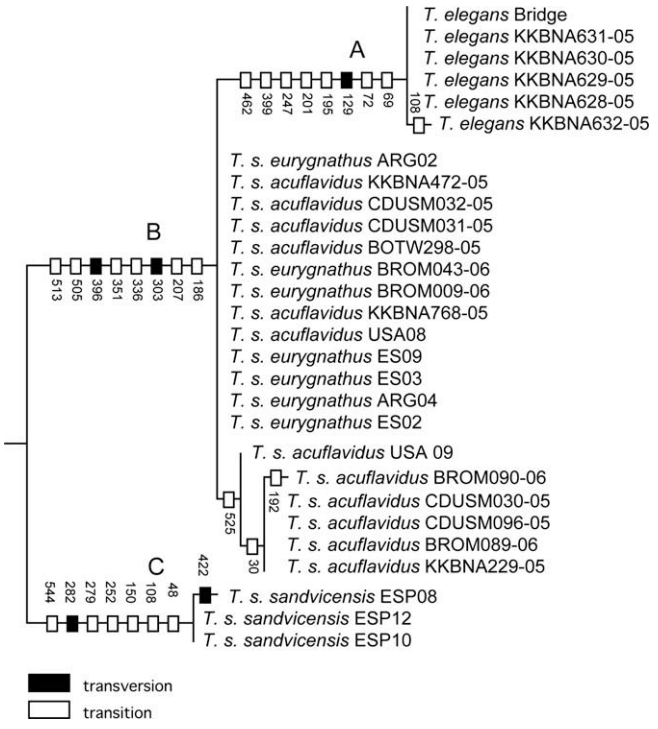

Fig. 2. Diagnostic substitutions in COI mapped on the Bayesian tree topology of the barcode sequences of 630 bp. Substitutions are numbered according to their position in the sequences. The branch lengths are proportional to the characters that change unambiguously on the branches. Nodes A, B, and C have posterior probabilities of 1.0.

phylogeny, splitting with high statistical support the European group (*T. s. sandvicensis*) from the other two subspecies of the complex (*T. s. acuflavidus*, and *T. s. eurygnathus*) and illustrating the efficacy of DNA barcoding in discovering potential new taxa of birds (Hebert et al., 2003). The advantage of complementing multigene phylogenetic evidence with DNA barcoding of the complex is that diagnostic substitutions characteristic of other well known sister species of birds are clearly revealed (Fig. 2), and can be tested statistically for taxonomic distinctiveness (Tavares and Baker, 2008; Rosenberg, 2007).

Our study shows that the North American/Caribbean (Cabot's tern) and the Caribbean/South American (Cayenne tern) populations are very similar genetically (Table 1). In a more extensive study on the genetic structure of the New World populations based on DNA sequence and microsatellite variability (M.A.E, S.L.B. unpublished results), populations from these two taxa share mtDNA and nuclear haplotypes and present low microsatellite differentiation with a complex genetic structure, with no evidence of complete reproductive isolation,. Therefore, our preliminary genetic results do not support the existence of subspecies in this taxon. We propose that the appropriate taxonomic treatment for the New World terns (*acuflavidus/eurygnathus* complex) should be as Cabot's Tern, *Thalasseus acuflavidus*, since *S. acuflavida* was nominated by Cabot in 1847 and *S. eurygnatha* by Sanders in 1876, in agreement with the grammatical arrangement suggested by David and Gosselin (2002).

What are the consequences of this new taxonomic treatment for the conservation efforts of these taxa? The IUCN currently classifies the *T. sandvicensis* complex as of Least Concern (LC) because of its large geographic range, with an estimated global extent of occurrence of 100,000–1,000,000 km^2 and a large global population (BirdLife International, 2008). Global population trends have not been quantified, but the species (s.l.) was not believed to approach the thresholds for the population decline criterion of the IUCN Red List (BirdLife International, 2008). A taxon is of Least Concern when it has been evaluated against these criteria and does not qualify for other categories (IUCN, 2001), but may require the same degree of attention that a more threatened taxon. The UK Joint Nature Conservation Committee considers the conservation status of *T. sandvicensis* in the UK to be unfavorable, and recommends general protection of breeding grounds (JNCC, 2008). In view of the new arrangement suggested here, in which the Old World and the New World populations are two distinct species, the conservation status of both *T. sandvicensis* and *T. acuflavidus* need to be revised.

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