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ORIGINAL ARTICLE

Multilocus barcoding confirms the occurrence of Elegant Terns in Western Europe

Paul Dufour[1] · Jean-Marc Pons[2,3] · J. Martin Collinson[4] · Julien Gernigon[5] · J. Ignacio Dies[6] · Patricia Sourrouille[1] · Pierre-André Crochet[1]

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Abstract We used sequences from one mitochondrial gene and six nuclear loci to confirm genetically the presumed identity of four large terns with an orange bill seen in Western Europe over the past decades. This multilocus genotyping (multilocus barcoding) approach confirmed that one bird was a Lesser Crested Tern *Sterna bengalensis*, as suspected based on its phenotype, and identified the three other birds as pure Elegant Terns *Sterna elegans*. This last result was again in accordance with the appearance of these birds even if their identity had long been considered as unproven. In comparison with traditional (single-locus) barcoding, our approach allowed us to unambiguously exclude that these birds were first-generation hybrids or backcrosses involving Elegant Terns or other species of orange-billed terns.

Keywords Genetic identification · Nuclear DNA · Hybridization · Long-range vagrancy

Zusammenfassung

Multi-locus DNS-Barcoding bestätigt das Vorkommen der Schmuckseeschwalbe (*Sterna elegans*) in Westeuropa

Wir benutzten Sequenzen eines mitochondrialen Gens und von Intron-Regionen aus sechs Kernloci, um mit molekulargenetischen Methoden die bereits vermutete Identität von vier großen Seeschwalben mit orangenen Schnäbeln zu bestätigen, die in den vergangenen Jahrzehnten in Westeuropa gesehen worden waren. Mit diesem "multi-locus Barcoding"-Ansatz zur Bestimmung des Genotyps konnte bestätigt werden, dass einer der Vögel eine Rüppellseeschwalbe (*Sterna bengalensis*), die anderen drei eindeutig Schmuckseeschwalben (*Sterna elegans*) waren. Dieses Ergebnis entsprach dem Auftreten dieser Vögel, wenngleich ihre Identität lange als unbewiesen gegolten hatte. Im Vergleich zum sonst üblichen (single-locus) Barcoding erlaubte uns unser Ansatz, eindeutig auszuschließen zu können, dass die Vögel Hybriden in erster Generation waren oder Rückkreuzungen von Schmuckseeschwalben oder anderen Seeschwalben-Arten mit orangenen Schnäbeln.

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✉ Paul Dufour
paul.dufour80@gmail.com

[1] CEFE UMR 5175, CNRS - Université de Montpellier - Université Paul-Valéry Montpellier - EPHE, 1919 Route de Mende, 34293 Montpellier Cedex 5, France

[2] Département Systématique et Evolution, Muséum National d'Histoire Naturelle, UMR 7205 Institut de Systématique, Evolution, Biodiversité, CNRS, MNHN, UPMC, EPHE, Sorbonne Universités, CP 51, 55 rue Buffon, 75231 Paris Cedex 05, France

[3] UMS MNHN/CNRS 2700 Outils et Méthodes de la Systématique Intégrative (OMSI), Muséum National d'Histoire Naturelle, 57 rue Cuvier, 75231 Paris Cedex 05, France

[4] School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, UK

[5] 5 Impasse de la Boguette, 17880 Les Portes-en-Ré, France

[6] Servicio Devesa-Albufera, CV-500 km 8.5, 46012 Valencia, Spain

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Introduction

Analysis of DNA sequences has been used since the 1990s either to discover or delimit species boundaries, or for identification of individual specimens (Saiki et al. 1988). Hebert et al. (2003) even suggested that the use of a single gene sequence could be enough to characterize the majority of animal biodiversity and proposed using the mitochondrial gene cytochrome c oxidase subunit 1 as a universal DNA barcode delimiting species based on mitochondrial sequence divergence. However, this approach is not without controversy (e.g. Moritz and Cicero 2004). To achieve universal and reliable species delimitation and specimen identification using single-locus barcoding, DNA sequences sampled within a species need to have their most recent common ancestor within that species, and levels of sequence divergence between species have to be much larger than within species. This is true in many cases but there are numerous exceptions, partly due to introgression (e.g. Whitworth et al. 2007). Moreover, recent hybridization events may be undetectable with a mitochondrial barcode because hybrids and backcrosses will only exhibit their mothers' mitochondrial DNA. It is thus of paramount importance to use multilocus nuclear genotyping to identify specimens in species groups where hybridization is suspected. The main difficulty of these multilocus barcoding approaches is that, unlike for mitochondrial DNA, individual nuclear loci have not yet been identified that are sufficiently variable to allow differentiation between species yet sufficiently conserved for use of 'universal' PCR primers that work in all species of deep taxonomic groupings.

Elegant Tern *Sterna elegans* and Sandwich Tern *Sterna sandvicensis* are two closely related species of "crested" terns (Efe et al. 2009) belonging to the subgenus *Thalasseus*, which is sometimes recognized as a distinct genus (Bridge et al. 2005) but not by the European taxonomic authorities we prefer to follow (BOURC and AERC, see http://www.bou.org.uk/thebritishlist/British-List.pdf and http://www.aerc.eu/tac.html). "Crested" terns have a worldwide distribution and are characterized by a black crown with elongated feathers on the rear forming a crest. Speciation within the group was inferred to be recent, with current species diversity originating within the last 3 million years (Bridge et al. 2005). Nearctic and Palearctic populations of the Sandwich Tern were long treated as conspecific due to their phenotypic similarity, but they were recently split into different species owing to their genetic divergence, the closer relationships of Nearctic populations to Elegant Tern than to Palearctic populations and to minute but likely diagnostic differences in plumage and structure (see Sangster et al. 2011). Currently, the Palearctic Sandwich Tern is treated as a monotypic species by most authorities while the North American Cabot's Tern (formerly treated as a Nearctic subspecies of Sandwich Tern) includes the subspecies *Sterna acuflavida acuflavida* and *Sterna acuflavida eurygnatha* (Cayenne Tern). Following this split, the distribution ranges of Elegant Tern and Sandwich Tern do not overlap any more (see Table 1).

In Europe, several birds presenting Elegant Tern characters have been seen either during the breeding period in Sandwich Terns colonies or along coasts of several European countries (e.g. Boesman 1992; Gutiérrez 1998; Milne and McAdams 2005; Fig. S1). This vagrancy pattern, involving several individuals of a species only breeding on the Pacific coast of America, is unprecedented in Europe. In addition, some of these birds were perceived as exhibiting features atypical for Elegant Tern. Although some apparent Elegant Terns in Europe have been morphologically indistinguishable from those on the Pacific coast of the USA, others, whose identification has remained controversial, have lacked the 'classic' long, richly bicoloured bill of Elegant Tern (see Table 1), showing instead a more uniform yellow bill.

Known occurrence of hybridisation between Sandwich Tern and the yellow-billed Lesser Crested Tern *Sterna bengalensis* in Europe (Dies and Dies 1998; Dies 2001), and the possibility of hybrids formed with the orange-billed Royal Tern *S. maxima*, have also confused the situation (see Table 1). Together, these lines of arguments raise the possibility that Elegant Tern-like birds seen in Europe might include, or entirely be, hybrids between various "crested" tern species. This hypothesis has precluded formal acceptance of recent records of Elegant Terns in several European countries (BOU 2014). Some presumed Elegant Terns have also been recorded along the Atlantic coast of North America, sometimes in Cabot's Terns colonies, although here again identification has been questioned (Paul et al. 2003). The 'hybrid hypothesis' was reinforced by the occurrence of several mixed pairs and suspected hybrids between Elegant Tern and Cabot's Tern along the Pacific (Collins 1997; Velarde and Rojo 2012) and Atlantic coasts of North America (Paul et al. 2003) and by the fact that some apparent Elegant Terns identified in Europe were paired with Sandwich Terns (own unpublished data).

In summary, the birds observed in Europe and showing characteristics of Elegant Terns (notably a combination of orange bill and white rump) might be genetically pure Elegant Terns or hybrids from mixed pairs involving Elegant Tern and Cabot's or Sandwich Tern. Some could even be offspring of Elegant Tern paired with Lesser Crested Tern (the last species has been seen in Western France in the same colony as Elegant-type birds) or with Royal Tern, or even hybrid Lesser Crested × Royal Terns, two species with yellow or orange bills which could produce offspring with orange bills and whitish rumps. To resolve these

Table 1 Adult phenotypic characters and breeding range of the sampled species

	Species	Size[a] (cm)	Bill characters[a]		Breeding range[b]
			Colour	Structure	
1.	Elegant Tern *Sterna elegans*	43	Reddish-orange with paler tip	Long with drooping bill tip	Pacific coast of North America
2.	Sandwich Tern *Sterna sandvicensis*	40	Black with small yellow tip	Slender bill	Europe from the Atlantic coast to the Caspian Sea
3.	Cabot's Tern *Sterna acuflavida acuflavida*	38	Black with small yellow tip	Slender bill, slightly shorter than 2	Atlantic coast of North America
4.	Cayenne Tern *Sterna acuflavida eurygnatha*	38	Yellow to black with small yellow tip	Slender bill, slightly shorter than 2	Central and South America
5.	American Royal Tern *Sterna maxima maxima*	45	Uniform orange to red	Heavy bill with curved culmen and marked gonys	Pacific coast of South America and Atlantic coast of America
6.	African Royal Tern *Sterna maxima albididorsalis*	45	Uniform paler orange	Heavy bill with flatter culmen and less obvious gonys than 5	Atlantic coast of Africa
7.	Lesser Crested Tern *Sterna bengalensis*	36	Uniform yellow–orange	Shorter than 2 with straight lower mandible	Mediterranean coast of Lybia, Red Sea, Persian Gulf, Indian Ocean and Australasia
8.	Greater Crested Tern *Sterna bergii*	46	Uniform green-yellow	Heavier than 7	Namibia, South Africa to East Africa, Red Sea, Indian Ocean, Southeast and East Asia to Australasia

[a] Source van Duivendijk (2010)

[b] Source http://www.iucnredlist.org

possibilities three adult European Elegant Tern-like birds, one in Spain and two from France, were caught, colour-ringed and sampled for DNA analysis. In addition, a bird assigned to Lesser Crested Tern, based on morphological cues, and seen in France in the same colony as Elegant-type birds was also caught and colour-ringed.

Preliminary genetic analyses performed in 2012 and 2013 by J. M. P. and J. M. C. using one nuclear intron (Beta Fibinogen intron 7; BFib7) and the mitochondrial gene ND2 revealed that all three Elegant Tern-like birds had *Sterna elegans* mtDNA but suggested that two of the three birds had mixed ancestry as they were heterozygous for a single nucleotide polymorphism (SNP) of the BFib7 locus that was believed to be species-diagnostic in the small sample of Elegant and Sandwich used as a reference (see below for details). This preliminary result generated considerable online debate and left us unsatisfied as it was based on a small sample of reference specimens and only one nuclear marker and because it was difficult to reconcile with the appearance of the birds (the supposedly pure individual having a phenotype far less typical of Elegant Tern than the suspected hybrids).

The aims of this study were thus to:

1. Increase the number of Elegant and Sandwich Terns sequenced for the BFib7 locus to verify its validity as a diagnostic marker between these two species.
2. Develop a multilocus barcoding approach for large "crested" terns (*Sterna* subgenus *Thalasseus*).
3. Apply multilocus barcoding to the identification of large terns with red or orange bills resembling Elegant Tern ('Elegant Tern-like birds' hereafter) and Lesser Crested Tern seen in Western Europe.

Methods

Sampling

Samples of birds for identification (four in total) were collected in Spain and in France as follows. One bird (*Sterna* 3) was caught in the Sandwich Tern colony of L'Albufera de Valencia on the Mediterranean coast of Spain (Valencia province, 39°20′N, 00°20′W) on 2 June 2006 by J. I. D. This bird was already ringed when caught in 2006, and the ring revealed it had been first ringed as a Lesser Crested Tern in the Marismas del Odiel, Huelva (Southwest Spain) on 8 October 2002 (M. Vázquez, personal communication). Its white rump suggested Elegant Tern, but its bill colour and shape were perceived as slightly atypical for that species and its identity was left unresolved. Colour rings (yellow ring) were added and a

blood sample taken from the bird (see http://www.freewebs.com/jidies/AlbuferaTern.pdf for details). The other two birds were caught and sampled by J. G. in the Sandwich Tern colony of the Banc d'Arguin on the Atlantic coast of France (Gironde). One of them (*Sterna 1*) was caught for the first time on 18 June 2007 and the second time on 15 June 2013. Several feathers were collected on each occasion. The other bird (*Sterna 2*) was caught on 3 July 2003 and a second time on 14 June 2013, several feathers were collected. Both birds were also colour-ringed (*Sterna* 1 with red/white rings and *Sterna* 2 with yellow/green rings). One of them (*Sterna* 1) perfectly matched the appearance of Elegant Tern in America but the other one (*Sterna* 2), with its relatively short and pale bill, was widely believed to be of hybrid origin. Lastly, one bird identified as Lesser Crested Tern (*Sterna* 4) was caught by J. G. on the Banc d'Arguin in the same colony as the Elegant Tern-like birds in July 2003 (see Supplementary Fig. 1 for photographs of all four birds).

Reference samples were obtained as follows: Sandwich Tern, breeding adults or chicks, Banc d'Arguin, Western France (44°35′N, 1°14′W) ($n = 10$) and near Agde, Southern France (43°23′N, 3°38′E) ($n = 4$); Elegant Tern, Bolsa Chica State Ecological Reserve, USA (33°41′N, 118°2′W) ($n = 5$) and Westport, Grays Harbor, USA (46°54′N, 124°7′W) ($n = 9$); Lesser Crested Tern (subspecies *emigrata*), Libya ($n = 8$); Royal Tern (subspecies *albididorsalis*), Cap Blanc peninsula, Mauritania, 20°49'N, 17°04'W ($n = 1$) and Tanji Bird Reserve, Gambia, 13°23'N, 16°48'W ($n = 1$); Royal Tern (subspecies *maxima*), Guadeloupe ($n = 1$); Crested Tern (subspecies *bergii*), Robben Island, South Africa, 33°49'S, 18°22'E ($n = 1$).

DNA extraction and genotyping

We selected 13 nuclear loci for initial screening (including BFib7, which had already been sequenced in the three Elegant Tern-like birds; see Introduction) that had already been found to be variable in birds (see Table 2 for details). Most of the samples were processed in Montpellier, but for some samples (including the 4 birds to identify) independent extractions, polymerase chain reaction (PCR) and sequencing were done in Aberdeen (by J. M. C.), Paris (J. M. P.) and Montpellier (P. D.) for a subset of the diagnostic loci.

In Montpellier, DNA was extracted from blood or feather base using the Qiagen Blood and Tissue Extraction Kit (Applied Biosystems, Foster City, CA), following the manufacturer's recommended procedures. Negative extraction blanks were made by processing tubes in exactly the same way as tissue samples. Standard amplification protocols were used. The annealing temperature was 55 °C for all loci except for ND2 (56 °C) and MYO2 (57 °C). Both strands of the PCR products were sent for sequencing at Eurofins Genomics (Ebersberg, Germany) using the same primers as for amplification (primers are reported in Table 2). Sequences were aligned with MEGA6 (Tamura et al. 2013) with further adjustment by eye. Point substitutions were spotted on the alignment and checked by visual inspection of the chromatographs using Chromas version 2.4.3 (Technelysium) but most heterozygous sites were missed by the program and had to be identified by visual inspection of the chromatographs.

In Aberdeen, DNA was extracted using the DNA Micro Kit (Qiagen, UK) according to the manufacturer's instructions, with addition of dithiothreitol to 0.1 M concentration in the digestion mix and elution in 80 µl of Qiagen buffer AE. PCR, DNA extraction and sequencing were performed using protocols as described in Shannon et al. (2014). The primers used were those described in Table 2. In addition, ND2 was amplified using universal primers L5216 and H6313 as described in Shannon et al. (2014).

All sequences were deposited in GenBank except for those of individuals that yielded incomplete sequences (GenBank accession nos. KU668666-681 for ND2, KU577493-506 for Myo2, KU577469-492 for BFib7, KU252681-712 for 3862, KU234225-256 for ACL, KU252713-745 for CRMIL, KU252780-812 for RGS4, KU252813-844 for TGF, KU252746-779 for FGB, KX131231-239 for 16264, KX131240-247 for 17483, KX131249-256 for 26187, and KX131257-265 for GAPD2; see "Appendix" for G3PDH). In addition, 11 ND2, 13 BFib 7 and four Myo2 sequences available in Genbank were included in our data set (see Fig. 1; Tables S1, S2). For illustrative purpose, a phylogenetic tree of the ND2 sequences was performed using Mega version 6 (Tamura et al. 2013). Briefly, we selected the best nucleotide substitution model selection (HKY) then performed a maximum-likelihood analysis with 1000 bootstrap replicates using this model of substitution.

Results and discussion

The molecular analyses clearly supported that our three Elegant Tern-like individuals were genetically pure Elegant Terns and suggested that the suspected Lesser Crested Tern was correctly identified.

Mitochondrial DNA

Results for the mitochondrial ND2 are reported in Fig. 1. Several diagnostic sites were found between all species. All three Elegant Tern-like birds presented without ambiguity a *S. elegans* mtDNA haplotype, whereas the Lesser Crested Tern from France presented, as expected, a *S. bengalensis* mtDNA haplotype.

Table 2 Genetic loci screened in this study

Short name	Length (base pairs)	Primers	Complete name	Source
16264	684	TTTATAGGCACATCCTTGAC GCATTGACCTCAAAGAAGGC	Post-synaptic protein CRIPT	Jackson et al. (2012)
17483	806	TTGCTCTTGGCACGATATGC GAAATGTGGTCTGAACAGTC	High-mobility group protein B2	Jackson et al. (2012)
26187	868	CATGTTCCAGAGGTTGTAGG GGTGGGAATGCAGTAGTAGA	ATPase, lysosomal accessory protein 2	Jackson et al. (2012)
3862[a]	742	CACCTCGTTGGAGATGTTCC CCCTCCGACTTCTTCAACCC	WD repeat protein 24	Jackson et al. (2012)1
ACL (16)[a]	458	CAGCAATAATGGCAATGGTG GCTCTGCTTATGACAGCACT	ATP citrate lyase	Jackson et al. (2012)1
BFIB (7)	948	TCCCCAGTAGTATCTGCCATTAGGGTT GGAGAAAACAGGACAATGACAATTCAC	Beta-fibrinogen	Jackson et al. (2012)
CRMIL (14)[a]	630	TGATGAGATCCACTCCATCG TCAATCATCCACAGAGACC	V-raf murine sarcoma viral oncogene	Jackson et al. (2012)
G3PDH (11)	380	ARRTCCACAACACGGTTGCTGTA GGCATTGCACTGARYGAYCATTT	Glyceraldehyde 3-phosphate dehydrogenase	Jackson et al. (2012)
RGS4[a] (3)	745	GTAGTCCTCACAACTGACC TCGCTGGAAAACTTGATCC	Regulator of G protein signaling 4	Jackson et al. (2012)
TGF[a] (5)	560	GAAGCGTGCTCTAGATGCTG AGGCAGCAATTATCCTGCAC	Transforming growth factor, beta 2	Kimball et al. (2009)
FGB[a] (5)	580	CGCCATACAGAGTATACTGTGACAT GCCATCCTGGCGATTCTGAA	Fibrinogen beta chain	Kimball et al. (2009)
GAPD2	380	GGCATTGCACTGAATGACCATT CTGGGGACAGAAACAGAAGTG	Glyceraldehyde 3-phosphate dehydrogenase-2	Gay et al. (2004)
MYO2	678	GCCACCAAGCACAAGATCCC GCAAGGACCTTGATAATGACTT	Myoglobin gene	Slade et al. (1993) Heslewood et al. (1998)
mtDNA				
ND2	1014	CCCATACCCCGAAAATGATG CTCTTATTTAAGGCTTTGAAGGC	NADH dehydrogenase 2	Sorenson et al. (1999)

Each locus is shown with intron number in brackets, annealing temperature, forward and reverse primers, complete name and source

[a] Loci presenting diagnostic single nucleotide polymorphisms

Nuclear introns: identification of diagnostic loci and reliability of BFib7

First, all nuclear introns except BFib7 were sequenced for five individuals of Elegant Tern and five individuals of Sandwich Tern. Among these 11 introns, five loci (16264, G3PDH, GAPD2, 17483 and 26187) did not reveal candidate diagnostic mutations between the two species and were thus discarded. For the other loci, at least one substitution separated all Elegant Tern from all Sandwich Tern individuals, and for these markers five additional individuals of Elegant Tern and five additional individuals of Sandwich Tern were sequenced to confirm suspected diagnostic sites.

For BFib7, we sequenced nine individuals of confirmed Elegant Tern and four individuals of Sandwich Tern in Montpellier. We added and compared them to the three Elegant Tern (two sequenced by J. M. P. and one unpublished sequence sent to us by E. S. Bridge), the two Sandwich Tern (sequenced by J. M. P.), and one Royal Tern (sequenced by M. C.) sequences which were already available. We also used additional GenBank sequences of Cabot's Tern (two *S. acuflavida* FJ356204-FJ356205 and five *S. eurygnatha* FJ356199-FJ356202), Royal Tern (AY695189), and Sandwich Tern (FJ356206-208). Adding more Elegant specimens to the small data set available to us previously revealed that this marker cannot be used to separate Elegant from Sandwich Tern as the substitution

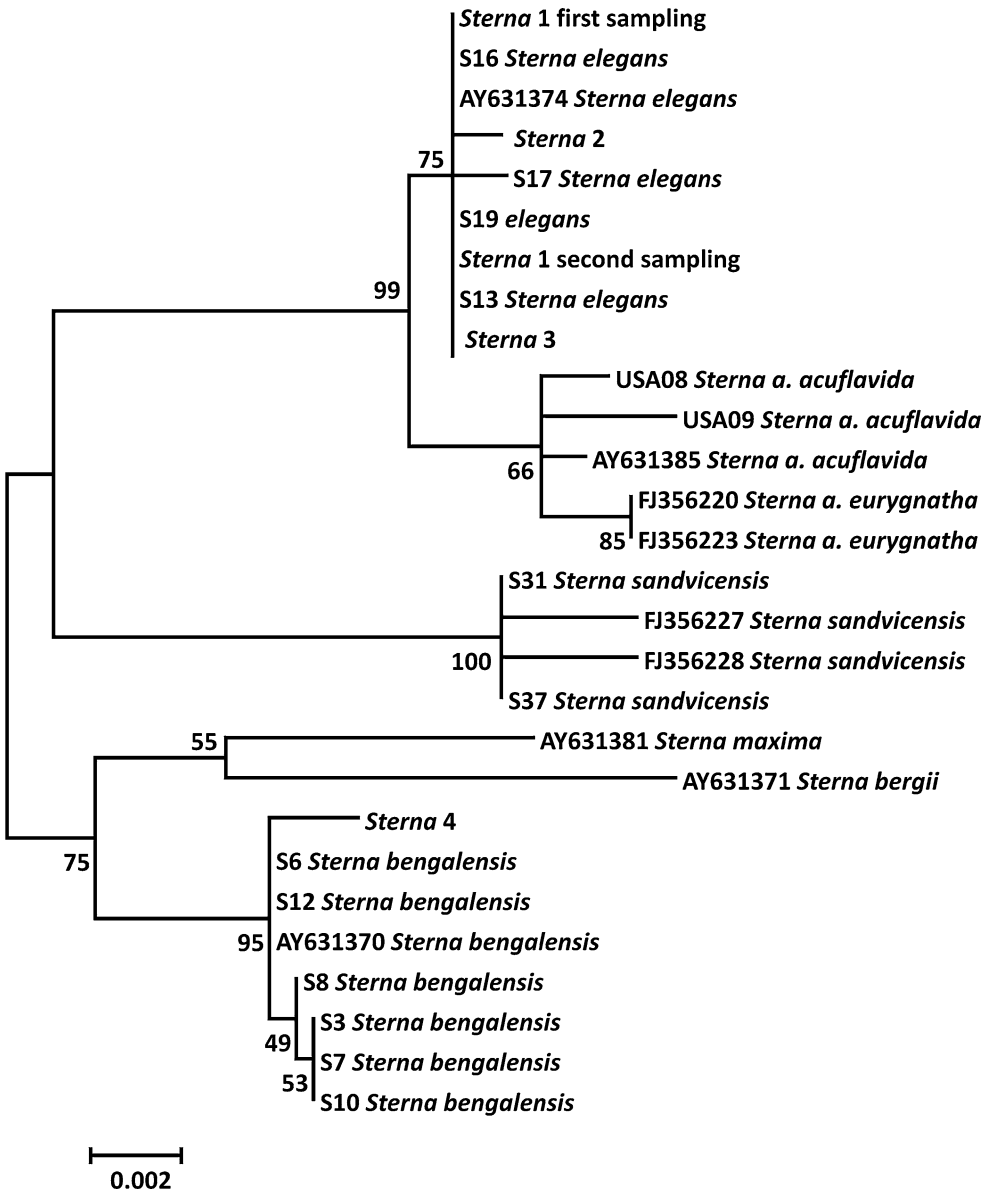

Fig. 1 Maximum-likelihood phylogenetic tree of mitochondrial ND2 sequences. Bootstrap values (1000 replicates) are indicated at *nodes*

we thought to be diagnostic for Sandwich Tern was in fact observed in two Elegant Terns from the USA in the heterozygous state (see Table S1).

The six others nuclear introns were retained as they presented at least one mutation fixed (ACL, FGB, TGF, 3862, CRMIL) or nearly so (RGS4) between our ten Elegant and ten Sandwich Tern specimens. The number of (near) diagnostic SNPs by intron varied between 1 and 2 (see Table 3). These six loci were thus sequenced on the three European Elegant Tern-like birds.

Nuclear introns: genotyping and identification

The three Elegant Tern-like birds were found to be homozygous for *S. elegans* alleles at all diagnostic SNPs in all six loci, excluding the possibility that they were F1 hybrid between Elegant and Sandwich Terns. The probability that an F2 backcross Elegant × Sandwich Tern with Elegant Tern would exhibit Elegant Tern alleles at all six loci is only $(0.5)^6 = 0.016$ (thus <2 %) and can be discarded as highly unlikely. Sequencing on some of these introns from independent extractions of the same birds by M. C. at Aberdeen University confirmed these results and allowed us to eliminate the risk of contamination or other mistakes in the laboratory.

As can be seen from Table 3, we can also exclude the possibility that any of these birds is a hybrid involving Lesser Crested Tern, Royal Tern or Greater Crested Tern, as none of them show any of the alleles of these species for three of the sic loci. This was also confirmed by examination of the

Table 3 Variable positions in nuclear introns of Sandwich and Elegant Terns and our Elegant Tern-like birds from Europe

	RGS4				FGB			TGF	ACL			3862		CRMIL
	256[a]	306[a]	338[a]	483[a]	174[a]	317[b]	385[a]	334[b]	104[b]	200[b]	219[a]	156[b]	387[b]	327[b]
1 *Sterna* 3 (S35-JMP)	T	T	T	G	C/T	*G*	T	*A*	*G*	*C*	G/A	*G*	*T*	*C*
1 *Sterna* 3 (S35)	?	?	?	?	?	*?*	?	*?*	*G*	*C*	G/A	*?*	*?*	*?*
2 *Sterna* 2 (S46-JMP)	T	T/C	T	T	C/T	*G*	T	*A*	*G*	*C*	G	*G*	*T*	*C*
2 *Sterna* 2 (S46)	?	?	?	?	?	*?*	?	*A*	*G*	*C*	G	*?*	*?*	*C*
3 *Sterna* 1 (S47-JMP)	T	T/C	T	T	C/T	*G*	T	*A*	*G*	*C*	G	*?*	*T*	*C*
3 *Sterna* 1 (S47)	T	T/C	T	T	C/T	*G*	T	*A*	*G*	*C*	G	*G*	*T*	*C*
Sterna elegans (S64)	T	T	T	T/G	C	*G*	T	*A*	*G*	*C*	G/A	*G*	*T*	*C*
S. elegans (S67)	?	?	?	?	C/T	*G*	T	*A*	*G*	*C*	G	*G*	*T*	*C*
S. elegans (S68)	T/C	T/C	T	T	C/T	*G*	T/C	*A*	*G*	*C*	G/A	*G*	*T*	*C*
S. elegans (S69)	T	T	T	T/G	C/T	*G*	T/C	*A*	*G*	*C*	G	*G*	*T*	*C*
S. elegans (S70)	T	T	T	T	C	*G*	T	*A*	*G*	*C*	G	*G*	*T*	*C*
S. elegans (S71)	T/C	C	T	T	T	*G*	C	*A*	*G*	*?*	?	*G*	*T*	*C*
S. elegans (S72)	T	T	T	G	C/T	*G*	T/C	*A*	*G*	*C*	G	*G*	*T*	*C*
S. elegans (S73)	T	T	T	G	T	*G*	C	*A*	*G*	*C*	G	*G*	*T*	*C*
S. elegans (S74)	T	C	T/A	G	C/T	*G*	T/C	*A*	*G*	*C*	G	*G*	*T*	*C*
S. elegans (S75)	T	T	T/A	T	T	*G*	C	*A*	*G*	*C*	G	*G*	*T*	*C*
Sterna sandvicensis (S48)	C	C	A	G	T	*A*	?	*G*	*A*	*T/C*	A	*A*	*C*	*T*
S. sandvicensis (S50)	C	C	A	G	T	*A*	C	*G*	*A*	*T*	A	*A*	*C*	*T*
S. sandvicensis(S53)	C	C	A	G	T	*A*	C	*G*	*A*	*T*	A	*A*	*C*	*T*
S. sandvicensis (S54)	C	C	A	G	T	*A*	C	*G*	*A*	*T*	A	*A*	*C*	*T*
S. sandvicensis (S55)	C	C	A	G	T	*A*	?	*G*	*A*	*C*	A	*A*	*C*	*T*
S. sandvicensis (S56)	C	C	A	G	T	*A*	C	*G*	*A*	*T*	A	*A*	*C*	*T*
S. sandvicensis (S59)	C	C	A	G	T	*A*	C	*G*	*A*	*T*	A	*A*	*C*	*T*
S. sandvicensis (S60)	C	C	A	G	T	*A*	C	*G*	*A*	*T*	A	*A*	*C*	*T*
S. sandvicensis (S61)	C	C	A	G	T	*A*	C	*G*	*A*	*T*	A	*A*	*C*	*T*
S. sandvicensis (S63)	?	?	?	?	?	*?*	?	*G*	*A*	*T/C*	A	*A*	*C*	*T*
Sterna bengalensis emigrata (S3)	C	C	A	G	T	*A*	T	*A*	*G*	*C*	A	*G*	*C*	*C*
S. bengalensis emigrata (S6)	C	C	A	G	T	*A*	T	*A*	*G*	*C*	A	*G*	*C*	*C*
S. bengalensis emigrata (S7)	C	C	A	G	T	*A*	T	*A*	*G*	*C*	A	*G*	*C*	*C*
S. bengalensis emigrata (S8)	C	C	A	G	T	*A*	T	*?*	*?*	*?*	?	*?*	*?*	*?*
S. bengalensis emigrata (S9)	C	C	A	G	T	*A*	T	*A*	*G*	*C*	A	*G*	*C*	*C*
S. bengalensis emigrata (S10)	C	C	A	G	T	*A*	T	*A*	*G*	*C*	A	*G*	*C*	*C*
S. bengalensis emigrata (S11)	C	C	A	G	T	*A*	T	*A*	*G*	*C*	A	*G*	*C*	*C*
S. bengalensis emigrata (S12)	?	?	?	?	T	*A*	T	*A*	*G*	*C*	A	*G*	*C*	*C*
Sterna maxima albididorsalis (S1)	?	?	?	?	T	*A*	T	*?*	*G*	*C*	A	*?*	*?*	*C*
Sterna maxima maxima (M02)	?	?	?	?	T	*A*	T	*A*	*G*	*C*	A	*?*	*?*	*C*
S. maxima maxima (S77)	C	C	A	G	T	*A*	T/C	*A*	*A*	*C*	A	*G*	*C*	*C*
Sterna bergii (S78)	C	C	A	G	T	*A*	C	*A*	*G*	*C*	G	*G*	*C*	*C*

Positions in sequences have been numbered relative to GenbankKU252780 (RGS4), KU252747 (FGB), KU252813 (TGF), KU234225 (ACL), KU252681 (3862) and KU252713 (CRMIL). The numbers in the second row refer to the base position in the intron

Question mark Missing base

[a] Near-diagnostic position

[b] Diagnostic position

complete alignment for these six loci (results not shown). We are thus confident that the three Elegant Tern-like birds sampled in Europe are indeed pure Elegant Terns.

One of the loci initially screened (MYO2) shows no fixed difference between Elegant and Sandwich Terns, but shows several sites that separate Lesser Crested from

Cabot's and Cayenne Terns, from Sandwich Tern and from most Elegant Terns (see Table S2). The MYO2 sequence for the French Lesser Crested Tern (*Sterna* 4) was typical of the species and, together with the mitochondrial data (see above), supported the field identification as pure Lesser Crested Tern.

Drawbacks of genotyping by sequencing

The multilocus barcoding approach that we have developed has thus proven very effective in the identification of individuals and exclusion of hybridization in a situation where it is suspected. However, it required a tedious step of marker selection involving sequencing multiple nuclear markers and selecting those showing diagnostic mutations on a subset of reference samples before genotyping additional reference samples. Only after this last step was it possible to genotype our target specimens. Furthermore, detection of heterozygous substitutions as double peaks on chromatographs depends on the quality of the sequenced DNA, and in most cases the software we used did not automatically recover these peaks as heterozygous. Reliable identification of heterozygous base positions thus relied entirely on visual inspection of individual chromatographs. Other genotyping methods, such as Sequenom (Bradić et al. 2011) or KASPar (Cuppen 2007), are available for large-scale genotyping of SNPs, but they are expensive for a small number of specimens and still rely on previous identification of target SNPs. Effective, simple and cheap multilocus barcoding approaches thus still need to be developed.

Morphological and plumage characters of the Elegant Tern-like birds

In spite of the initial confusion surrounding the identification of the three Elegant Tern-like birds, morphological and plumage characters are consistent with our genetic conclusions. Compared with Elegant Terns photographed in the native range, our three birds fit well into the phenotypic variability of the species (personal observation). Moreover, a putative hybrid raised by of one of our three genotyped male Elegant Terns and a female Sandwich Tern, colour-ringed before fledging in the Banc d'Arguin colony and photographed as an adult, is similar to a Sandwich Tern with orange spots on the dark bill and a more extensive yellow bill tip (personal observation).

Origin of the Elegant Terns seen in Europe

We have demonstrated here that three pure Elegant Terns currently reside in Europe where they have been seen every year between 2001 and 2015. Because these three birds do not differ in phenotype from the other Elegant Tern-like birds seen in Europe that have not been sampled in the present study, it is reasonable to assume that most Elegant Tern-like birds seen in Europe are indeed pure Elegant Terns, unless there are plumage or bare-part irregularities that argue against such identification. A total of 25 sightings of birds presenting Elegant Tern characteristics have been noted in Europe since 1974 (P. D., unpublished data) but this probably includes repeated records of the same individuals. Whether all Elegant Tern-like birds seen in Europe were hatched in America and reached Europe via transatlantic vagrancy or some of them were hatched in Europe remains an open question. Most records concern adult birds and several pertain to birds observed in Sandwich Tern colonies for several breeding seasons, usually paired with Sandwich Tern. These birds have sometimes adopted the migratory behaviour of European Sandwich Terns, as illustrated by sightings of both colour-ringed birds from France on their wintering sites in South Africa and Namibia (J. G. unpublished data). Moreover, the Spanish bird that we analysed has previously paired with unsampled Elegant Tern-like birds in Spain in 2011, 2012, 2013, 2014 and 2015, fledging a probably pure Elegant Tern chick in 4 years (J. I. D., unpublished data; see also http://www.rarebirdspain.net). No Elegant Tern-like birds were seen paired together before 2011, but it is conceivable that pure pairs could have escaped detection prior to then.

Extreme vagrancy as a source of interspecific gene flow and long-distance range colonisation

However unlikely long-distance vagrancy might seem to be, our results highlight that it can have evolutionary important consequences. The fact that Elegant Terns and Sandwich Terns are reciprocally monophyletic in mtDNA and several nuclear loci demonstrates that interspecific gene flow has not regularly occurred in the past, knowing that even low levels of gene flow (a few successful hybridization events per century) would result in extensive lineage sharing at neutral markers (Wright 1940). This is clearly not due to pre-mating mechanisms as Elegant Terns regularly mate with Sandwich Terns in Europe and Cabot's Tern in North America, where several mixed pairs have been observed in Florida and California since 1980 (McCarthy 2006). To date all mixed pairs observed in France, Spain (personal observation) and North America (McCarthy 2006) involved *S. elegans* males. We have no information on breeding success of Elegant × Sandwich Tern hybrids. Although complete post-zygotic isolation is theoretically possible, it is unlikely given the low genetic divergence of these species (see Efe et al. 2009). Whatever the reason for a lack of historical gene flow between Elegant and Sandwich Terns, the current records of Elegant

Terns in Europe and of Sandwich Terns in North America illustrate that allopatric ranges, even when normal breeding range are separated by 10,000 km, are not necessarily sufficient alone to totally prevent interspecific gene flow in seabirds. The recent reproduction of homospecific pairs of Elegant Tern in Europe (in Spain, see above) also provides a possible mechanism for long-range colonization in seabirds, including trans-Atlantic colonization, that does not necessarily proceed from gradual range expansion followed by fragmentation, but could originate from occasional natural long-distance vagrants [as in the case of the recent colonization of America by the Cattle Egret *Bubulcus ibis* (see Moralez-Silva and Del Lama 2014)].

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Appendix: sequences of the nuclear intron glyceraldehyde-3-phosphodehydrogenase (G3PDH)

>Seq1 (organism = *Sterna sandvicensis*) S50, France, G3PDH
GAAGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTC
TGCCCCATCTCACACAACTGAACCACTCAGCTTCC
CATCCACTTCTAGTAAAGTAAGTAGGAAAAATTCC
ATACACCCTTCAAATACGGTAAGGAAAAGGCTAC
AGTCATTTCAGATAAGCAGCAACTTCACTCCACAG
AAACTTCATAATATGTTGGAGCCACCCTACACAGC
AGGGGTCTACGTTATGACCCCACACTGCCAACCTG
GCAGTGATGAACAGGACAGAAGCCTGCAACTTGC
CTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCC
CACCACCCCTTAAGGCTGCACCTACCAGGAAACCA
GCTTGACAAAATGATC

>Seq2 (organism = *S. sandvicensis*) S54, France, G3PDH
GAAGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTC
TGCCCCATCTCACACAACTGAACCACTCAGCTTCC
CATCCACTTCTAGTAAAGTAAGTAGGAAAAATTCC
ATACACCCTTCAAATACGGTAAGGAAAAGGCTACA
GTCATTTCAGATAAGCAGCAACTTCACTCCACAGAA
ACTTCATAATATGTTGGAGCCACCCTACACAGCAG
GGGTCTACGTTATGACCCCACACTGCCAACCTGGC
AGTGATGAACAGGACAGAAGCCTGCAACTTGCCT
GTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCAC
CACCCCTTAAGGCTGCACCTACCAGGAAACCAGCT
TGACAAAATGATC

>Seq3 (organism = *S. sandvicensis*) S60, France, G3PDH
GAAGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCT
GCCCCATCTCACACAACTGAACCACTCAGCTTCCCA
TCCACTTCTAGTAAAGTAAGTAGGAAAAATTCCAT
ACACCCTTCAAATACGGTAAGGAAAAGGCTACAG
TCATTTCAGATAAGCAGCAACTTCACTCCACAGAA
ACTTCATAATATGTTGGAGCCACCCTACACAGCAG
GGGTCTACGTTATGACCCCACACTGCCAACCTGGC
AGTGATGAACAGGACAGAAGCCTGCAACTTGCCTG
TGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACC
ACCCCTTAAGGCTGCACCTACCAGGAAACCAGCTTG
ACAAAATGATC

>Seq4 (organism = *S. sandvicensis*) S61, France, G3PDH
GACGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTC
TGCCCCATCTCACACAACTGAACCACTCAGCTTCCC
ATCCACTTCTAGTAAAGTAAGTAGGAAAAATTCCA
TACACCCTTCAAATACGGTAAGGAAAAGGCTACAG
TCATTTCAGATAAGCAGCAACTTCACTCCACAGAA
ACTTCATAATATGTTGGAGCCACCCTACACAGCAG
GGGTCTACGTTATGACCCCACACTGCCAACCTGGCA
GTGATGAACAGGACAGAAGCCTGCAACTTGCCTGT
GTCAGCTCCTCATCCCCCCCAGTGTCTCCCCCACCA
CCCCTTAAGGCTGCACCTACCAGGAAACCAGCTTG
ACAAAATGATC

>Seq5 (organism = *Sterna elegans*) S64, USA, G3PDH
GGCGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTC
TGCCCCATCTCACACAACTGAACCACTCAGCTTCC
CATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCC
ATACACCCTTCAAATACGGTAAGGAGAAGGCTACA
GTCATTTCAGATAAGCAGCAACTTCACTCCACAGA
AACTTCATAATATGTTGGAGCCACCCTACACAGCA
GGGGTCTACGTTATGACCCCACACTGCCAACCTGG
CAGTGATGAACAGGACAGAAGCCTGCAACTTGCCT
GTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCA
CCACCCCTTAAGGCTGCACCTACCAGGAAACCAG
CTTGACAAAATGATC

>Seq6 (organism = *S. elegans*) S67, UWBM69602, USA, G3PDH
GACGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTC
TGCCCCATCTCACACAACTGAACCACTCAGCTTCC
CATCCACTTCTAGTAAAGTAAGTAGGAAGAATTC

CATACACCCTTCAAATACGGTAAGGAGAAGGCTA
CAGTCATTTCAGATAAGCAGCAACTTCACTCCACA
GAAACTTCATAATATGTTGGAGCCACCCTACACA
GCAGGGGTCTACGTTATGACCCCACACTGCCAAC
CTGGCAGTGATGAACAGGACAGAAGCCTGCAACT
TGCCTGTGTCACCTCCTCATCCCCCCCAGTGTCTC
CCCCACCACCCCTTAAGGCTGCACCTACCAGGAA
ACCAGCTTGACAAAATGATC

>Seq7 (organism = *S. elegans*) S68, UWBM70563, USA, G3PDH
GACGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCT
GCCCCATCTCACACAACTGAACCACTCAGCTTCCC
ATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCCAT
ACACCCTTCAAATACGGTAAGGAGAAGGCTACAGT
CATTTCAGATAAGCAGCAACTTCACTCCACAGAAA
CTTCATAATATGTTGGAGCCACCCTACACAGCAGGG
GTCTACGTTATGACCCCACACTGCCAACCTGGCAGT
GATGAACAGGACAGAAGCCTGCAACTTGCCTGTGT
CAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCGCC
CCTTAAGGCTGCACCTACCAGGAAACCAGCTTGAC
AAAATGATC

>Seq8 (organism = *S. elegans*) S69, UWBM69603, USA, G3PDH
GACGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTC
TGCCCCATCTCACACAACTGAACCACTCAGCTTCCC
ATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCCAT
ACACCCTTCAAATATGGTAAGGAGAAGGCTACAG
TCATTTCAGATAAGCAGCAACTTCACTCCACAGAA
ACTTCATAATATGTTGGAGCCACCCTACACAGCAG
GGGTCTACATTATGACCCCACACTGCCAACCTGGC
AGTGATGAACAGGACAGAAGCCTGCAACTTGCCT
GTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCA
CCACCCCTTAAGGCTGCACCTACCAGGAAACCAG
CTTGACAAAATGATC

>Seq9 (organism = *S. elegans*) S70, UWBM70562, USA, G3PDH
GACGAACAGAAGTGCTGTCAGGACTGACCCATT
TCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCT
GCCCCATCTCACACAACTGAACCACTCAGCTTCCC
ATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCCA
TACACCCTTCAAATACGGTAAGGAGAAGGCTACA
GTCATTTCAGATAAGCAGCAACTTCACTCCACAG
AAACTTCATAATATGTTGGAGCCACCCTACACAG
CAGGGGTCTACGTTATGACCCCACACTGCCAACC
TGGCAGTGATGAACAGGACAGAAGCCTGCAACT
TGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTC
CCCCACCACCCCTTAAGGCTGCACCTACCAGGAA
ACCAGCTTGACAAAATGATC

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