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Multi-locus barcoding confirms the occurrence of Elegant Tern in Western Europe

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Dear Editor,

Please find thesecond revised version of our manuscript JORN-D-16-00029 entitled “Multi-locus barcoding confirms the occurrence of Elegant Tern in Western Europe”. We have followed all suggested changes.

All the very best

Instead of using the ring-colour code to describe your birds, please use a better code, such as Sterna I, Sterna II etc.
Done in the text and figures (although we have retained the correspondence with the color codes in one place as this is the information that will allow readers to link our results to field data).
In Fig. 1, please include the genus name
Done.

Multi-locus barcoding confirms the occurrence of Elegant Terns in Western Europe

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Summary: We used sequences from one mitochondrial gene and from intronic regions of six nuclear loci to confirm genetically the presumed identity of four large terns with 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 *S. 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, multilocus barcoding, nuclear DNA, hybridization, long range vagrancy.

Introduction

The 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 to use the mitochondrial gene cytochrome c oxidase subunit 1 (COI) as a universal DNA ʻbarcodeʼ delimiting species based on mitochondrial sequence divergence. However this approach is not without controversy (e.g. Moritz & Cicero 2004). For barcoding to allow universal and reliable species delimitation and specimen identification, 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 mother's mitochondrial DNA. It is thus of paramount importance to use multilocus nuclear genotyping to identify specimens in species groups where hybridization is suspected or proven. The main difficulty of these "multilocus barcoding" approaches is that, unlike for mitochondrial DNA, we do not have nuclear loci which are variable enough to differ between most species but have primers

conserved enough to be used in all species of a deep taxonomic grouping.

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, seehttp://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 as different species owing to their genetic divergence and 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 *S. acuflavida acuflavida* and *S. a. eurygnatha* (Cayenne Tern). Following this split, the distribution range of Elegant Tern and Sandwich Tern do not overlap (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 & MacAdams 2005) (see Figure S1). This vagrancy pattern, involving several individuals of a species only breeding on the Pacific coast of America occurring in Europe, 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 *S. bengalensis* in Europe (Dies & Dies, 1998; Dies 2001), and the possibility of hybrids with the orange-billed Royal Tern *S. maxima*, also confused the situation (see Table 1). Together, these lines of arguments raised the possibility that Elegant-like terns 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 & 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 W France in the same colony as Elegant-type birds) or with Royal Tern, or even hybrids Lesser Crested x Royal Terns, two species with yellow or orange bills which could produce offspring with orange bills and whitish rumps. To resolve these possibilities three adult European Elegant-like birds, one in Spain and two from France were

caught, colour-ringed and sampled for DNA analysis. In addition, a bird assigned to the Lesser Crested Tern on morphological cues 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 JMP and JMC using one nuclear intron (Beta Fibinogen intron 7, BFib7 hereafter) and the mitochondrial gene ND2 revealed that all three Elegant-like birds had *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 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 1) to 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) to develop a "multilocus barcoding" approach for large "crested" terns (*Sterna* subgenus *Thalasseus*) and 3) to apply it to the question of the identification of large terns with red or orange bill resembling Elegant Tern (Elegant-like birds hereafter) and Lesser-crested Tern seen in Western Europe.

METHODS

Sampling

Samples of birds to identify (4 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 02

June, 2006 by JID. This bird was already ringed when caught in 2006, and the ring revealed it had been first ringed as Lesser Crested Tern in the Marismas del Odiel, Huelva (SW Spain) on 8 October 2002 (M. Vázquez, pers. comm.). Its white rump suggested Elegant Tern but its bill colour and shape were perceived as slightly untypical for that species and its identity was left unresolved. Colour-rings (yellow ring) were added and the bird was blood sampled (see http://www.freewebs.com/jidies/AlbuferaTern.pdf for details). The other two birds were caught and sampled by JG in the Sandwich Tern colony of Banc d'Arguin on the Atlantic coast of France (Gironde department, 44°35' N, 1°14' W). One of them was caught a first time on June 18, 2007 and a second time on June 15, 2013. Several feathers were collected on each occasion. The other was caught on July 3, 2003 and several feathers were collected. Both birds were colour-ringed as well (Sterna 1 with red/white rings and Sterna 2 with yellow/green rings). One of them perfectly matched the appearance of Elegant Tern in America but the other one, with its relatively short and pale bill, was widely believed to be of hybrid origin. Last, one bird identified as Lesser Crested Tern (Sterna 4) was caught by JG on Banc d'Arguin in the same colony as Elegant-like birds on July, 2003 (see Supplementary Figure 1 for photos of all four birds).

Reference samples were obtained as follows: Sandwich Tern, breeding adults or chicks, Banc d'Arguin, W France, 44°35'/-1°14' (n=10) and Agde, S France, 43°23'/3°38' (n=4); Elegant Tern, Bolsa Chica State Ecological Reserve, USA, 33°41'/-118°2' (n=5) and Westport, Grays Harbor, USA, 46°54'/-124°7' (n=9); Lesser Crested Tern (subspecies *emigrata*), Libya (n=8); Royal Tern (subspecies *albididorsalis*), Cap Blanc peninsula, Mauritania, 21°0'/-17°4' (n=1) and Tanji Bird Reserve, Gambia, 13°23'/-16°48' (n=1); Royal Tern (subspecies *maximus*), Guadeloupe (n=1); Crested Tern (subspecies *bergii*), Robben Island, South Africa, 33°49'/-18°22' (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-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 independent extractions, PCR and sequencing were done in Aberdeen (by JMC), Paris (JMP) and Montpellier (PD).

In Montpellier, DNA was extracted from blood or feather base using the Qiagen Blood and Tissue extraction kit (Applied Biosystems, Foster City, CA, USA), 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 the amplification (primers are reported in Table 2). Sequences were aligned with MEGA6 (Tamura et al. 2013) with further adjustment by eye. Heterozygous sites and point substitution were spotted on the alignment and checked by visual inspection of the chromatographs using Chromas v2.4.3 (Technelysium Pty Ltd).

In Aberdeen, DNA was extracted using the 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 was performed using protocols as described in Shannon et al. (2014). 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 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, KX131257-265 for GAPD2, see Appendix 1 for G3PDH). In addition, 11 ND2, 13 BFib 7 and 4 Myo2 sequences available in Genbank were included in our data set (see Figure 1 and Table S1 and 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& DISCUSSION

The molecular analyses clearly supported that our three Elegant-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 on Figure 1. Several diagnostic sites were found between all species. All three Elegant-like birds presented without ambiguity an *elegans* mtDNA haplotype whereas the Lesser Crested Tern from France presented as expected a *bengalensis* mtDNA haplotype.

Nuclear introns: identification of diagnostic loci and reliability of BFib7

First, all nuclear introns except BFib7 were sequenced on 5 individuals of Elegant Tern and 5 individuals of Sandwich Tern. Among these11 introns, 5 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 from all

Sandwich individuals and for these markers 5 additional individuals of Elegant Tern and 5 additional individuals of Sandwich Tern were sequenced to confirm suspected diagnostic sites.

For BFib7, we sequenced in Montpellier nine individuals of confirmed Elegant Tern and four individuals of Sandwich Tern. We added and compared them to the 3 Elegant (2 sequenced by JMP and one unpublished sequence sent to us by E.S. Bridge), the 2 Sandwich (sequenced by JMP), and one Royal (sequenced by MC) sequences were already available. We also used additional GenBank sequences of Cabot's Tern (2 *acuflavida* FJ356204-FJ356205 and 5 *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 as the substitution we thought to be diagnostic for Sandwich Terns was in fact observed in two Elegant Terns from 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 10 Elegant and 10 Sandwich 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-like birds.

Nuclear introns: genotyping and identification

The three Elegant-like birds were found to be homozygous for *elegans* alleles at all diagnostic SNPs in all 6 loci, excluding the possibility that they were F1 hybrid between Elegant and Sandwich. The probability that an F2 backcross Elegant x Sandwich Tern with Elegant Tern would exhibit Elegant alleles at all 6 loci is only $(0.5)^6 = 0.016$ (thus less than 2%) and can be discarded as highly unlikely. Sequencing on some of these introns from independent extractions of the same birds by MC 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 Crested Tern as none of them show any of the alleles of these species for 3 of the 6 loci. This was also confirmed by examination of the complete alignment for these 6 loci (results not shown). We are thus confident that the three Elegant-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, from Sandwich and from most Elegant (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 to identify individuals and exclude hybridization in a situation where it was 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, it was 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 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-like birds

In spite of the initial confusion surrounding the identification of the three Elegant-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 (pers. obs.). Moreover, a putative hybrid raised by of one of our 3 genotyped male Elegant Terns and a female Sandwich Tern, colour-ringed before fledging in the Banc d'Arguin colony and photographed as adult, is similar to a Sandwich Tern with orange spots on the dark bill and a more extensive yellow bill tip (pers. obs.).

Origin of the Elegant Terns seen in Europe

We have demonstrated here that three pure Elegant Terns currently reside in Europe where they were seen every year between 2001 and 2015. Because these three birds do not differ in phenotype from most other Elegant-like birds that have not been sampled in the present study, it is reasonable to assume that most Elegant-like terns 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 birds presenting Elegant Tern characteristics were noted in Europe since 1974 (PD, unpublished data) but this probably includes repeated records of the same individuals. Whether all Elegant-like Terns 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 (JG unpublished data). Moreover, the Spanish bird that we analysed has previously paired with unsampled Elegant-like birds in Spain in 2011, 2012, 2013, 2014 and 2015, fledging a probably pure Elegant Tern chick in four years (JID unpublished data, see also www.rarebirdspain.net). No Elegant-like birds were seen paired together before2011, but it is conceivable that pure pairs could have escaped detection prior to that.

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 demonstrate 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 not only in Europe but also 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 (pers. obs.) and North America (McCarthy 2006) involved *elegans* males. We have no information on breeding success of Elegant x Sandwich hybrids. Although complete post-zygotic isolation is theoretically possible, it is unlikely given that the low genetic divergence of these species suggests a recent speciation event (see Efe et al. 2009). Whatever the reason for 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, is not necessarily sufficient alone to totally prevent interspecific gene flow in seabirds. The recent reproduction of Elegant Tern in pure pairs in Europe (in Spain, see above) also provides a possible mechanism for long range colonization in seabirds, including trans-Atlantic colonization, that do 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 &Del Lama 2014).

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394 Proceedings of the Royal Society of London B: Biological Sciences 274: 1731-1739
395 Wright S (1940) Breeding Structure of Populations in Relation to Speciation. The American
396 Naturalist. 74: 232-248.
397
398

399

400 **Table 1:** Phenotypical characters in adults and breeding range of the sampled species.
401 Sources: [1] : van Duivendijk (2010) [2] : www.iucnredlist.org
402

	Species	Size[1] (cm)	Bill characters[1]		Breeding range[2]
			Color	Structure	
1.	Elegant Tern *Sterna elegans*	43	Reddish-orange with paler tip	Long with dropping bill-tip	Pacific coast of North America
2.	Sandwich Tern *S.sandvicensis*	40	Black with small yellow tip	Slender bill	Europe from the Atlantic coast to the Caspian Sea
3.	Cabot's Tern *S. acuflavida acuflavida*	38	Black with small yellow tip	Slender bill, slightly shorter than 2.	Atlantic coast of North America
4.	Cayenne Tern *S. a. eurygnatha*	38	Yellow to black with small yellow tip	Slender bill, slightly shorter than 2.	Central and South America
5.	American Royal Tern *S. 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 *S. m. albididorsalis*	45	Uniform paler orange	Heavy bill with flatter culmen and lesser obvious gonys than 5	Atlantic coast of Africa
7.	Lesser Crested Tern *S. 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.	Great Crested Tern *S. bergii*	46	Uniform green-yellow	Heavier than 7	Namibia, South Africa to East Africa, Red Sea, Indian Ocean, SE and E Asia to Australasia

403

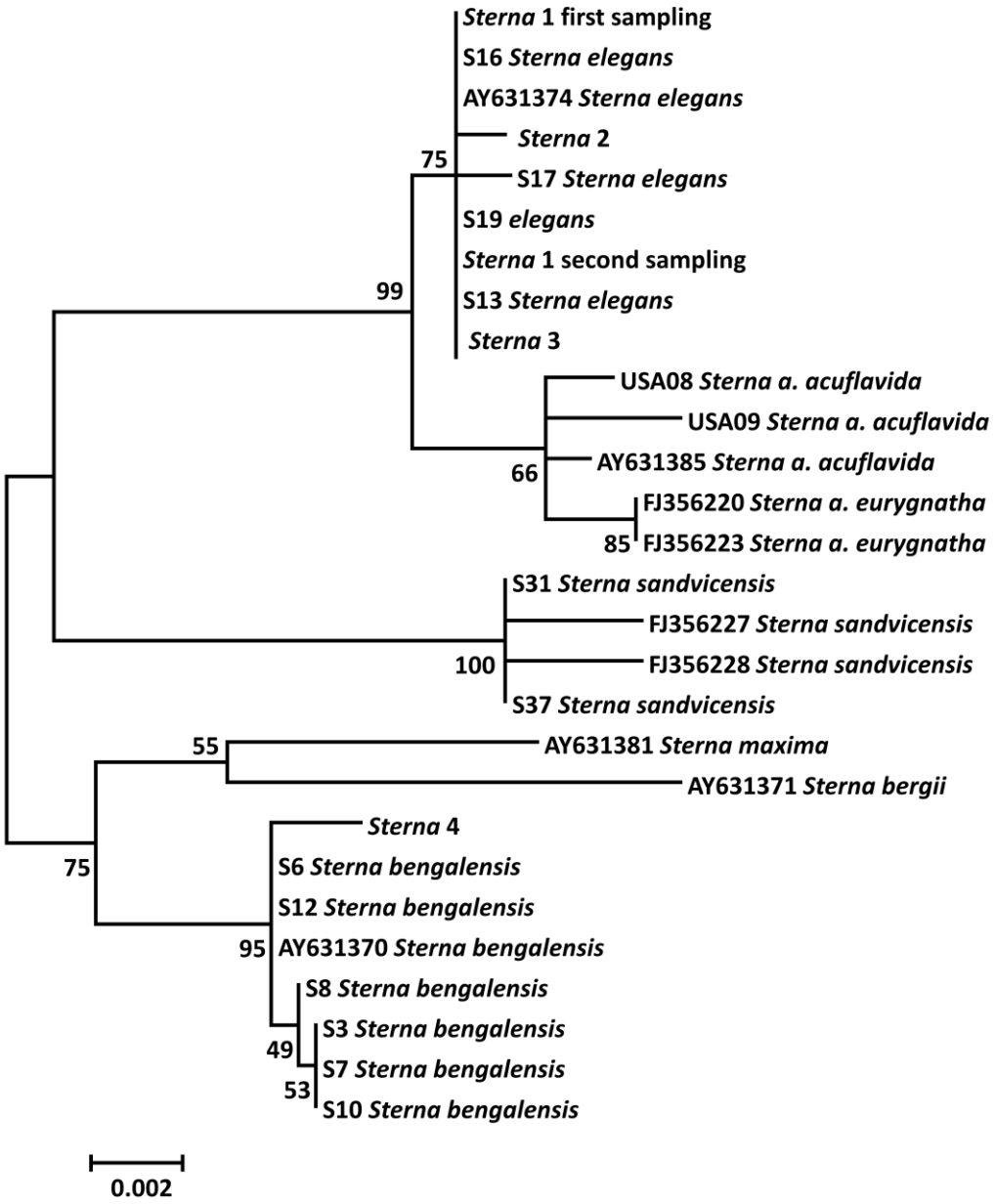
Table 2: Genetic loci screened in this study. Each locus is shown with intron number in parentheses, annealing temperature, forward and reverse primers, gene function and source. Loci in bold are recognised as presenting diagnostic SNPs. Sources: [1] Jackson *et al.*(2012) [2] Kimball *et al. (*2012) [3] Gay *et al.* (2004) [4] Slade*et al.* (1993) [5] Heslewood *et al.*(1998) [6] Sorenson *et al.*(1999).

Name	Length (bp)	Primers	Function	Source
16264	684	TTTATAGGCACATCCTTGAC GCATTGACCTCAAAGAAGGC	Postsynaptic protein CRIPT	1
17483	806	TTGCTCTTGGCACGATATGC GAAATGTGGTCTGAACAGTC	High mobility group protein B2	1
26187	868	CATGTTCCAGAGGTTGTAGG GGTGGGAATGCAGTAGTAGA	ATPase, lysosomal accessory protein 2	1
3862	742	CACCTCGTTGGAGATGTTCC CCCTCCGACTTCTTCAACCC	WD repeat protein 24	**1**
ACL (16)	458	CAGCAATAATGGCAATGGTG GCTCTGCTTATGACAGCACT	ATP citrate lyase	**1**
BFIB (7)	948	TCCCCAGTAGTATCTGCCATTAGGGTT GGAGAAAACAGGACAATGACAATTCAC	Beta-fibrinogen	1
CRMIL (14)	630	TGATGAGATCCACTCCATCG TCAATCATCCACAGAGACC	V-raf murine sarcoma viral oncogene	**1**
G3PDH (11)	380	ARRTCCACAACACGGTTGCTGTA GGCATTGCACTGARYGAYCATTT	Glyceraldehyde 3-phosphate dehydrogenase	1
RGS4 (3)	745	GTAGTCCTCACAACTGACC TCGCTGGAAAACTTGATCC	Regulator G-protein signaling 4	**1**
TGF (5)	560	GAAGCGTGCTCTAGATGCTG AGGCAGCAATTATCCTGCAC	Transforming growth factor, beta 2	**2**
FGB (5)	580	CGCCATACAGAGTATACTGTGACAT GCCATCCTGGCGATTCTGAA	Fibrinogen beta chain	**2**
GAPD2	380	GGCATTGCACTGAATGACCATT CTGGGGACAGAAACAGAAGTG	Glyceraldehyde 3-phosphate dehydrogenase-2	3
MYO2	678	GCCACCAAGCACAAGATCCC GCAAGGACCTTGATAATGACTT	Myoglobin gene	4 5
<u>mtDNA</u>				
ND2	1014	CCCATACCCCGAAAATGATG CTCTTATTTAAGGCTTTGAAGGC	NADH Dehydrogenase 2	6

Table 3: Variable positions in nuclear introns of Sandwich and Elegant Terns and our Elegant-like terns from Europe. Columns in grey are diagnostic position, in white near-diagnosis. The number in the top row refer to the base position in the intron. "?" = missing base. Positions in sequences have been numbered relative to GenbankKU252780 (RGS4), KU252747 (FGB), KU252813 (TGF), KU234225 (ACL), KU252681 (3862) and KU252713 (CRMIL).

	RGS4				FGB			TGF	ACL			3862		CRMIL
	256	306	338	483	174	317	385	334	104	200	219	156	387	327
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
S. 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
S. 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
S. 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
S. maximus albididorsalis (S1)	?	?	?	?	T	A	T	?	G	C	A	?	?	C
S. maximus maximus (M02)					T	A	T	A	G	C	A			C
S. maximus maximus (S77)	C	C	A	G	T	A	T/C	A	A	C	A	G	C	C
S. bergii (S78)	C	C	A	G	T	A	C	A	G	C	G	G	C	C

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



Supplementary Materials:

Figure S1 : A: Sterna 2 Elegant Tern - "Yellow/green" - Banc d'Arguin, Atlantic coast-France 11/07/2006 (Julien Gernigon) **B: Sterna 3** Elegant Tern - "Yellow" - Salins de Bagnas, Mediterranean coast, France 15/07/2008 (X. Rufray) **C: Sterna 1** Elegant Tern - "Red/white" - Banc d'Arguin, Atlantic coast, France 08/08/2007 (Edouard Dansette) **D: Sterna 4** Lesser-crested Tern - "Orange" - Banc d'Arguin, Atlantic coast, France 06/07/06 (Julien Gernigon)

Table S1: Variable positions in the Bfib7 nuclear intron. The numbers in the top row refer to the base position numbered relative to Genbank KU577469.

	BFIB7						
	414	**519**	**639**	**640**	**645**	**660**	**727**
Sterna 3 / MC	T/C	T/G	A/G	T/G	A/G	T/C	T/G
Sterna 3 (S35) / JMP	T/C	T/G	A/G	T/G	A/G	T/C	T/G
Sterna 2 / MC	C	T	G	T	A	T/C	G
Sterna 2 (S46) / JMP	C	T	G	T	A	T/C	G
Sterna 1 (S2) / JMP	T/C	T/G	A/G	T/G	A/G	T	T/G
Sterna 1 (S47) / JMP	T/C	T/G	A/G	T/G	A/G	T	T/G
Sterna 1 / MC	T/C	T/G	A/G	T/G	A/G	T	T/G
S. elegans (B5788-Bridge)	C	T	G	T	A	T	G
S. elegans (S13)	C	T	G	T	A	T	G
S. elegans (S19)	C	T	G	T	A	?	?
S. elegans (S67)	T/C	T/G	A/G	T/G	A/G	T	T/G
S. elegans (S68)	C	T	G	T	A	T	G
S. elegans (S69)	C	T	G	T	A	C	G
S. elegans (S70)	C	T	G	T	A	T	G
S. elegans (S71)	C	T/G	A	G	G	T	T
S. elegans (S72)	C	T	A/G	T/G	A/G	T	T/G
S. elegans (S73)	C	T	G	T	A	C	G
S. elegans (S74)	C	T	G	T	A	T	G
S. elegans (S75)	T/C	T/G	A/G	T/G	A/G	T/C	T/G
S. sandvicensis (ESP12/FJ356208)	T	G	G	T	A	T	G
S. sandvicensis (ESP10/FJ356207)	T	G	A	G	G	T	T
S. sandvicensis (ESP08/FJ356206)	T	G	A	G	G	T	T
S. sandvicensis (S44)	T	G	A	G	G	T	T
S. sandvicensis (S45)	T	G	A	G	G	T	T
S. sandvicensis (S50)	T	G	A/G	T/G	A/G	T	T/G
S. sandvicensis (S54)	T	G	A	G	G	T	T
S. sandvicensis (S60)	T	G	A	G	G	T	T
S. sandvicensis (S61)	T	G	A	G	G	T	T
S. a. acuflavida (USA09/FJ356205)	C	T	G	T	A	C	G
S. a. acuflavida (USA08/FJ356204)	C	T	G	T	A	C	G
S. a. eurygnatha (ES03/FJ356202)	C	T	G	T	A	C	G
S. a. eurygnatha (ES09/FJ356203)	C	T	G	T	A	C	G
S. a. eurygnatha (ES02/FJ356201)	C	T	G	T	A	C	G
S. a. eurygnatha (ARG04/FJ356200)	C	T	G	T	A	C	G
S. a. eurygnatha (ARG02/FJ356199)	C	T	G	T	A	C	G
S. bengalensis emigrata (S3)	C	T	G	T	A	T	G
S. bengalensis emigrata (S7)	C	T	G	T	A	T	G
S. bengalensis emigrata (S10)	C	T	G	T	A	T	G
S. bengalensis emigrata (S12)	C	T	G	T	A	T	G
S. maximus maximus (AY695189)	C	T	G	T	A	T	G
S. maximus maximus (M02)	C	T	G	T	A	T	G

Table S2: Variable positions in the Myo2 nuclear intron. The numbers in the top row refer to the base position numbered relative to Genbank KU577500.

	MYO2				
	110	**112**	**124**	**372**	**528**
Sterna 3 / MC	T	C	A	T	T
Sterna 2 / MC	T/G	C	A/G	T/G	T
Sterna 1 (S2) / JMP	T	C/T	A	T	T
Sterna 1 / MC	T	C/T	A	T	T
Sterna 4 (C44) / JMP	G	C	G	T	C
S. elegans (S13)	T/G	C	A/G	T/G	T
S. elegans (S16)	T	C	A	T	T
S. elegans (S19)	T/G	C/T	A/G	T/G	T
S. sandvicensis (C41)	G	C	G	G	T
S. sandvicensis (C42)	G	C	G	G	T
S. sandvicensis (C43)	G	C	G	G	T
S. a. acuflavida (USA09/FJ356216-26)	T	C	G	T	T
S. a. acuflavida (USA08/FJ356215-25)	T	C	G	T	T
S. a. eurygnatha (ARG02/FJ356210-20)	T	C	G	T	T
S. a. eurygnatha (ES03/FJ356213-23)	T	C	A	T	T
S. bengalensis emigrata (S6)	G	C	G	T	T/C
S. bengalensis emigrata (S7)	G	C	G	T	T/C
S. bengalensis emigrata (S8)	G	C	G	T	T
S. bengalensis emigrata (S10)	G	C	G	T	T/C
S. bengalensis emigrata (S12)	G	C	G	T	T/C

Appendix 1: Sequences of the nuclear intron Glycéraldéhyde-3-phosphodehydrogenase (G3PDH).

>Seq1 [organism=Sterna sandvicensis] S50,France,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GAAGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAAAATTCCATACACCCTT
CAAATACGGTAAGGAAAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT
GTTGGAGCCACCCTACACAGCAGGGGTCTACGTTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCACCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC

>Seq2 [organism=Sterna sandvicensis] S54,France,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GAAGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAAAATTCCATACACCCTT
CAAATACGGTAAGGAAAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT
GTTGGAGCCACCCTACACAGCAGGGGTCTACGTTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCACCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC

>Seq3 [organism=Sterna sandvicensis] S60,France,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GAAGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAAAATTCCATACACCCTT
CAAATACGGTAAGGAAAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT
GTTGGAGCCACCCTACACAGCAGGGGTCTACGTTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCACCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC

>Seq4 [organism=Sterna sandvicensis] S61,France,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GACGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAAAATTCCATACACCCTT
CAAATACGGTAAGGAAAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT
GTTGGAGCCACCCTACACAGCAGGGGTCTACGTTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCACCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC

>Seq5 [organism=Sterna elegans] S64,USA,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GGCGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCCATACACCCTT
CAAATACGGTAAGGAGAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT
GTTGGAGCCACCCTACACAGCAGGGGTCTACGTTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCACCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC

>Seq6 [organism=Sterna elegans] S67,UWBM69602,USA,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GACGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCCATACACCCTT
CAAATACGGTAAGGAGAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT
GTTGGAGCCACCCTACACAGCAGGGGTCTACGTTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCACCTCCTCATCCCCCCCCAGTGTCTCCCCCACCACCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC

>Seq7 [organism=Sterna elegans] S68,UWBM70563,USA,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GACGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCCATACACCCTT
CAAATACGGTAAGGAGAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT
GTTGGAGCCACCCTACACAGCAGGGGTCTACGTTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCGCCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC

>Seq8 [organism=Sterna elegans] S69,UWBM69603,USA,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GACGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCCATACACCCTT
CAAATATGGTAAGGAGAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT

GTTGGAGCCACCCTACACAGCAGGGGTCTACATTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCACCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC

>Seq9 [organism=Sterna elegans] S70,UWBM70562,USA,Glycéraldéhyde-3-phosphodehydrogenase(G3PDH)
GACGAACAGAAGTGCTGTCAGGACTGACCCATTTCTTGCATCCCCTTCGTCCTAATTTTCCTGCTCTTCTGCCCC
ATCTCACACAACTGAACCACTCAGCTTCCCATCCACTTCTAGTAAAGTAAGTAGGAAGAATTCCATACACCCTT
CAAATACGGTAAGGAGAAGGCTACAGTCATTTCAGATAAGCAGCAACTTCACTCCACAGAAACTTCATAATAT
GTTGGAGCCACCCTACACAGCAGGGGTCTACGTTATGACCCCACACTGCCAACCTGGCAGTGATGAACAGGAC
AGAAGCCTGCAACTTGCCTGTGTCAGCTCCTCATCCCCCCCCAGTGTCTCCCCCACCACCCCTTAAGGCTGCACC
TACCAGGAAACCAGCTTGACAAAATGATC