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Morphology vs Genetics: the hybrid origin of a sea turtle disproved by DNA

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Abstract

A putative hybrid sea turtle juvenile was evaluated with discriminant DNA markers. When compared with standard values for sea turtles, the general morphological features assigned the specimen to *Caretta caretta*, while the shape and coloration of the head and the beak profile fell within the *Eretmochelys imbricata* range; the front flippers were instead like those of a *Chelonia mydas*. Moreover, prefrontal scale number was outside the putative parental species' ranges. The mitochondrial D-loop sequence was from *C. caretta*, and matched haplotype CC-A2.1, the most common in the Mediterranean. Sequence profiles at three nuclear loci with species-specific substitutions (Cmos, BDNF and R35) revealed only *C. caretta* variants, thus excluding that the individual was an F1 hybrid. This study highlights the importance of integrating different methodological approaches to understand reproductive animal biology and to set the boundaries for specific morphological traits. In particular, we propose the genetic analysis of a new combination of mitochondrial and nuclear markers as a standard procedure which can be adopted in the identification of sea turtle hybrids.

Keywords: Genetics, hybrid, morphology, mtDNA, nuclear DNA, sea turtle.

Inter-specific hybridization in sea turtles has been reported for the Pacific and the Atlantic, but it was never documented for the Mediterranean. In this latter basin three species are usually present: the most common is the loggerhead turtle (*Caretta caretta*), followed by the green turtle (*Chelonia mydas*), found mainly in the eastern Mediterranean, while the least frequent is the leatherback turtle (*Dermochelys coriacea*). Two other species have been occasionally observed in central-western Mediterranean waters: the hawksbill (*Eretmochelys imbricata*) and the Kemp's ridley (*Lepidochelys kempii*) turtles (Casale & Margaritoulis, 2010). Hybrids *C. caretta* x *C. mydas, C. mydas* x *E. imbricata, C. caretta* x *E. imbricata, L. kempii* x *C. caretta*, *L. olivacea* x *E. imbricata* and *L. olivacea* x *C. caretta* are all reported in the literature. The study of these hybrid individuals were based either on morphological analysis alone (Kamezaki *et al*., 1983, 1996; Frazier, 1988; James *et al*., 2004), or the use of allozymes (Wood *et al*., 1983; Coincecão *et al*., 1990), or the genetic analysis of the mitochondrial (mt) and single-copy nuclear (scn) DNA with Restriction Fragment Length Polymorphisms (RFLPs) (Karl *et al*., 1995; Seminoff *et al*., 2003; Witzell & Schmid, 2003), or the combined use of the morphological and mtDNA analyses (Lara-Ruiz *et al*., 2006, Reis *et al*., 2010).

In August 2003 a juvenile sea turtle (Curved Carapace Length = 40 cm) was found stranded in Torre Faro (Messina, Sicily) and rescued by the "Centro Regionale Recupero Fauna Selvatica e Tartarughe Marine" (Comiso, Ragusa). The specimen, nicknamed Matilde, was in good general health conditions and, at a glance, it seemed a loggerhead turtle (Fig. 1). Going into more depth, its morphology and lepidosis departed from those typical for *C. caretta*. In order to assess whether the turtle was the result of hybridization, a double step procedure was carried out, which included morphometric evaluation and genetic typing.

Morphological traits of the animal were recorded and compared with standard values for sea turtles (Table 1) (Márquez, 1990; Wyneken, 2001; Kamezaki, 2003). Most of Matilde's features were in line with those commonly observed in *C. caretta*. Variations were neverthe-

Fig. 1: Picture of Matilde in the rescue center "Centro Regionale Recupero Fauna Selvatica e Tartarughe Marine" (Comiso, Ragusa).

Fig. 2: Detail of the shape and coloration of the head.

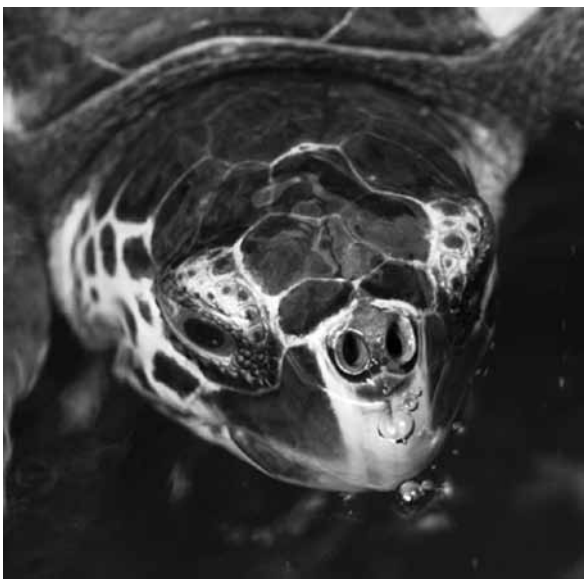

Fig. 3: Detail of the number of prefrontal scales.

less found in the shape and coloration of the head and in the beak profile, resembling those of *E. imbricata* (Fig. 2). Moreover, the animal showed only one claw on the front flippers, like *C. mydas* and unlike both *C. caretta* and *E. imbricata*. Finally, the number of prefrontal scales did not fit the value for any of the putative parental species (Fig. 3). In summary, the morphological analysis alone could not determine whether the individual was a hybrid and, if so, which parental species (*E. imbricata* or *C. mydas*) contributed in addition to *C. caretta*.

In order to determine both maternal and paternal contribution to Matilde's genome, genetic analysis consisting in the characterization of maternally inherited mtDNA and biparentally inherited nuclear loci was carried out. DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN). We amplified and sequenced a fragment of 815 bp in the mitochon-

Table 1. Morphological features of the putative hybrid compared to those usually reported for *E. imbricata, C. mydas* and *C. caretta.*

Morphological features	*E. imbricata*	*C. mydas*	*C. caretta*	Matilde
Prefrontal scales	4	2	5	6
Marginal scutes	12	11	12 - 13	12
Supracaudal scutes	2	2	2	2
Intergular scute	yes	yes	yes	yes
Postanal scute	no	no	no	no
Nuchal scute	yes	yes	yes	yes
Lateral scutes	4	4	5	5
Vertebral scutes	5	5	5	5
Inframarginal scutes	4	4	3	3
Beak	upper part strongly curved	rounded, large	rounded	upper part strongly curved
Anterior claws	2	1	2	1

Table 2. Summary of the nuclear loci analysed, gene lengths, number of sea turtle species (out of the three putative parents hereby considered) distinguishable with each marker and diagnostic variations for each gene. Abbreviations: *C.c. = Caretta caretta*; *E. i. = Eretmochelys imbricata; C. m .= Chelonia mydas.*

Locus	Gene length (bp)	Distinguishable species	N° of diagnostic variations	Transitions	Transversions	Three-state sites	Gaps
R35	942	*C.c.* vs *E. i.* vs *C. m.*	42	7	5	1	29
Cmos	602	*C.c.* vs *E. i.* vs *C. m.*	7	5	2	/	/
BDNF	718	*C.c.* and *E. i.* vs *C. m.*	4	3	1	/	/

drial Control Region with primers LCM15382 and H950 (Abreu-Grobois *et al*., 2006), and three nuclear loci: Cmos, BDNF and R35 (Naro-Maciel *et al*., 2008). These loci can be amplified with universal primers for marine turtles, and contain variable positions specific for *C. caretta*, *C. mydas* and *E. imbricata* (Table 2). Sequencing was carried out with standard protocols on to ABI3130 Avant automated sequencer (Applied Biosystems) on both strands, with the same primers used for the amplification. Electropherograms were visually inspected and sequences were aligned using the BioEdit software (Hall, 1999). Mitochondrial haplotype nomenclature followed the one reported by the Archie Carr Center for Sea Turtle Research (http://accstr.ufl.edu/). Nuclear sequences obtained for the putative hybrid were aligned at each locus with those registered for sea turtle species at http://www.ncbi.nlm.nih.gov/.

The mitochondrial sequence was unequivocally from *C. caretta*. In particular, it matched the most common Mediterranean haplotype CC-A2.1, shared by individuals from nesting colonies of this basin, and also present at lower frequencies in some Atlantic rookeries (Garofalo *et al*., 2009, Monzón-Argüello *et al*., 2010). This haplotype is not therefore indicative of a specific colony of origin.

Sequence profiles at the three nuclear loci revealed contributions from a single species, with no overlapping peaks in the discriminating nucleotide positions (i.e. those which distinguish *C. caretta* from the other sea turtle species). Multispecies alignment at all loci showed that Matilde's sequences were identical to those of Atlantic and Pacific loggerhead turtles.

In conclusion, early morphological measurements taken on this turtle highlighted values compatible with contributions from three different sea turtle species. Then, a genetic analysis was carried out based on a new combination of mitochondrial and nuclear sequences never applied before for the detection of sea turtle hybrids. In fact, the three nuclear genes used in this study have been applied previously only to reconstruct turtle phylogeny (Fujita *et al*., 2004; Le *et al*., 2006; Naro-Maciel *et al*., 2008).

Mitochondrial DNA allowed the assignment to a *C. caretta* matriline. At three nuclear loci we did not find alleles from species other than *C. caretta* and thus we excluded that Matilde was a first generation (F1) hybrid. Finally, as independent segregation of parental *C. caretta* alleles at 3 out of 3 marker loci could not be excluded, we reduced the likelihood that Matilde was a backcross between a F1 hybrid and *C. caretta* to 0.125 (0.5 x 0.5 x 0.5). In the absence of evidence of a contribution from other species, Matilde's features are better described as morphological traits seldom observed in this species.

These results prompt for a cautionary use of morphological parameters for species identification due to the presence of large intra-specific variation, as pointed out by other authors as well (e.g. Kamezaki, 2003). In particular, two pairs of prefrontal scales, occasionally subdivided, were reported for the loggerhead turtle by McCann (1966). Moreover, the number of claws on the fore flipper is already considered an inconsistent diagnostic feature (for example to distinguish a Pacific subspecies *C. caretta gigas*). On the other hand, it is here confirmed the usefulness of the scalation pattern as a first morphological screening in the species assignment, which has to be followed anyway by more detailed genetic analyses.

Although in the past hybridization among sea turtles has been reported sometimes for the Atlantic Ocean and seldom for the Pacific one, this phenomenon remains still unobserved in the Mediterranean Sea. Probably, this is due to the rare occurrence of species other than *C. caretta* in the basin. Nevertheless, the possibility to encounter in Mediterranean foraging areas sea turtle hybrids originated there, as well as hybrid individuals of Atlantic provenance, cannot be excluded.

This study highlights the importance of integrating different methodological approaches to understand reproductive animal biology. In particular, genetics is a valid tool in the identification of hybrids, and is capable to prove/disprove hypotheses based on morphology.

As compared to the less expensive RFLPs, sequences from mitochondrial and nuclear markers do not require

any reference samples of the putative parental species for comparison, but can be simply matched to the already web-registered sequences. Furthermore, besides the identification of the maternal species, mtDNA haplotypes can also help in the assignment of unknown individuals to specific source populations. Finally, the three nuclear markers chosen contain on the whole 53 informative positions (Table 2), which allow distinction among the three species considered. In summary, we propose the new methodological approach hereby tested as a standard procedure to be adopted in the identification of sea turtle hybrids, which can also be extended to other crosses between species not analysed in this work.

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