

Resolving taxonomic uncertainty in vulnerable elasmobranchs: are the Madeira skate (*Raja maderensis*) and the thornback ray (*Raja clavata*) distinct species?

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Received: 1 July 2015 / Accepted: 25 December 2015 / Published online: 11 February 2016
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Abstract Skates and rays constitute the most speciose group of chondrichthyan fishes, yet are characterised by remarkable levels of morphological and ecological conservatism. They can be challenging to identify, which makes monitoring species compositions for fisheries management purposes problematic. Owing to their slow growth and low fecundity, skates are vulnerable to exploitation and species exhibiting endemism or limited ranges are considered to be the most at risk. The Madeira skate *Raja maderensis* is endemic and classified as ‘Data Deficient’ by the IUCN, yet its taxonomic distinctiveness from the morphologically similar and more wide-ranging thornback ray *Raja clavata* is unresolved. This study evaluated the sequence divergence of both the variable control region and cytochrome oxidase I ‘DNA barcode’ gene of the mitochondrial genome to elucidate the genetic differentiation of specimens identified as *R. maderensis* and *R.*

clavata collected across much of their geographic ranges. Genetic evidence was insufficient to support the different species designations. However regardless of putative species identification, individuals occupying waters around the Azores and North African Seamounts represent an evolutionarily significant unit worthy of special consideration for conservation management.

Keywords Phylogeography · Population genetics · Phylogenetics · D-Loop · Fisheries management · Marine conservation

Introduction

Speciation, or the origin of species, was famously referred to as that “mystery of mysteries” (Darwin 1859; Cannon 1961) and understanding its process remains one of the greatest challenges in evolutionary biology. The biological species concept (BSC) regards species as “groups of

Electronic supplementary material The online version of this article (doi:10.1007/s10592-015-0806-1) contains supplementary material, which is available to authorized users.

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interbreeding natural populations which are reproductively isolated from other such groups” (Mayr 1963, 1995). Geographic isolation is considered to play a significant role in the process of speciation, which in this case can be considered a dynamic continuum throughout which a number of stages of divergence may be observed (Mayr 1954). Where populations are only recently diverged they can appear morphologically indistinguishable. This is more likely to occur in taxa that demonstrate a high degree of morphological conservatism and do not rely on visual cues for reproductive interactions (Bickford et al. 2007).

Skates (Order Rajiformes, Class: Chondrichthyes) are characterised by a highly conserved morphology. The identification of species boundaries, fundamental for effective conservation management, is therefore challenging. This issue is reflected in market mis-labelling of commercial landings (Iglésias et al. 2010; Griffiths et al. 2013). Complementary molecular approaches can assist with defining these boundaries and verifying species identifications. In particular, the mitochondrial ‘barcoding’ gene, cytochrome c oxidase subunit I (COI), can reveal differentiation at fine taxonomic levels, yet usefully diverges little within species (Hebert et al. 2003). COI barcoding has recently proven a successful tool for validating skate identification in a number of fisheries (Spies et al. 2006; Serra-Pereira et al. 2011; Coulson et al. 2011; Mabrugaña et al. 2011; Lynghammar et al. 2014).

Like many elasmobranchs worldwide, skate populations in Europe have experienced significant declines, and in some cases even local extirpation, owing to historic and current high levels of fishing pressure (Brander 1981; Dulvy et al. 2000). The reproductive strategy of skates is characterised by late maturity and low fecundity, which coupled with susceptibility to capture, makes them arguably some of the most vulnerable of all exploited marine fishes (Dulvy et al. 2014). Their commercial importance is often low compared to other demersal fisheries, resulting in historically poor management of this group (Dulvy et al. 2000). Despite this, catches remain significant, with the total weight of all landings of the most common European

skate species amounting to 16,600 tonnes (FAO 2009). Reporting species-specific landings became European law in 2009 (EC No 43/2009) however, such protective legislation requires an unambiguous definition of a species, which formally encompasses all natural variation.

Raja maderensis (Lowe, 1838) is a poorly known endemic species, reportedly restricted to waters close to the island of Madeira, however a similar form has also been recorded from the Azores (Stehmann 2009). It is classified as ‘Data Deficient’ by the IUCN red list of threatened species (Stehmann 2009), which considers factors such as geographic extent of range and abundance to determine extinction risk. Its vulnerability is therefore currently unknown and more data concerning its biology and ecology is required to establish responsible management. In particular, the taxonomic status of skates identified as *R. maderensis* in the Azores needs to be resolved, especially as recent molecular evidence suggested little difference between *R. clavata* and a specimen identified as *R. maderensis* landed in a Portuguese fish market (Serra-Pereira et al. 2011). The distinct dorsal pattern (Fig. S1) of *R. maderensis* reportedly distinguishes it from the morphologically highly similar thornback ray (*R. clavata*). However, the thornback ray is noticeably variable with regard to its general morphology when compared to many other European rajiids, with polychromatism recorded in the Mediterranean (Mnasri et al. 2009) and a number of recognised patterns relatively widely known in the UK (Pritchard 1977), as well as documented cases of albinism (Ball et al. 2013). It also occupies a much greater geographical range, particularly in terms of latitude, than many other skates. The thornback ray is distributed from Norway and Iceland to Northwest Africa, including the Mediterranean and Black Seas, and until recently was considered to inhabit waters as far south as southern Africa (Froese and Pauly 2011). The latter records most likely represented mis-identifications of the recently diverged and morphologically similar biscuit skate, *Raja straeleni*, distributed in southern Africa (Pasolini et al. 2011). Similarly, the rough ray (*Raja radula*), restricted to the Mediterranean (Morey et al. 2009) is regarded as a sister species to *R. clavata*. This species is considered to have split from the main lineage (Valsecchi et al. 2005), following re-colonisation of the western Mediterranean basin by *R. clavata* after the Late Miocene Messinian salinity crisis, around 5 Myr ago (Rogl 1998; Duggen et al. 2003). The question of whether *R. maderensis* represents a distinct species, or merely another morphological variant of the polytypic thornback ray, therefore remains unanswered. Clearly, there is a need to sample *R. clavata* and *R. maderensis* across their geographical range and assess their genetic relatedness in comparison to other closely related species, such as *R. straeleni* and *R. radula*.

DNA barcoding using COI has proven successful in distinguishing commonly captured species of European skate,

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but showed little difference between *R. clavata* and a single specimen of *R. maderensis* (Serra-Pereira et al. 2011). This suggests the need for further investigation using additional samples and a more variable genetic marker that affords better resolution. The mitochondrial control region (CR) has been shown to have a higher degree of nucleotide polymorphism in skate species (Valesecchi et al. 2005) than COI. In the present study both markers were used to determine the genetic distinctiveness of specimens identified as *R. maderensis* and *R. clavata* over a considerable portion of their geographic range. Their taxonomic and evolutionary status (based on the COI gene) was also examined in the context of 67 other Atlantic species of the family Rajidae.

Materials and methods

Sample collection

The majority of tissue samples were collected between June 2006 and December 2013 from a range of scientific research cruises throughout Europe and the Mediterranean (Fig. 1 and summarised in Table S1, S2). A minority of tissue samples, most notably all those from Portugal and South Africa, were collected at landing or at fish markets.

Photographs of specimens were not collected with tissue samples. However, sampling was undertaken by experienced researchers following detailed biological descriptions.

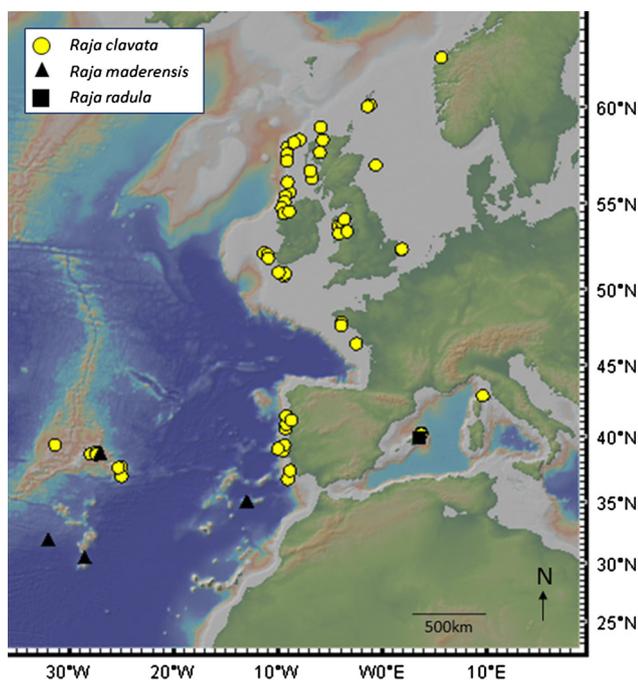


Fig. 1 Map of sampling locations for *R. clavata*, *R. maderensis* and *R. radula* (samples of *R. straeleni* collected from a fish market in Cape Town, South Africa are not shown)

Samples of *R. maderensis* collected by the Universidade dos Açores and by Instituto Português do Mar e da Atmosfera were identified following Stehmann and Burkel (1984). Photographs of reference specimens are included in Fig. S1. In particular, the lack of dark crossbars on the tail and the smoothness of the skin were considered distinguishing features of *R. maderensis*, relative to the common *R. clavata* caught off the Azores (G. Menezes, pers. comm).

DNA sequencing

Genomic DNA was isolated using the ‘Wizard’ protocol (Promega Madison, WI). Approximately 1000 bp of the mitochondrial control region was amplified using primers RayDloopFor (5′-CATTAAATCGACTRTCAACTATTTCA TT-3′) and Ray12s (5′-TACTGAGGCTAGGACCAAAC-3′), following Griffiths et al. (2010). A subset of individuals, with particular focus on *R. maderensis* and *R. clavata* from around the Azores and Northern Africa, were also amplified for COI (the ‘DNA barcode’) using the primers FishF2 (5′-TCGACTAATCATAAAGATATCGGCAC-3′) and FishR2 (5′-ACTTCAGGGTGACCGAAGAATCA-GAA-3′) (Ward et al. 2005). Reaction conditions followed Serra-Pereira et al. (2011). PCR products were sequenced by Macrogen Inc (Korea) and Source Bioscience (UK), and results checked by eye in BIOEDIT version 7.1.11 (Hall 1999) for errors and ambiguous base calls.

Control region analysis: divergence between species

Sequences of the CR from *R. maderensis* (n = 37), *R. clavata* (n = 196), *R. straeleni* (n = 6) and *R. radula* (n = 3) were aligned in MEGA version 5 (Tamura et al. 2011). Nucleotide diversity indices: number of segregating sites (S), number of haplotypes (Nh), haplotype diversity (Hd) and nucleotide diversity (p) were calculated using DnaSP version 5.10 (Rozas et al. 2003). The evolutionary divergence between pairs of species was estimated in Mega 5 using the K80 + I substitution model (Kimura 1980), determined as appropriate by JModelTest ($\Delta AICc = 3.86$, Darriba et al. 2012). Standard error estimates were obtained by bootstrapping (1000 replicates). Haplotype genealogy was reconstructed from trees using a maximum likelihood (ML) approach with the above substitution model implemented in PHYML 3.0 (Guindon and Gascuel 2003) and visualised using Haploviewer (Salzburger et al. 2011). To assist with interpretation, samples were labelled on the haplotype network according to species. In the case of *R. clavata*, they were additionally grouped by broad geographic sampling region based on previous findings (Chevolot et al. 2006): Northern north east Atlantic; Portuguese coast; Mediterranean and Azores.

Control region analysis: differentiation among sampling regions

To compare genetic differentiation between geographically distinct sample collections of *R. clavata* (grouped as above) with that observed between species (*R. maderensis* and *R. clavata*), the pairwise fixation index Φ_{ST} (which takes into account nucleotide diversity) was computed using the Kimura 2-P distance model in Arlequin 3.5 (Excoffier et al. 2005). In addition, a hierarchical analysis of molecular variance (AMOVA) was computed using the same model in Arlequin 3.5 (Excoffier et al. 2005). Using this analysis, total variance is divided into variance components representing: (1) differences among groups (Φ_{CT}), (2) differences among populations within groups (Φ_{SC}) and (3) differences within populations (Φ_{ST}). The best geographic/species subdivisions are assumed to have maximum among group variance (Φ_{CT} values), with a low within-group component (Φ_{SC}), which significantly differs from a random distribution. A number of hypothesised groupings of samples were therefore considered in the AMOVA (summarised in Table 1) with samples identified as *R. maderensis* along with the *a priori* geographic sampling regions of *R. clavata* used as a basis for comparison.

Cytochrome oxidase I analysis

Sequences produced from tissue samples of *R. maderensis* ($n = 37$), *R. straeleni* ($n = 6$) and a subset of 30 *R. clavata* were aligned alongside those from the closely related *R. radula* downloaded from BOLD database. A haplotype genealogy was constructed from trees using a ML approach. A HKY (Hasegawa et al. 1985) nucleotide substitution model determined by JModelTest ($\Delta AICc = 1.42$, Darriba et al. 2012) was implemented in PHYML 3.0, and genealogy was visualised using Haploviewer (Salzburger et al. 2011).

To build a comparative phylogeny, sequences of 65 additional Atlantic skate species were downloaded from the Barcode of Life (BOLD) and Genbank databases. Only sequences originating from the following specialist publications were utilised to minimise inaccuracy with species identification: Spies et al. (2006), Serra-Pereira et al. (2011),

Coulson et al. (2011), Mabrugaña et al. (2011) and Lynghammar et al. (2014). Additional sequences from the starry ray (*R. asterias*, a Mediterranean endemic species) were also downloaded, as this species is considered closely related to *R. clavata* (summarised in Table S3). Evolutionary divergence between pairs of species and genera was estimated using the TrN + I + Γ substitution model (Tamura and Nei 1993). Standard error estimates were obtained by bootstrapping (1000 replicates) in Mega 5. The number of sequences was subsequently collapsed so only unique haplotypes were represented using online fasta toolbox software FaBox (Villesen 2007). Phylogenetic relationships were then inferred using maximum-likelihood (ML) tree methods with the HKY + I + Γ model (Hasegawa et al. 1985) This was determined as the most appropriate by JModeltest ($\Delta AICc = 35.11$) and implemented in PhyML 3.0 with 1000 bootstrap replications to establish the robustness of internal tree branches. The tree was rooted using sequences of ancestral chimaera species: *Chimaera monstrosa*, *Chimaera opalescens* and *Chimaera fulva*.

Results

Control region analysis: divergence between species

A total of 242 samples of *R. clavata*, *R. maderensis*, *R. straeleni* and *R. radula* were sequenced for the CR (Table S1). According to the diversity measures calculated, *R. clavata* and *R. maderensis* showed comparable haplotype diversity (Table 2). Of the four haplotypes identified for *R. maderensis*, three were shared with *R. clavata* and the fourth, which was unique, differed only by a single base. The estimated evolutionary distance between *R. clavata* and *R. maderensis* was very low (0.0076 ± 0.0029). In contrast, all haplotypes for *R. straeleni* and *R. radula* were species-specific. The calculated genetic distance between *R. clavata* and *R. straeleni* was $0.018 (\pm 0.0046)$ and between *R. clavata* and *R. radula*, $0.023 (\pm 0.0052)$, Table 3).

The CR haplotype network revealed five main haplogroups, with the vast majority of samples found in two of these haplogroups (Fig. 2). The first haplogroup (group A)

Table 1 Analysis of molecular variance (AMOVA) for five hypothesised groupings comprising *Raja maderensis* and four *a priori* geographic sampling regions of *Raja clavata*

Hypothesised groupings	Φ_{SC}	Φ_{ST}	Φ_{CT}	% Among groups (%)	$P \Phi_{CT}$
[NNEA] [PC] [MED] [AZ] [MAD]	0.005	0.651	0.649	64.95	0.083
[NNEA, PC] [MED] [AZ] [MAD]	0.174	0.684	0.617	61.70	0.064
[NNEA, MED] [PC] [AZ] [MAD]	0.005	0.707	0.705	70.57	0.039
[NNEA, MED, PC] [AZ] [MAD]	0.155	0.765	0.722	72.27	0.02
[NNEA, MED, PC] [AZ, MAD]	0.147	0.777	0.739	73.90	0.02

MAD individuals identified as *Raja maderensis*. Four geographic sampling regions of *Raja clavata*: *NNEA* Northern north east Atlantic, *PC* Portuguese coast, *MED* Mediterranean Sea, *AZ* Azores Islands

Table 2 Summary of genetic parameters for the mtDNA control region by species

Species	Common name	<i>n</i>	<i>S</i>	<i>Nh</i>	<i>Hd</i>	π
<i>Raja clavata</i>	Thornback ray	196	18	21	0.443 (0.044)	0.00343
<i>Raja maderensis</i>	Madeira skate	37	4	4	0.482 (0.072)	0.00134
<i>Raja straeleni</i>	Biscuit skate	6	2	2	0.333 (0.215)	0.00049
<i>Raja radula</i>	Rough ray	3	2	3	1.00 (0.272)	0.00194

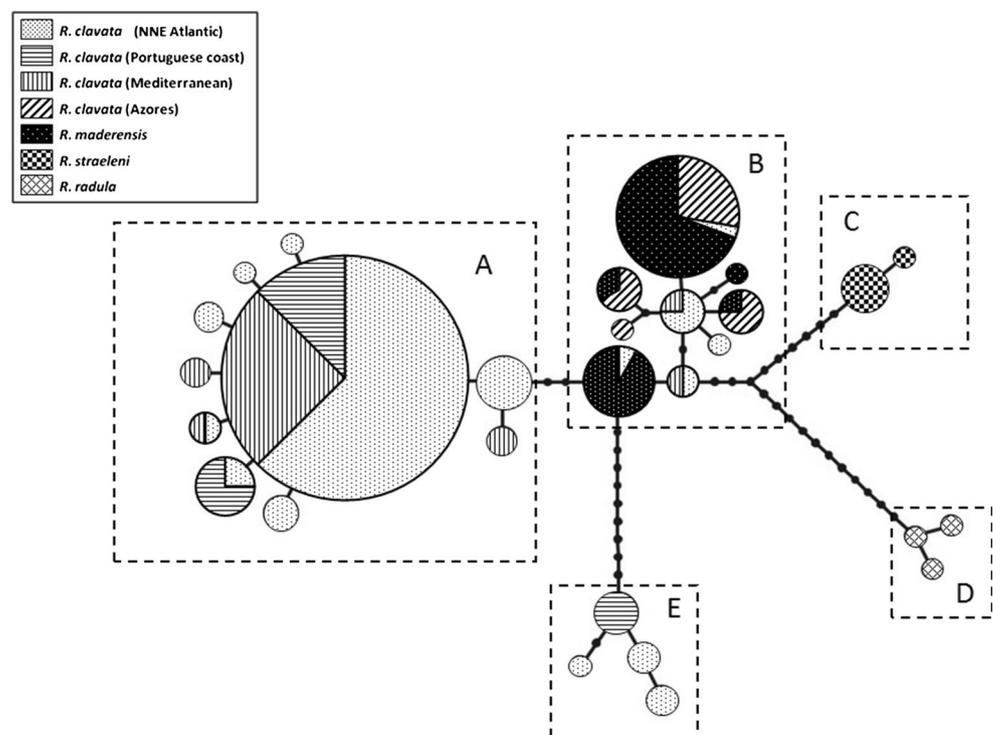
n number of samples, *S* number of polymorphic sites, *Nh* number of haplotypes, *Hd* haplotype diversity (\pm SD), π nucleotide diversity

Table 3 Estimates of evolutionary divergence between species based on mtDNA control region sequences

	<i>R. clavata</i>	<i>R. maderensis</i>	<i>R. straeleni</i>
<i>R. maderensis</i>	0.0076 (\pm 0.0026)		
<i>R. straeleni</i>	0.0182 (\pm 0.0046)	0.0165 (\pm 0.0044)	
<i>R. radula</i>	0.0233 (\pm 0.0052)	0.0218 (\pm 0.0051)	0.0265 (\pm 0.0059)

Standard error is given in parenthesis

Fig. 2 Haplotype genealogy based on mtDNA control region sequences for *R. clavata*, *R. maderensis*, *R. straeleni* and *R. radula*. Samples are patterned according to species and those from *R. clavata* are also labelled according to geographic region. Haplotype size is proportional to the number of individuals represented. Solid black dots represent mutational steps, dashed boxes represent separate haplogroups



contained the most common *R. clavata* haplotype (68 % of individuals), which was found in every region sampled, except the Azores. The second haplogroup (group B) contained the second most common *R. clavata* haplotype (6 % of individuals), found only in the Azores and the west coast of Ireland (one individual). This haplotype also represented the majority of individuals identified as *R. maderensis* (57 %) and all haplotypes of *R. maderensis* were contained within this haplogroup. Haplogroups A and B were only separated by two stepwise mutations. An

additional haplogroup of *R. clavata* was also identified that included nine individuals from three different sampling locations. The individuals in this group, which was separated by nine stepwise mutations from the next nearest *R. clavata* haplotype (group E), originated from the southern North Sea, the west coast of Scotland and the Portuguese coast. The remaining two haplogroups represented specimens of *R. straeleni* (group C) and *R. radula* (group D), separated by nine and 14 mutational steps, respectively, from the nearest *R. clavata* haplotype.

Table 4 Phi (Φ_{ST}) for four sampling regions of *R. clavata* (NNEA Northern North East Atlantic, PC Portuguese coast, MED Mediterranean Sea), contrasted with *R. maderensis* (MAD)

	NNEA	PC	MED	AZ
PC	0.061			
MED	0.025	0.181		
AZ	0.776	0.642	0.956	
MAD	0.736	0.600	0.907	0.218

All comparisons were statistically significant for Φ_{ST} except between the Portuguese coast and the Mediterranean which was only marginally non-significant ($P = 0.056$)

Control region analysis: differentiation among sampling regions

Little genetic divergence was evident between the Northern north east Atlantic and the Portuguese coast (pairwise $\Phi_{ST} = 0.061$, $P = 0.026$), or the Mediterranean ($\Phi_{ST} = 0.025$, $P = 0.055$, Table 4). The Portuguese coast and the Mediterranean were more strongly differentiated ($\Phi_{ST} = 0.18$, $P < 0.001$). However, pairwise comparisons of Azores *R. clavata* samples with other sample sites demonstrated a highly significant degree of separation ($\Phi_{ST} = 0.64$ – 0.95 , $P < 0.0001$). More importantly, samples identified as *R. maderensis* were not so strongly differentiated from proximate Azorean *R. clavata*, ($\Phi_{ST} = 0.21$, $P = 0.02$, Fig. 3) despite being similarly differentiated from Atlantic shelf and Mediterranean groups. Overall the AMOVA results best supported the existence of only two groups, with among group variation constituting 73.9 % of the total ($P = 0.02$). The first group was represented by *R. clavata* distributed across the northern north east Atlantic, the Portuguese coast and the Mediterranean; and the second group was represented by *R. clavata* collected from the Azores and individuals identified as *R. maderensis* (Table 1).

Cytochrome oxidase I analysis

Sequences of the COI gene for *R. clavata* ($n = 30$) and *R. maderensis* ($n = 38$) revealed substantial overlap, with haplotypes shared between species. Only one specimen identified as *R. maderensis* had a unique haplotype, separated by a single mutation from *R. clavata* (Fig. 4). Specimens of *R. straeleni* and *R. radula* were clearly separated by six and 15 stepwise mutations, respectively from the nearest *R. clavata* haplotype.

Considering sequences of 69 species from the Atlantic, the overall mean sequence divergence between species was 0.157 ± 0.035 , but within the genus *Raja* it was only 0.1045 ± 0.0642 . Samples from closely related species *R. clavata*, *R. maderensis* and *R. straeleni* showed high levels

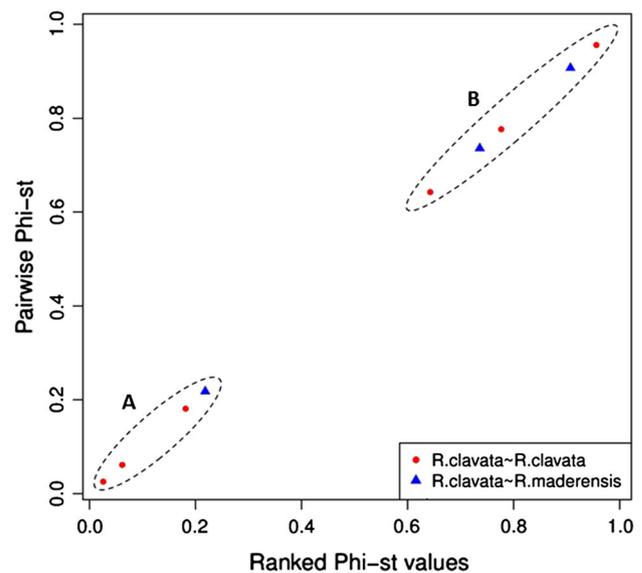


Fig. 3 Pairwise Φ_{ST} (Phi) plotted against ranked Φ_{ST} for population and species comparisons of *R. clavata* and *R. maderensis* established from sequence variation at the mtDNA control region. Cluster A = contrasts: MAD ~ AZ, MED ~ PC, MED ~ NNEA, NNEA ~ PC, Cluster B = contrasts: MAD ~ PC, MAD ~ NNEA, MAD ~ MED, AZ ~ PC, AZ ~ NNEA, AZ ~ MED where MAD = individuals identified as *Raja maderensis* and for geographic sampling regions of *R. clavata*: NNEA Northern north east Atlantic, PC Portuguese coast, MED Mediterranean Sea, AZ Azores Islands

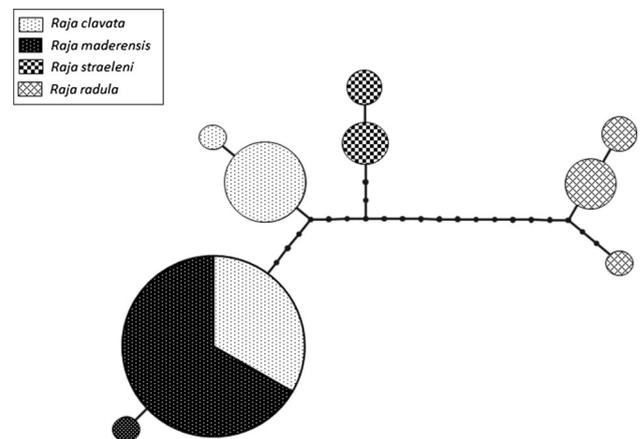


Fig. 4 Haplotype genealogy based on mtDNA COI region sequences for *R. clavata*, *R. maderensis*, *R. straeleni* and *R. radula*. Haplotype size is proportional to the number of individuals represented. Solid black dots represent mutational steps

of similarity (Table 5). In particular, among specimens of

Table 5 Estimates of evolutionary divergence between species based on mtDNA COI sequences calculated by Tamura-Nei nucleotide substitution model

	<i>R. clavata</i>	<i>R. maderensis</i>
<i>R. maderensis</i>	0.0056 (± 0.0023)	
<i>R. straeleni</i>	0.0144 (± 0.0046)	0.0175 (± 0.0056)

Standard error is given in parenthesis

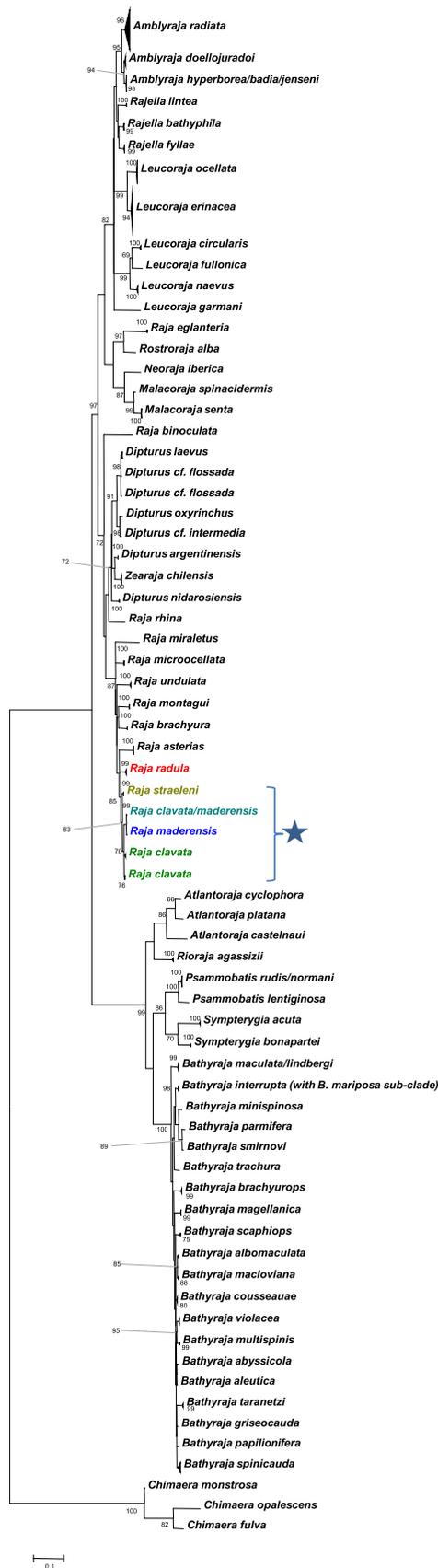


Fig. 5 Maximum likelihood tree using a HKY substitution model based on a common 556 bp COI sequence from 69 skate species of the Atlantic. Only bootstrap values (based on 1000 replicates) >70 % are shown. Branches where species formed monophyletic groups were collapsed and the shape of the triangle represents the number of haplotypes (vertically) and their diversity (horizontally). Individuals highlighted as mis-identifications in the original studies were relabelled accordingly. If identities were not resolved, they were removed from the analysis. *Star* denotes area of the tree detailing *R. clavata* and *R. maderensis*

R. maderensis and *R. clavata*, divergence ranged from 0.00 to 0.016. Average divergence between species within genera ranged considerably, with the highest levels seen in the *Leucoraja* (0.1094 ± 0.0165 , Table S4).

The phylogenetic analysis produced a tree topology supported by high bootstrap values. In particular the main evolutionary lineages (families Rajidae, Blainville 1816 and Arhynchobatidae, Fowler 1934) were clearly identifiable (Ebert and Compagno 2007). Within Rajidae, clades with strong support were the Amblyrajini, Rajini, and a third containing *Raja eglanteria* and *Rostroraja alba* of the Rostrorajini (Chiquillo et al. 2014), as well as genera *Neoraja* and *Malacoraja* of the Gurgesiellini (McEachran and Dunn 1998). Within the Rajini, the genus *Raja* was not monophyletic, although there was strong support for reciprocal monophyly of most species. In particular, *R. radula*, *R. straeleni* and *R. clavata* were clearly separated, while *R. maderensis* and *R. clavata* had overlapping haplotypes (Fig. 5).

Discussion

One of the key issues in evolutionary biology lies in identifying the patterns and causes of reproductive isolation and speciation among closely-related taxa (Mayr 1963; Coyne and Orr 2004). Skates represent a particularly speciose group constituting nearly a quarter of all known chondrichthyan fishes (Ebert and Compagno 2007). Perhaps somewhat paradoxically, they are extremely morphologically conserved, maintaining a dorso-ventrally flattened body form typical of all batoids and brown-grey coloration. Despite evidence of speciation in the form of accumulated genetic differences (Pasolini et al. 2011), species identification remains challenging, potentially due to the selection of a well-adapted phenotype (Futuyma 1979). In this study it was considered whether the endemic *R. maderensis* truly denotes an individual species or whether it represents a morphotype of the polytypic and widely distributed *R. clavata*. Using molecular approaches, there is little evidence to suggest they are distinct species, but *R. maderensis* may constitute part of a diverging population.

Control region analysis

Based on sequence variation of the CR, there were no fixed differences between *R. clavata* and *R. maderensis*. Only a single *R. maderensis* specimen possessed a unique haplotype, and this differed from a *R. clavata* haplotype by a single base. This sharply contrasts with *R. straeleni* and *R. radula*, where species boundaries were clearly defined in the haplotype network and numerous mutational steps separated these groups. In fact, the separation of clades for *R. clavata* and *R. maderensis* appears to more closely follow geographic origin of samples, rather than species identity. This meant individuals identified as *R. maderensis* actually grouped with geographically proximate *R. clavata* captured around the Azores. Conversely, comparisons of population sub-division between *R. clavata* from the Azores or *R. maderensis* to continental shelf or Mediterranean groups, demonstrated concordant high levels of divergence. If *R. maderensis* has been reproductively isolated from *R. clavata* for an extended period, it would generally be expected that levels of divergence between the species would greatly exceed those observed between populations from within the same species (reviewed in Mallet 1995). This is clearly not the case and data suggest that individuals inhabiting areas around islands and seamounts in the eastern Atlantic (Macaronesia) potentially represent a distinct demographic unit, which is isolated from populations inhabiting the continental shelf of Europe. The low nuclear allelic diversity in *R. clavata* from the Azores region and its high genetic differentiation is certainly consistent with a strong bottleneck and physical isolation (Chevolot et al. 2006). Similarly, Naylor et al. (2012) also observed genetic differences between *R. clavata* from the Azores and the continental shelf of mainland Europe. A number of studies considering the population genetic structure of European fish species illustrate the distinctiveness of this region, such as the white sea bream, *Diplodus sargus*, (González-Wangüemert et al. 2010) and the shanny, *Lipophrys pholis* (Stefanni et al. 2006). This is perhaps not surprising when considering the Azores islands represent the most remote archipelago of the North Atlantic, some 1300 km from Portugal suggesting both depth and oceanic distance are significant dispersal barriers for a variety of fish species. This is likely to be even more of an obstacle in demersal species such as skate that lack larval dispersal and have a restricted depth distribution.

Significantly, the haplotype most commonly associated with *R. maderensis* was also isolated in a small number of *R. clavata* from Ireland (with other highly similar haplotypes from within the same clade also identified in *R. clavata* from around the North Sea, Scotland, Ireland

and Portugal). Previously, it has been suggested that the Azores represented a refugium for *R. clavata* during the last glacial maxima (Chevolot et al. 2006) and that waters around the British Isles were directly re-colonised by fish from this region. Our results demonstrate that these areas share haplotypes, yet the exact route of re-colonisation is unclear, given that very similar haplotypes are represented along the African seamounts and Portuguese coast.

A highly divergent group of CR haplotypes was also identified in nine of the specimens of *R. clavata* from across the continental shelf, specifically the southern North Sea; western Scotland; the Irish Sea and the Portuguese coast. Given the large number of stepwise mutations that separate these from other haplotypes, the possibility of mis-identification was considered. This seems unlikely as the closest known species to *R. clavata*, *R. straeleni* and *R. radula* are also represented in the network. Other species that could have been mistaken for *R. clavata* in British waters include *Raja brachyura* and *Raja montagui*. These species, along with representatives of all the commonly captured species of skate around the UK, have been previously sequenced at the same CR region and *R. clavata* remains the closest match in terms of sequence homology (Griffiths et al. 2010). Hybridisation can also be ruled out for the same reason, as the haplotypes do not appear to originate from any other contemporary sympatric species of skate. No photographs accompany these tissue samples, but cryptic speciation is also doubtful as five of the individuals were also genotyped at a suite of microsatellite loci and all had alleles falling within the range identified for *R. clavata* (Griffiths, unpublished data). Interestingly, the majority of the mutations accounting for this group are located within a 50 bp region (~340–390 bp), suggesting that these changes could have arisen through a single gross mutational event. Alternatively, this group of haplotypes could have arisen within an additional, unidentified refugium, where *R. clavata* persisted during the last glacial maxima. There is growing evidence from a number of marine species that many organisms survived in isolated populations much closer to the ice sheets than previously thought (Provan et al. 2005; Hoarau et al. 2007; Olsen et al. 2010; Finnegan et al. 2013). This has been suggested in previous studies of *R. clavata* to explain the diversity of haplotypes present in northern Europe (Chevolot et al. 2006), putatively suggesting Hurd Deep (i.e. the region of the western English Channel) as the refugium. However, the sequences identified in the current study remain highly divergent (separated from the most common *R. clavata* haplotypes by a similar genetic distance as *R. radula* or *R. straeleni*), they are extremely rare and do not appear to be exclusive to any specific region of the continental shelf that was sampled.

Cytochrome oxidase I analysis

The numbers of COI haplotypes identified in *R. maderensis* and *R. clavata* were low (2 and 6 respectively), with a high frequency shared haplotype common to both species in the Azores and surrounding seamounts. This essentially supports the results of the CR sequencing, that individuals identified as *R. maderensis* and *R. clavata* from around Macaronesia represent a single demographic unit, which is distinct from *R. clavata* inhabiting areas of the European continental shelf and Mediterranean.

The substantial phylogeny reconstructed here reveals that *R. clavata* and *R. maderensis* do not form monophyletic groups, yet the closely related *R. straeleni* and *R. radula* both branch with strong bootstrap support (81 and 86 % respectively), with *R. straeleni* demonstrating the closest relationship with the *R. clavata* and *R. maderensis*. This analysis extends the previous work of Chiquillo et al. (2014) by including genera of both hard-nosed (upper clade) and soft-nosed (lower clade) skates and supports the polyphyletic nature of the genus *Raja*. In particular, *Raja eglanteria* certainly requires re-arrangement as it aligns closely with *Rostroraja alba*, forming part of a newly proposed tribe: Rostrorajini (Chiquillo et al. 2014). These taxonomic issues highlight how understudied the phylogenetics of batoids remains and the tree presented here represents one of the most comprehensive phylogenetic reconstructions of skates to date.

The COI gene has proven to be a useful species diagnostic tool owing to its diversity between, but conservation within, species (Hebert et al. 2003). As a result, numerous attempts have been made at establishing boundaries which usefully define species. An extensive analysis of chordates demonstrated that 98 % of species pairs were separated by a COI gene sequence divergence greater than 2 %, although this work also highlighted that the appropriate cut-off should be calibrated with known species of the taxon under study (Hebert et al. 2003). A detailed analysis of fish suggested that 3.5 % divergence was appropriate for the group (Ward et al. 2009), however rates of nucleotide substitution have been shown to be lower in elasmobranchs (Martin et al. 1992). Using a sample set of 69 skate species from the Atlantic, the average genetic distance between species was 15.7 % and within the genus *Raja*, 10.4 % (or 8.6 % if *R. eglanteria* was not included). This sharply contrasts with that calculated between *R. maderensis* and *R. clavata* (0.4 %). Meier et al. (2008) considered that the lowest distance to the nearest neighbour might be a more appropriate cut-off. In this case, comparison with the levels of divergence between *R. clavata* and its close relatives *R. straeleni* and *R. radula*, may be informative. We calculated genetic distances of 1.1 and 2.2 % between these species. Therefore, the distance between *R. maderensis* and *R.*

clavata is considerably below any of the species cut-offs considered, even the most conservative and taxonomically appropriate. Nevertheless, such cut-offs remain contentious as taxonomic groups exist on an evolutionary continuum, and are unlikely to be fixed (Trewick 2008).

Distinctiveness of *Raja maderensis*

The combined analysis of both CR and COI sequences failed to support the genetic distinctiveness of *R. maderensis*. New animal species most commonly evolve through allopatric speciation, which results from geographic isolation, beginning with highly structured populations (Coyne and Orr 2004; Gavrilets 2004). This had been suggested as the predominant mode of evolution in European skates (Pasolini et al. 2011). *Raja clavata* and *R. straeleni* are thought to have separated during the early Pleistocene as a result of glacial activity in equatorial Africa around 1.3 Ma. Using the highly variable CR, we calculated a mean sequence divergence of 1.8 % between these two species, which equals that demonstrated by Pasolini et al. (2011). Similarly, the sequence divergence we calculated between *R. clavata* and the endemic Mediterranean skate *R. radula* (2.3 %) compares well to previous estimates used to date the evolution of *R. radula* to less than 5 million years ago (2.9 %, estimated from data from Valsecchi et al. 2005). This result supports the possibility that the independent demographic unit comprising specimens identified as *R. maderensis* and geographically proximate Azorean *R. clavata* represents an earlier stage of such a process of divergence.

The lack of genetic distinctiveness of *R. maderensis* is difficult to reconcile with the morphological differences ascribed to it (Stehmann and Burkel 1984; Ebert and Stehmann 2013). Perhaps this distinct morphology corresponds to examples of local adaptation or phenotypic plasticity in response to varying environmental conditions, indeed *R. clavata* is known to exhibit considerable variation in coloration and skin texture across its distribution in the UK and Mediterranean Sea (Pritchard 1977; Mnasri et al. 2009). It is acknowledged that difficulties distinguishing between *R. clavata* and *R. maderensis* may have potentially led to misidentification, which would contribute to apparent overlap of shared haplotypes between species. Despite this, wholesale misidentification seems very unlikely given the number of samples analysed and the experience of the researchers. Morphological identification remains challenging, although appropriate taxonomic guides were utilised (Stehmann and Burkel 1984; Ebert and Stehmann 2013), suggesting the conclusions are robust. Further biological and ecological information concerning *R. maderensis* is required to assess the taxonomic validity of this group. However, this work is the result of many

years of focused sample collection, and represents a substantial effort to collect data from remote and inaccessible areas of Europe and Africa. This difficulty is also reflected in the fact that no samples were collected from the coastal waters of Madeira, a key part of the described range of *R. maderensis*. The region remains difficult to trawl due to its rough topography, but samples were collected in near-by waters, approximately 100 nautical miles north east of the archipelago. Indeed, further sampling along the coast of north Africa is not only important for assessing the genetic distinctiveness of *R. maderensis* and establishing the distribution of *R. clavata* and *R. straeleni*, but also the elasmobranch fauna of the whole region generally remains rather poorly described (Bonfil 1994; Compagno 1994; Kroese and Sauer 1998).

A recent review by Dulvy et al. (2014) highlights that most chondrichthyan catches worldwide are unregulated, misidentified, unrecorded, aggregated or discarded at sea, resulting in a lack of species specific landing information. Skates are particularly challenging to identify and a lack of species composition monitoring has masked severe population declines (Brander 1981; Walker and Hislop 1998; Iglésias et al. 2010). They are slow growing, late maturing fishes with a relatively low fecundity; a set of life history traits which renders them highly susceptible to over-exploitation (Dulvy and Reynolds 2002). Endemic species with restricted ranges are of particular concern and there is a growing need to gather more information pertaining to their biology and ecology to assess their risk of extinction, used as a factor in determining IUCN classification. Therefore, determining the taxonomic status of *R. maderensis* has important implications for conservation and management. The application of genetic markers to this question seems especially appropriate, given that this approach has been highly successful in unambiguously distinguishing skates across Europe and elucidating taxonomic issues in the group (Spies et al. 2006; Griffiths et al. 2010; Iglésias et al. 2010; Serra-Pereira et al. 2011; Coulson et al. 2011; Mabrugaña et al. 2011; Lynghammar et al. 2014). In terms of IUCN assessment this study highlights polytypic nature of *R. clavata*, further complicating its assessment for red-listing. While these results do not appear to support the occurrence of *R. maderensis* as a distinct species, our extensive sampling suggests that if it does exist it must have an extremely restricted range and small population size, rendering it extremely vulnerable to extinction.

Regardless of their morphological differences, the individuals collected from across the Azores and seamounts proximal to North Africa appear to belong to a single demographic unit, distinct from other populations on the European continental shelf and Mediterranean. However, our analysis of the CR revealed these groups were not

entirely reciprocally monophyletic, which remains an important criterion in defining Evolutionary Significant Units (reviewed in Moritz 1994). Despite this, previous analysis with nuclear markers has also revealed the genetic distinctiveness of Azorean *R. clavata* (Chevolot et al. 2006). Recognising the evolutionary significance and conservation value of this group, would certainly serve to protect the unique morphology associated with *R. maderensis*, regardless of its underlying origin.

Acknowledgments We are grateful to all those who helped with sample collection. This includes the MEDITIS survey programme (IEO Mallorca) for Mediterranean samples. Portugal mainland samples were collected under the EU Data Collection Framework (DCF, PNAB). Azores specimens from the Department of Oceanography and Fisheries (DOP) of the University of the Azores (UAc) were collected under the project DEMERSAIS “Monitorização das espécies demersais dos Açores” financed by the Azorean government, and the project DEECON “Unravelling population connectivity for sustainable fisheries in the Deep Sea” project approved by the European Science Foundation (ESF) under the EUROCORES programme (proposal No 06-EuroDEEP-FP-008 & SFRH-EuroDEEP/0002/2007). This study was funded by the UK Natural Environment Research Council (NERC) Oceans 2025 Strategic Research Programme Theme 6 (Science for Sustainable Marine Resources). REB was supported by the Fisheries Society of the British Isles (FSBI), BSP was funded by the Fundação para a Ciência e a Tecnologia, SFRH/BPD/72351/2010 and JL was supported by The Alasdair Downes Marine Conservation Fund.

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