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Population connectivity and phylogeography of the Mediterranean endemic skate *Raja polystigma* and evidence of its hybridization with the parapatric sibling *R. montagui*

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ABSTRACT: The genetic structure and population connectivity of the Mediterranean endemic speckled skate *Raja polystigma* were investigated in 10 population samples (N = 232) at 7 exon-primed nuclear microsatellites and at 3 mitochondrial DNA sequence markers. The phylogeographic and population genetic analyses revealed that *R. polystigma* in the western and central Mediterranean represents a near-panmictic population, with a subtle but significant mitochondrial divergence of the Adriatic deme. Nuclear genotypes revealed that 2.5% of the total individuals exhibited an admixed ancestry with the sibling species *R. montagui* (spotted ray). Individuals with admixed ancestry were detected along with purebred individuals in the Algerian, southern Tyrrhenian, Sicilian and Adriatic *R. polystigma* population samples, but they were absent or rare in Sardinian and northern Tyrrhenian ones. Since the 2 species co-occur in the southwestern Mediterranean, we suggested that this area may act as a secondary hybrid zone.

KEY WORDS: Speckled skate · Spotted ray · Species misidentification · Population connectivity · Admixed ancestry · Mediterranean · Mitochondrial DNA · Microsatellites · Secondary hybrid zone

INTRODUCTION

Despite representing only 0.32% of the surface of the oceans, the Mediterranean Sea is estimated to contain on average 6.4% of the world's marine species, of which approximately 20.2% are endemic (Coll et al. 2010). The Mediterranean Sea is also reported to be 1 of 3 regions where chondrichthyans are most threatened (Field et al. 2009). Fifteen coastal species of skate (Rajidae) have been identified in the Mediter-

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ranean Sea, 4 of which are considered endemic: *Raja polystigma*, *R. asterias*, *R. radula* and *Leucoraja melitensis* (Serena 2005, Cavanagh & Gibson 2007, Serena et al. 2010). Incidental by-catch in mixed-species trawl fisheries and habitat degradation are the primary threats to coastal skates in this region (Dulvy et al. 2000, Stevens et al. 2000). Population declines and local extinctions of skates have been reported in some Mediterranean areas over the last 25 yr and have been attributed to the overexploitation of demersal resources (Jukic-Peladic et al. 2001, Dulvy & Reynolds 2002, Ferretti et al. 2013).

Inaccurate morphology-based species identification is a significant issue for the conservation and management of skates. The pronounced inter-specific conservatism of morphological characteristics in closely related species and the overlap in distribution ranges (McEachran & Dunn 1998) hampers correct species identification (Iglésias et al. 2010). The resulting lack of accurate species-specific fisheries landing data can mask declines and/or local extinctions in particular species (Dulvy et al. 2000, Iglésias et al. 2010). Rare and poorly studied species, such as endemic Mediterranean skates, are therefore prone to high rates of extinction and threat (McKinney 1999) because of non-optimal reporting and sampling, which reduce the power of analyses (Roberts & Hawkins 1999, Dulvy et al. 2003).

Speckled skate *R. polystigma* (Regan, 1923) is a small-sized skate (maximum total length [TL_{max}] = 60 cm) predominantly found in coastal, shallow-water areas with soft-bottom habitats (minimum depth limit 13 m; depth range 100 to 400 m; Serena 2014, Froese & Pauly 2016). *R. polystigma* occurs mainly in the western Mediterranean Sea, though it is also reported in the Sicilian Channel and Adriatic Sea (Serena 2005, Serena et al. 2010). Phylogenetic analyses indicate that *R. polystigma* is a sister species of spotted ray *R. montagui* (Fowler, 1910), with the 2 species diverging approximately 3.5 million years (Myr) ago at the end of the Messinian Salinity Crisis (Valsecchi et al. 2005).

R. montagui (TL_{max} = 60 cm) is widespread in inshore waters and shallow shelf areas of the northeast Atlantic and western-central Mediterranean Sea on sandy sediments, at depths between 100 and 500 m (Ellis et al. 2007). Due to the high level of external morphological similarity displayed by juveniles and adults, *R. polystigma* and *R. montagui* should be considered cryptic siblings (sensu Bickford et al. 2007). Specimens of *R. polystigma* have been frequently misidentified as *R. montagui* in past Mediterranean scientific trawl surveys (Serena 2005, 2014, Serena et al. 2010). Mitochondrial DNA (mtDNA)-based identification of putative *R. polystigma* specimens collected in the fish market of Annaba (Algeria), revealed some specimens to be *R. montagui*, indicating that the 2 species are sympatric in the southwestern Mediterranean fishing grounds (Cannas et al. 2008). This study highlights the need for testing for the existence of interspecific hybrids and/or introgressed individuals, using suitable bi-parentally inherited molecular markers.

The occurrence of inter-specific hybridization and gene introgression has been documented in several marine taxa (Gardner 1997), with some relevant examples recently documented in marine fish (Alvarado Bremer et al. 2005b, Hobbs et al. 2009, Arlyza et al. 2013, Kimura-Kawaguchi et al. 2014, Pujolar et al. 2014). This phenomenon is less common in chondrichthyans than in bony fish because of additive prezygotic barriers to hybrid formation, such as mate choice and internal fertilization (Morgan et al. 2012). Within elasmobranchs, detection of hybrids may be difficult due to high levels of morphological stasis in closely related species (Heist 2004). Genetic studies based on nuclear molecular markers (e.g. microsatellites and single nucleotide polymorphisms [SNPs]) in sister species with overlapping distributions may enable the extent of hybridization in this group to be assessed (Portnoy & Heist 2012). To date, interspecific hybridization has been documented in closely related and morphologically similar carcharhinid sharks (Morgan et al. 2012), but it has not been reported in skates.

Previous studies on skates and rays have shown significant genetic subdivision at global and regional scales (Chevolot et al. 2006b, 2007, Plank et al. 2010, Schluessel et al. 2010, Griffiths et al. 2011, Borsa et al. 2012, Frederico et al. 2012, Le Port & Lavery 2012, Li et al. 2013, 2015, Newby et al. 2014, Sellas et al. 2015). Skate-tagging studies, including *R. montagui*, conducted in the English Channel have indicated that adults have small home ranges, with 85% of individuals remaining within a range of 110 km (Walker et al. 1997). Further, their small body size and oviparous reproduction with large benthic eggs also suggest a limited potential for dispersal. Thus, *R. montagui* and *R. polystigma* may be expected to exhibit a high level of structure and reduced gene flow among populations due to their high evolutionary and ecological similarity.

The current study addresses 2 issues of importance to the conservation genetics of the Mediterranean endemic skate *R. polystigma*. Firstly, the population structure and connectivity in *R. polystigma* will be assessed in order to evaluate genetic diversity pat-

terns which affect population dynamics and resilience to environmental changes, including fisheries overexploitation (Field et al. 2009). Secondly, the reproductive and evolutionary relationships between *R. polystigma* and the parapatric sibling *R. montagui* will be investigated by empirically estimating the proportion and spread of individuals with admixed ancestry in native populations of parental species.

MATERIALS AND METHODS

Sampling

Skate tissue samples were collected from 2000 to 2010 and were handled and stored according to the guidelines provided by Serena et al. (2010). Samples were mainly obtained from scientific trawl surveys or were provided by contracted commercial fishermen. The only exceptions were the Algerian individuals, which were collected at the Annaba fish market by Farid Hemida. This market receives mainly commercial landings from Algerian trawlers fishing in national waters (F. Hemida pers. obs.). Tissues were sampled whenever possible directly on board or immediately after landing, and ethanol-preserved.

A total of 232 skates were collected from 9 Mediterranean areas and from the western Irish Sea (Fig. 1, Table 1). Specimens were identified based on morphological characteristics (Stehmann & Bürkel 1984, Serena et al. 2010). Individuals were categorized as (1) 'putative *polystigma*' (n = 187): specimens morphologically identified as *Raja polystigma* occurring in the Mediterranean, including 14 specimens collected in the Algerian fishing grounds and morphologically identified as *R. polystigma*; and (2) 'putative *montagui*' (n = 45): specimens morphologically identified as *R. montagui* including 35 individuals collected in the western Irish Sea and 10 specimens collected in the Algerian fishing grounds (Table 1 and Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m554p099_supp.pdf).

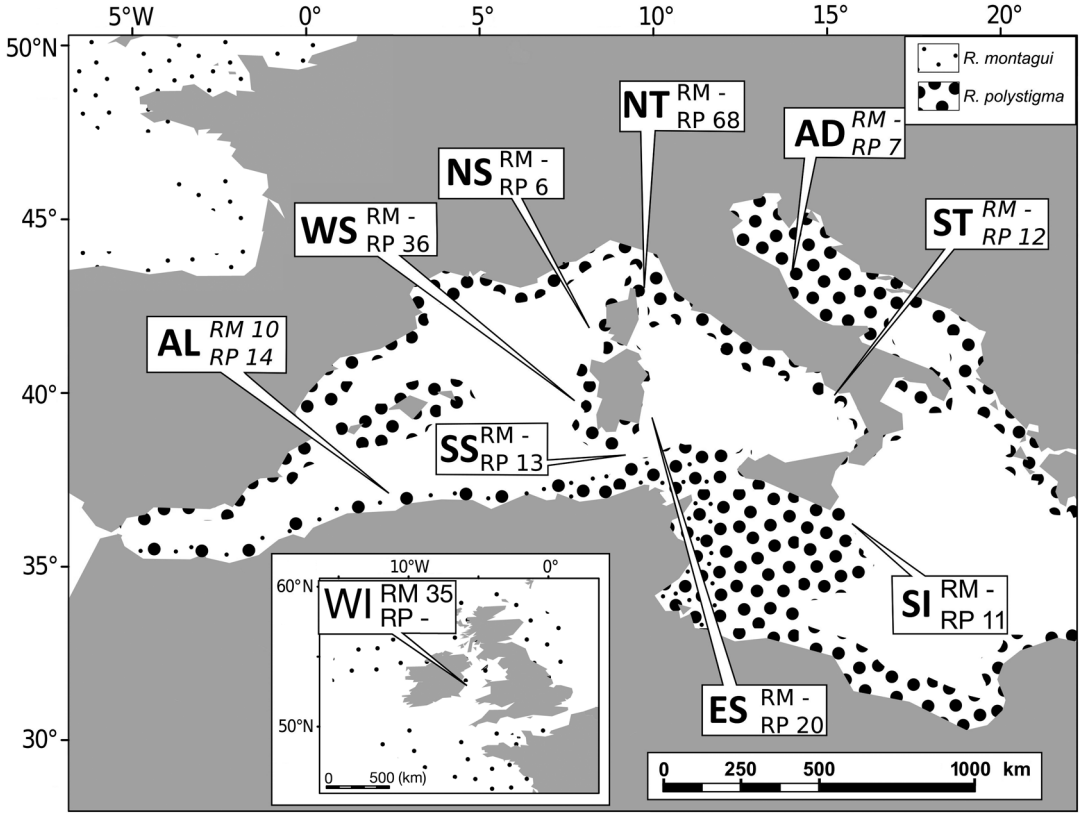

Fig. 1. Sampling areas and species distribution of *Raja polystigma* and *R. montagui*. Species distribution ranges are represented according to literature (Ellis et al. 2007, Serena et al. 2010, Ungaro et al. 2015). For each sampling area, the number of individuals assigned to *R. polystigma* (RP) and *R. montagui* (RM) based on morphology is provided. Sampling locations where molecular data indicated the occurrence of introgressed or admixed individuals are reported in italics. See Table 1 for sampling area codes

Table 1. Sampling of *Raja polystigma* and *R. montagui* in the Mediterranean and North Eastern Atlantic. RP: individuals morphologically identified as *R. polystigma*; RM: individuals morphologically identified as *R. montagui*. Individual data are detailed in Table S1

Sampling area	Code	Sample size	RP	RM	Year	Collection details
Adriatic Sea	AD	7	7	–	2000, 2001, 2004	Scientific trawl surveys
Sicilian Strait	SI	11	11	–	2001, 2008	Contracted fishermen
South Tyrrhenian	ST	12	12	–	2003, 2004, 2007	Scientific trawl surveys
North Tyrrhenian	NT	68	68	–	2002, 2003, 2004	Scientific trawl surveys
Eastern Sardinia	ES	20	20	–	2005	Scientific trawl surveys
Northern Sardinia	NS	6	6	–	2005	Scientific trawl surveys
Western Sardinia	WS	36	36	–	2002, 2005	Scientific trawl surveys
Southern Sardinia	SS	13	13	–	2005	Scientific trawl surveys
Algerian Coasts	AL	14	14	10	2003, 2010	Annaba fish market
Western Irish Sea	WI	35	–	35	2007	Scientific trawl surveys, RV 'Celtic Voyager'
Total		232	187	45		

Molecular methods

Total genomic DNA (gDNA) was extracted using a standard cetyltrimethyl ammonium bromide (CTAB) procedure (Winnepenninckx et al. 1993).

A panel of 7 exon-primed microsatellite loci (El Nagar et al. 2010) was used to genotype 128 individuals, after identifying optimal PCR conditions for *R. polystigma* and *R. montagui* (Table S2 in Supplement 1). The amplicons were sized by a commercial provider (Macrogen), using the GS-500LIZ internal size standard. Alleles were sized using the GeneMarker® software (SoftGenetics). A total of 231 specimens were analysed at 3 polymorphic mtDNA markers, whose nucleotide polymorphism enables discrimination between skate species and for which primers and reference sequences for both target species were available (Tinti et al. 2003, Valsecchi et al. 2005, Cannas et al. 2008, Griffiths et al. 2010, Pasolini et al. 2011, Serra-Pereira et al. 2011, Costa et al. 2012, Knebelsberger et al. 2014, Landi et al. 2014, Lynghammar et al. 2014). An approximately 600 bp fragment of the cytochrome oxidase I (COI) gene was amplified by PCR using the primer set FishF2/FishR2 (Ward et al. 2005). An approximately 240 bp hypervariable fragment of the 16S rDNA (16S) gene was amplified according to a modified protocol from Tinti et al. (2003), detailed in Supplement 2 at www.int-res.com/articles/suppl/m554p099_supp.pdf. A mitochondrial control region hypervariable fragment of approximately 360 bp (CR) was amplified according to Valsecchi et al. (2005). PCR products were sequenced by a commercial sequence service provider (Macrogen Europe).

Species identification

The morphological identification as putative *R. polystigma* and *R. montagui* was provided by specimen collectors using species-specific characters reported in the available identification guidelines and dichotomous keys (Stehmann & Bürkel 1984, Serena et al. 2010) and individual data are reported in Table S1. The morphological assignment of 128 individuals from the Atlantic and Mediterranean sampling locations was verified by correspondence analysis of nuclear genotypes as implemented in GENETIX 4.05 (Belkhir et al. 1996–2004). Individuals were clustered according to their microsatellite multilocus genotypes and by reducing the multidimensional allelic frequency space to a bidimensional space. Subsequently, the agreement between species identification based on morphology and that based on the clustering of nuclear genotypes with mtDNA clades was assessed for each specimen.

Admixture, gene introgression and hybridization

Individual multilocus genotypes of 128 individuals were clustered using the Bayesian algorithm implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000, Hubisz et al. 2009). Three different tests (Test1, Test2 and Test3) were performed with a stepwise approach to assess evidence of hybridization and introgression between *R. polystigma* and *R. montagui*, applying more stringent models and priors in each test in order to verify the robustness of the results produced. In addition (Test4), estimates of admixture proportions

and hybrid ancestry were inferred with NEWHYBRIDS (Anderson & Thompson 2002). A detailed description of approaches and tests is provided in Supplement 2.

Genetic diversity, phylogeographic and population structure analyses

The allele frequencies, number of alleles and allelic richness of population samples at the microsatellite loci were calculated using FSTAT v.2.9.3.2 (Goudet 2001). The observed (H_o) and the expected (H_e) heterozygosity were calculated using GENETIX 4.05. Estimates of null allele frequencies for each locus and population were computed following the expectation maximization (EM) algorithm as implemented in FreeNA (Chapuis & Estoup 2007). Deviation from Hardy-Weinberg equilibrium (HWE) was tested using the online software GENEPOP 4.2 (Rousset 2008).

The population differentiation was assessed by estimating overall and pairwise F_{ST} and by analysis of molecular variance (AMOVA) as implemented in Arlequin v.3.5 (Excoffier & Lischer 2010). The software STRUCTURE was also used to investigate the presence of genetic structuring among population samples of *R. polystigma* using a total of 97 multilocus genotypes, originating from 7 sampling locations. Settings were defined as in the first STRUCTURE test on the complete dataset.

Polymorphism of COI, CR, 16S, and concatenated sequence datasets was estimated by using DNAsp v.5 (Librado & Rozas 2009) and MEGA 6 (Tamura et al. 2013). Haplotype relationships were inferred using a median-joining clustering algorithm (Bandelt et al. 1999) implemented in the software PopART (http://popart.otago.ac.nz), and Bayesian and maximum likelihood approaches using MrBayes v.3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) and PhyML v.3.0 (Guindon et al. 2010), respectively. The population differentiation was assessed by estimating overall and pairwise Φ_{ST} as implemented in Arlequin v.3.5. The interspecific genetic distance was estimated by AMOVA, pooling all the sampling populations bearing *R. polystigma* haplotypes in 1 group and comparing them with a group made up from the *R. montagui* clade. Pairwise Φ_{ST} between population samples were used in principal coordinates analysis (PCA) using the packages ade4 (Dray & Dufour 2007) and ape (Paradis et al. 2004) in R environment 3.0.2 (R Core Team 2013). Past population demography of *R. polystigma* was inferred from each mtDNA marker through the coalescent Bayesian skyline plot approach (Drummond et al. 2005) as implemented in BEAST 1.75 (Drummond & Rambaut 2007). Details of the methods and settings used in the data analysis are provided in the Supporting Information.

RESULTS

The microsatellite dataset included 128 individual multilocus genotypes from 8 population samples (Table S3 in Supplement 1; Table 2). The COI, CR and 16S datasets included 138, 180 and 193 sequences, respectively, obtained from 8 to 10 popu-

Table 2. Summary results of the analyses performed to assess evidence of hybridization and introgression between *Raja polystigma* and *R. montagui*. A: purebred *R. montagui* individuals; B: putative purebred *R. montagui* individuals; C: purebred *R. polystigma* individuals; D: putative purebred *R. polystigma* individuals; E: unclassified and/or admixed individuals. Classifications were based on a threshold value of 0.90 applied to Q (in Test1 to Test3) and q_n (in Test4) coefficients. See further details in supplementary Table S4 for the assignment criteria and detailed result for each individual. The numbers in parentheses refer to the individuals assigned to the categories that are supported by strict credibility intervals. See Table 1 for sampling area codes

Sample	N	STRUCTURE															NEWHYBRIDS				
		Test1					Test2					Test3					Test4				
		A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
AD	6				2(2)	4					4(3)	2			5	1				5	1
SI	7			3	3(3)	1			2	4(4)	1			6	1				7		
ST	11			7		4			5	2(2)	4			9	1	1			8	1	2
NT	35			35(35)					35(35)					35					35		
ES	12			12(12)					12(12)					12					12		
WS	12			12(12)					12(11)					12					12		
AL	20	3	1(1)	11(5)		5			4(4)	10(2)	1(1)	5	5	1	12		2[a]	5		12	1	2[a]
WI	25	25(25)							25(24)				25					25				

[a]Occurrence of admixed individuals

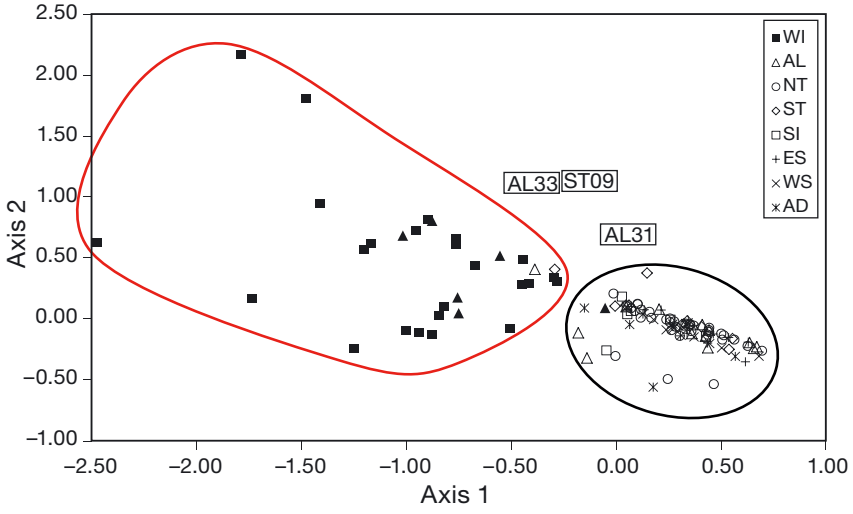

Fig. 2. Correspondence analysis of individuals defined by their genotypes at polymorphic microsatellite loci. Mitochondrial haplotypes are indicated by empty symbols for Clade P and filled ones for Clade M. The black line encircles individuals with concordant morphological and nuclear assignment to *Raja polystigma*, while the red one encircles those assigned to *R. montagui*. Individuals with discordant species assignment and mitochondrial clade are indicated by labels. Details about these individuals are provided in the main text and in supplementary Table S4. See Table 1 for sampling area codes

lation samples depending on the marker (Table 3). The combination of 16S+COI+CR was available for 95 individuals from 8 locations (Table 3). Each individual was characterized at the microsatellite and mitochondrial markers whenever possible.

Species identification and distribution

The clustering of individuals, obtained from the correspondence analysis on nuclear loci genotypes, revealed 2 groups (Fig. 2) and the distinction between species could be mainly ascribed to the values of Axis 1. The mtDNA Clade P was largely characterized by the group corresponding to *Raja polystigma*, while the mtDNA Clade M mainly contained the group assigned to *R. montagui*. Species identification based on nuclear markers was highly concordant with the morphological assignment. Only 2 out of the 128 genotyped individuals were not coherently assigned to the same species (Table S1). Specimen AL31, sampled in the Algerian area and classified morphologically as *R. montagui*, clustered within the genetic group of *R. polystigma* individuals, while individual

Table 3. Mitochondrial gene polymorphism of the *Raja polystigma* and *R. montagui* population samples subdivided according to Clades P and M. COI: cytochrome oxidase I fragment; CR: control region hypervariable fragment; 16S: 16S rDNA hypervariable fragment; N: number of individuals; Nh: number of haplotypes; *h*: haplotype diversity; π: nucleotide diversity; SD: standard deviation; na: not analysed. See Table 1 for sampling area codes

mtDNA clade	Location	COI				CR			
		N	Nh	*h* ± SD	π ± SD	N	Nh	*h* ± SD	π ± SD
Clade P									
	AD	7	1	–	–	5	1	–	–
	SI	10	4	0.778 ± 0.091	0.00195 ± 0.00029	6	3	0.800 ± 0.122	0.00453 ± 0.00102
	ST	10	3	0.644 ± 0.103	0.00263 ± 0.00038	10	3	0.644 ± 0.101	0.00460 ± 0.00067
	NT	22	6	0.758 ± 0.060	0.00196 ± 0.00027	64	14	0.765 ± 0.045	0.00568 ± 0.00080
	ES	18	5	0.791 ± 0.052	0.00262 ± 0.00021	16	8	0.85 ± 0.075	0.00782 ± 0.00172
	NS	na				3	2	0.667 ± 0.314	0.00205 ± 0.00097
	WS	19	7	0.860 ± 0.051	0.00243 ± 0.00038	24	8	0.848 ± 0.045	0.00547 ± 0.00106
	SS	na				8	5	0.857 ± 0.108	0.00747 ± 0.00255
	AL	13	4	0.654 ± 0.106	0.00187 ± 0.00030	11	4	0.709 ± 0.099	0.00492 ± 0.00090
Total/mean		99	11	0.848 ± 0.015	0.00255 ± 0.00013	147	19	0.781 ± 0.028	0.00566 ± 0.00052
Clade M									
	AL	3	1	–	–	3	1	–	–
	WI	30	5	0.193 ± 0.095	0.00034 ± 0.00017	26	3	0.151 ± 0.093	0.00047 ± 0.00030
Total/mean		33	5	0.176 ± 0.078	0.00030 ± 0.00015	29	3	0.135 ± 0.085	0.00041 ± 0.00026
Grand total/mean		132	16			176	22		

ST09, assigned by morphology to *R. polystigma*, presented the inverse pattern, grouping with the *R. montagui* genetic group. Both individuals displayed a mtDNA haplotype corresponding with the morphological assignment. In addition, individual AL33, identified as *R. montagui* by both morphology and nuclear data, possessed a *R. polystigma* mtDNA haplotype. These 3 cases suggested that reciprocal mtDNA introgression between the 2 species has occurred.

As expected, the parapatric distribution of the 2 species was confirmed, with *R. polystigma* widespread in the western-central Mediterranean and *R. montagui* in the adjacent eastern Atlantic. The latter also penetrates into the Mediterranean and co-occurs with *R. polystigma* in the Algerian coastal area (Table 1, Fig. 1).

Admixture, gene introgression and hybridization

The number of purebred, putative purebred, admixed and unclassified individuals inferred by STRUCTURE and NEWHYBRIDS are summarized in Table 2 and displayed as bar plots in Fig. 3 (fully detailed results are provided in Table S4). In Test1 of STRUCTURE, both Evanno's and Pritchard's methods indicated $K = 2$ as the most likely number of clusters, which is coherent with the presence of 2 species in the nDNA dataset.

Classification of individuals as purebred (Q and $q_n \geq 0.90$, where Q is the proportion of membership assigned by STRUCTURE to each individual and q_n is the Bayesian posterior probability assigned by NEWHYBRIDS to each individual), putative purebred (Q and q_n between 0.90 and 0.80), or of admixed ancestry (Q and $q_n < 0.80$) was largely comparable among tests. In particular, Test1 and Test2 indicated WI as the *R. montagui* 'reference sample', while the *R. polystigma* 'reference samples' were assigned to locations NT, ES and WS, with all individuals assigned to the purebred clusters (Table 2, Table S4, Fig. 3). In the remaining samples (AL, ST, SI and AD), the great majority of individuals were recognized as purebred or putative purebred, with a maximum of 14 unclassified individuals (Test1, Table 2, Table S4, Fig. 3). Test3, in which we used STRUCTURE with the USEPOPINFO model, categorized individuals AL33 and AL34 as admixed, and specimen ST09 as unclassified (Table 2, Table S4, Fig. 3). Similar results were obtained with NEWHYBRIDS (Test4), in which AL34 was classified as admixed and 3 individuals (AL30, ST09, ST10) as unclassified. In agreement with the results of the correspondence analysis (Fig. 2), specimen AL31 was recognized as purebred *R. polystigma* despite its '*montagui* mtDNA' and AL33 was recognized as purebred *R. montagui* despite its '*polystigma* mtDNA' (Table S4).

Both STRUCTURE (with more stringent conditions; Test3) and NEWHYBRIDS (Test4) failed in assigning samples to either F1 or F2 hybrid categories (Fig. 3, Table S4).

Table 3 (continued)

16S				Concatenated			
N	Nh	$h \pm$ SD	$\pi \pm$ SD	N	Nh	$h \pm$ SD	$\pi \pm$ SD
4	1	–	–	4	1	–	–
7	2	0.286 ± 0.196	0.00241 ± 0.00166	5	5	1.000 ± 0.126	0.00332 ± 0.00065
10	2	0.200 ± 0.154	0.00084 ± 0.00065	10	4	0.711 ± 0.117	0.00286 ± 0.00044
64	6	0.548 ± 0.030	0.00263 ± 0.00030	18	11	0.856 ± 0.079	0.00289 ± 0.00035
18	2	0.471 ± 0.082	0.00199 ± 0.00035	14	10	0.923 ± 0.060	0.00358 ± 0.00059
6	4	0.800 ± 0.172	0.00534 ± 0.00134	na			
26	5	0.668 ± 0.081	0.00345 ± 0.00060	9	9	1.000 ± 0.052	0.00304 ± 0.00040
13	4	0.679 ± 0.089	0.00346 ± 0.00072	na			
11	1	–	–	8	4	0.750 ± 0.139	0.00207 ± 0.00050
159	10	0.558 ± 0.035	0.00277 ± 0.00024	68	34	0.937 ± 0.016	0.00321 ± 0.00018
3	1	–	–	2	1	–	–
27	1	–	–	21	4	0.271 ± 0.124	0.00024 ± 0.00012
30	1	–	–	23	4	0.249 ± 0.116	0.00022 ± 0.00011
189	11			91	38		

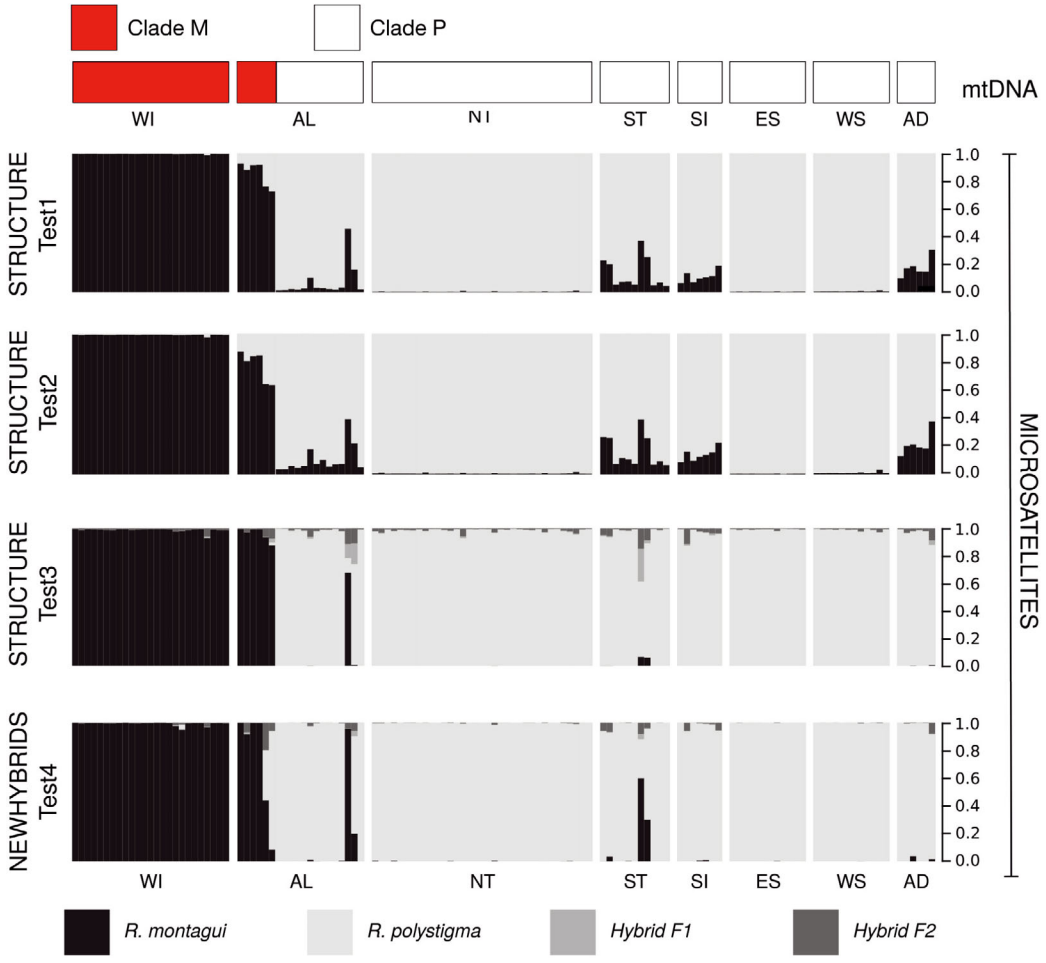

Fig. 3. Assignment test results with evidence of hybridization and introgression between *Raja polystigma* and *R. montagui*. The first row shows the distribution of the 2 mtDNA clades in the population samples: (red bars) Clade M and (empty bars) Clade P haplotypes. The second to fourth rows illustrate the results of the Bayesian clustering using STRUCTURE, while the fifth row is the ancestry inference from NEWHYBRIDS (see also Table 2 for summarized results). Each vertical bar represents 1 individual. The proportion of the bar assigned to each cluster is defined by the proportion of membership to each cluster, ranging from 0 to 1. See supplementary Table S4 for the detailed results for each individual and Table 1 for sampling area codes

The 6 individuals classified as putative admixed, introgressed or unclassified in Test3 and Test4 (AL30, AL31, AL33, AL34, ST09 and ST10) were removed from the datasets used in the subsequent analyses.

Genetic diversity, phylogeographic and population structure analyses

The mean allelic richness was homogeneous across all samples, although slightly higher in *R. montagui* samples. Only 3 sample sites (*R. montagui*: WI; *R. polystigma*: NT and ST; Table S3) showed an overall significant deficiency in heterozygous genotypes. The observed deviations from equilibrium could be only partially explained by the presence of null alleles, as the estimated frequency of null alleles did not follow the same pattern of genotypic imbalance. Due to the high frequency of missing genotypes at the LERI50 locus, all subsequent analyses were performed both including and excluding this locus, in order to assess the influence of missing data on the results produced. Since results obtained with 6 and 7 loci (without and with LERI50, respectively) were largely comparable, only results obtained when excluding the LERI50 locus are presented.

The mean interspecific genetic distance between *R. polystigma* and *R. montagui* was very low but significant (F_{CT} = 0.137, p = 0.035 ± 0.006; Table S5 in Supplement 1). However, the pairwise F_{ST} values between *R. polystigma* and *R. montagui* samples were in general low, and significant only for locations

WI and AL (Table S6 in Supplement 1). When considering the *R. polystigma* samples alone, the overall F_{ST} value was still significant (F_{ST} = 0.053, p = 0; Table S5); however, *R. polystigma* samples showed low and non-significant pairwise genetic differentiation, with the exception of sampling location AL (Table S6). The Bayesian clustering analysis carried out with STRUCTURE failed to identify any genetically distinct cluster within *R. polystigma*. In fact, Pritchard's method indicated K = 1 as the most likely number of clusters, suggesting the presence of a near-panmictic population within this Mediterranean endemic species (data not shown).

A total of 191 individuals, belonging to all Mediterranean samples (Table S1), showed *R. polystigma* mtDNA haplotypes identified by nucleotide variation of COI (n = 103), CR (n = 149) and 16S (n = 161) sequence markers (Table 3). A total of 41 individuals showed *R. montagui* mtDNA haplotypes (COI: n = 35; CR: n = 31; 16S: n = 32; Table 3, Table S1) and they included 35 individuals from WI and 6 individuals from AL (Table S1). A complete agreement between sequence markers in defining the mtDNA haplotypes was obtained when multiple gene fragments were sequenced for the same specimen.

The COI sequences gave a final alignment of 616 bp, containing 24 (3.90%) variable sites, of which 19 (3.08%) were parsimony informative. A total of 17 transitions and 7 transversions were recorded, involving the third codon position in 22 cases and the second codon position in 2 cases. The 16S sequences gave a final alignment of 237 bp, containing 11 (4.64%) variable sites of which 7 (2.95%) were parsimony informative. The CR sequences gave a final alignment of 354 bp, containing 29 (8.25%) variable sites, of which 23 (6.50%) were parsimony informative. A total of 16 COI, 22 CR and 11 16S sequence variants were identified, with well-differentiated species-specific variants for the 3 markers (Table 3, Fig. 4, Tables S7–S10 in Supplement 1).

In *R. polystigma*, the haplotype diversity (h) was generally high with overall values of 0.848 ± 0.015, 0.781 ± 0.028 and 0.558 ± 0.035, at the COI, CR and 16S gene fragments, respectively (Table 3). An exception was the AD population sample, in which unique variants were found with consequent null polymorphism. Similarly, both the *R. montagui* samples (AL and WI) showed low or null haplotype diversity. The nucleotide diversity (π) was extremely low across species, population samples and sequence markers (Table 3). The mean intraspecific sequence divergence was low at all markers (COI: 2.5%; CR: 5.7%; 16S: 2.7%).

The median-joining network built with each of the 3 mtDNA markers resolved the haplotypes of Clades P and M into 2 distinct groups (Fig. 4). In Clade P, the 4 most frequent COI sequence variants (COI/02 to COI/04 and COI/11) were found in 74.76% of the individuals and in all sampled areas except the Adriatic Sea, which in contrast possessed the unique and private COI/09 variant (Fig. 4, Table S7). The most common CR and 16S variants (CR/07 and 16S/07) were found in 40.94 and 61.49% of the individuals belonging to Clade P, respectively, and in all population samples (Fig. 4, Tables S8 & S9). The second most frequent 16S variant (16S/09) was shared only by north Tyrrhenian and Sardinian samples. Rare private sequence variants of each mitochondrial marker were also found in Tyrrhenian and Sardinian samples (Fig. 4, Tables S7–S9). The network of the concatenated sequences (Fig. 4D) was more complex, with a total of 38 haplotypes. Only a few of them were shared among populations (CM/02, CM/03, CM/05, CM/07 and CM/13), while the majority were private haplotypes. The 4 haplotypes of Clade M (CM/06 and CM/36 to CM/38) were clearly distinct from those of Clade P (Fig. 4D, Table S10).

The trees in Fig. S1 in Supplement 1, obtained with Bayesian and maximum likelihood methods, show the relationships among sequence variants, and confirm the sharp separation between the 2 clades.

The mean interspecific genetic distance between *R. polystigma* and *R. montagui* was very high and significant at all sequence markers (COI: Φ_{CT} = 0.900, p = 0.035 ± 0.006; CR: Φ_{CT} = 0.900, p = 0.012 ± 0.003; 16S: Φ_{CT} = 0.860, p = 0.016 ± 0.001; Table S5). Similarly, the pairwise Φ_{ST} values among *R. polystigma* and *R. montagui* population samples were in general high and significant (range 0.820–1; Tables S11 & S12 in Supplement 1). When considering only the *R. polystigma* samples, the overall Φ_{ST} value was mostly significant (COI: Φ_{ST} = 0.20, p = 0; CR: Φ_{ST} = 0.02 NS; 16S: Φ_{ST} = 0.120, p = 0; Table S5). However, *R. polystigma* population samples showed low and non-significant pairwise genetic differentiation, with the exceptions of AD and ST, which displayed significant COI and CR Φ_{ST} values in the comparisons with WS and NT (Tables S11 & S12). The PCAs based on the Euclidean-transformed Φ_{ST} matrices (Fig. S2 in Supplement 1) clearly showed the relationships among species and samples detected at each marker.

Demographic history analysis

The inferred past demography of the Mediterranean *R. polystigma* based on COI, CR, 16S and con-

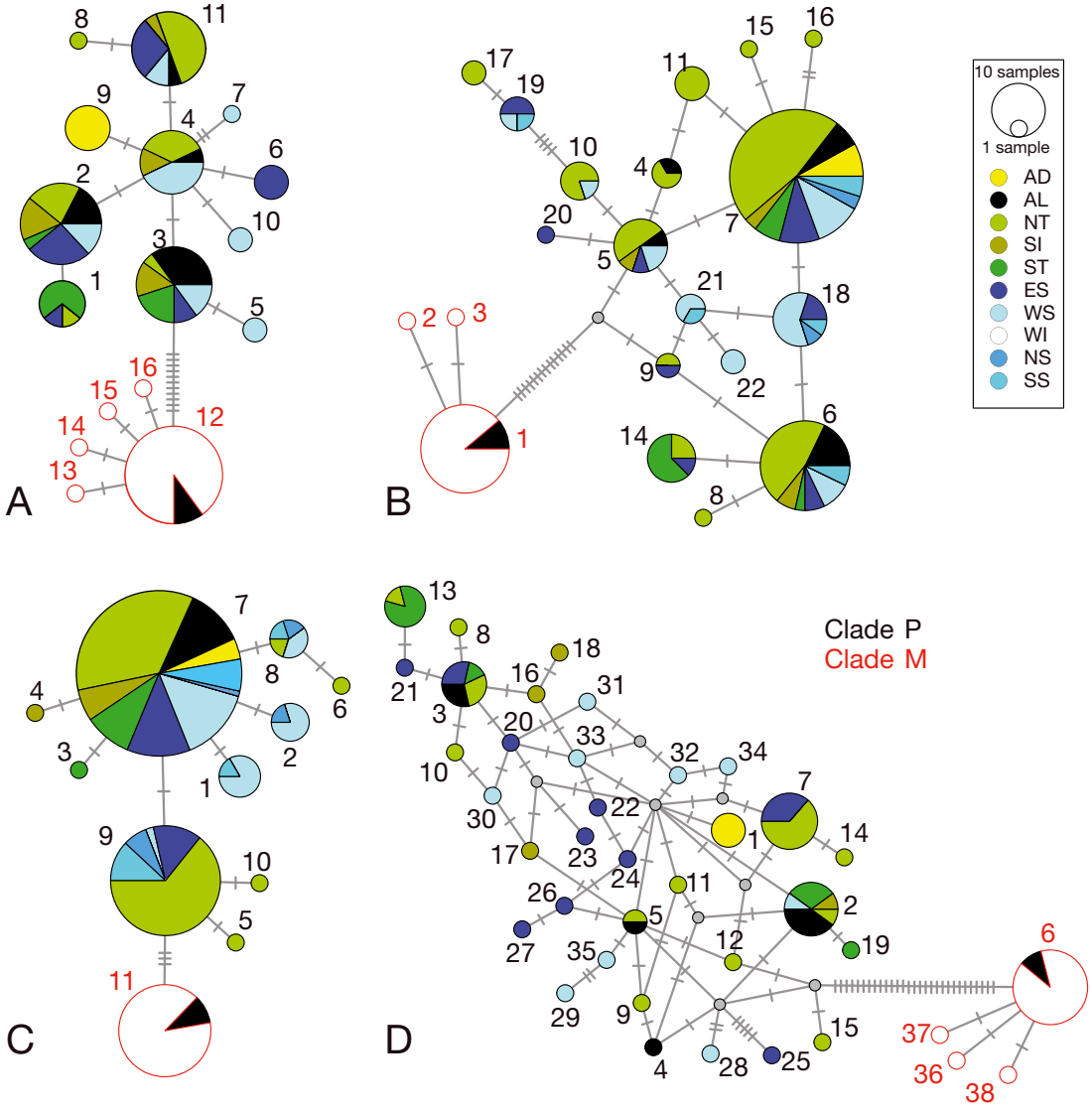

Fig. 4. Median-joining network based on the mitochondrial haplotypes as detected by the sequence variation of the (A) cytochrome oxidase I (COI), (B) mitochondrial control region hypervariable fragment (CR), (C) 16S and (D) concatenated markers. Nucleotide substitutions are represented by orthogonal bars. The mtDNA variants and haplotypes belonging to Clades P and M are numbered in black and in red, respectively. See Table 1 for sampling area codes and supplementary Tables S7–S10 for full details of the frequency of the mtDNA clades in the population samples

catenated markers revealed stable trends without any detectable expansion or bottleneck events (Fig. S3 in Supplement 1). Despite an apparent weak increase in female effective population sizes with all markers except COI (Fig. S3), the estimated values are of the same order of magnitude throughout time, and hence the changes are not substantial.

DISCUSSION

The current study improves the knowledge of the population structure and connectivity of *Raja polystigma*, a vulnerable, endemic skate of the Mediterranean Sea, and of its reproductive interactions with its sibling *R. montagui*.

Species identification and distribution

Reliable taxonomic identification is a significant issue in skate conservation, as ineffective species identification hampers monitoring and management (Tinti et al. 2003), and may prevent the recognition of local reductions in abundance or extinctions (Dulvy & Reynolds 2009, Iglésias et al. 2010). Species identifi-

cation using molecular methods such as DNA barcoding has been increasingly applied to skates (Alvarado Bremer et al. 2005a, Ward et al. 2005, 2008, Spies et al. 2006, Griffiths et al. 2010, 2013, Iglésias et al. 2010, Serra-Pereira et al. 2011, Costa et al. 2012, Knebelsberger et al. 2014, Landi et al. 2014, Lynghammar et al. 2014). Further, molecular taxonomy methods employing both nuclear and mitochondrial data have proven useful in assessing relationships between pairs of morphologically similar taxa (Morgan et al. 2012, Arlyza et al. 2013).

The results of the current study confirm the importance of accurate species identification in skates, as misidentified individuals of both species were found by employing an integrated morphological and molecular identification methodology. Moreover, the Bayesian assignment to species of several individuals based on nuclear genotypes remained ambiguous, suggesting they were hybrid or introgressed.

Phylogeography and population structure of *Raja polystigma*

R. polystigma is relatively rare and its sampling involved a number of caveats. For certain areas, where the species occurred rarely in the scientific surveys, the samples analysed were obtained by pooling individuals collected during different years. It is important to note that small sample sizes decrease the power of the analyses and consequently reduce the ability to detect significant population structure (Chevolot et al. 2006b); therefore, caution should be used in interpreting the results. However, similar experimental designs and analytical approaches have shown geographical population structure and genetic differentiation at multiple taxonomic levels in other skates (Chevolot et al. 2006a, Plank et al. 2010, Pasolini et al. 2011).

Analyses of microsatellite markers in *R. polystigma* suggests the presence of a single, almost panmictic population inhabiting the western-central Mediterranean, with high levels of connectivity and genetic diversity. This near-panmictic pattern was unexpected considering the early benthic phase of skates and the limited potential for adult migration as observed for example in *R. montagui* by tagging experiments (Walker et al. 1997). This high level of population connectivity was supported by several mtDNA haplotypes shared among samples collected over a wide geographical range in the western and central Mediterranean.

Given the behavioural preferences of *R. polystigma* for shallow-water habitats linked to life-history functions (i.e. breeding and sexual segregation) and the lack of a pelagic dispersal phase (Capape et al. 1980), the major physical constraint to dispersal in this small-sized skate may be bathymetry, as shown in a number of skates and rays (Chevolot et al. 2006a, b, Plank et al. 2010, Pasolini et al. 2011, Le Port & Lavery 2012). The presence of a continuous shelf along the north African coasts connected to the Sicilian shelf by a shallow plateau approximately ranging from 200 to 800 m in depth (IOC, IHO & BODC 2003) provides the potential for a certain degree of biological connectivity between the south-western and the north-western Mediterranean demes. The narrow shelf along the southern Tyrrhenian coast plausibly represents a dispersal corridor, whereas the northern Tyrrhenian–western Sardinian area, characterized by a continuous, shallow shelf, might have acted as a recent centre of expansion. The northern Tyrrhenian–western Sardinian demes correspond to the areas including approximately 75% of the total lineage diversity, thus featuring the bulk of the species' evolutionary potential. As such, they represent important priority populations and areas for conservation purposes, providing potential sources for recolonization in cases of strong bottlenecks or local extinctions.

In the present study, the mitochondrial DNA was more informative than nuclear loci in detecting the subtle but significant differentiation of the population sampled in the Adriatic Sea, which possessed a private mitochondrial haplotype characterized by a single mutation in the COI sequence fragment (i.e. a T > C transition at position 142 of the COI alignment). This may be due to the small sample size rather than a real lack of genetic differentiation in the Adriatic Sea. However, it could be consistent with a recent population history related to the rising sea level following the lowstand during the Last Glacial Maximum (Koblmüller et al. 2015) and a partial connectivity of this area with the bulk of the species' range, which is mainly located in the western-central Mediterranean. Subtle genetic divergence in marine fish populations with shallow evolutionary histories can be better assessed by mtDNA markers than nuclear ones (Hoarau et al. 2004). Haploid maternal inheritance of mtDNA can lead to smaller effective population size (Birky et al. 1989) and thus faster genetic drift.

Admixture, gene introgression and cross-species hybridization

The identification of a number of individuals with admixed nDNA ancestry suggests that hybridization

has occurred between *R. polystigma* and *R. montagui.* Hybridization is known for several vertebrates, including bony fish (Dowling & Secor 1997), but it has only recently been documented among chondrichthyans in 2 cases, both represented by closely related species of Australian carcharhinid sharks (Morgan et al. 2012) and Indo-Pacific dasyatid stingrays (Arlyza et al. 2013). Purebred *R. montagui* individuals in the Mediterranean were observed only in the Algerian sample, although the south Tyrrhenian, Sicilian and Adriatic *R. polystigma* samples were characterized by the presence of some individuals with nuclear contribution from *R. montagui*. Even though the number and variation of microsatellite loci used in this study cannot substantiate clear-cut evidence of F1 or F2 interspecific hybrids, such preliminary evidence of purebred individuals of both species together with individuals of admixed ancestry in the Algerian shelves seems to identify the south-western Mediterranean as a bimodal hybrid zone (Seehausen 2004), where both parental species and hybrids coexist and purebreds outnumber hybrids. Bimodal hybrid zones appear to be common in nature and they have likely arisen from secondary contact between recently diverged or incipient species (Seehausen 2004, Arias et al. 2012). On the contrary, no *R. polystigma* individuals with admixed ancestry were found in the north-western Mediterranean samples (e.g. NT, ES and WS), which is probably linked to the distance of these demes from the secondary hybrid zone.

Natural hybridization between *R. polystigma* and *R. montagui* in sympatric areas may be likely over an evolutionary time scale, considering the species' life-history traits and their recent divergence (<2 Myr ago; Valsecchi et al. 2005). Therefore, their recent splitting could have allowed the evolution of divergent mitochondrial lineages, but may not yet have led to a strong reproductive isolation between the species. Skates typically exhibit low evolutionary rates (McEachran & Dunn 1998), so inter-specific reproductive barriers might take longer to develop. However, it cannot be ruled out that the pattern detected might be the result of incomplete lineage sorting of the mtDNA and microsatellite gene pools (Avise 1994, 2000). This does appear unlikely since the signature of this process is still evident in only a few demes of a nearly panmictic Mediterranean population.

Further analyses with a higher number of polymorphic nDNA and mtDNA loci are required to comprehensively assess the relationships and cross-specific dynamics between *R. polystigma* and *R. montagui.* Several nuclear genes have been screened in Rajidae (Pasolini et al. 2006, Rocco et al. 2007) and a suitable level of polymorphism could be determined in order to clearly differentiate the recently diverged species as *R. polystigma* and *R. montagui.*

The present study confirmed the parapatric distribution of the 2 species and further assessed their geographic ranges. It provides important novel zoogeographical data which will contribute to the updating of the available reference literature data and maps (Ellis et al. 2007, Serena et al. 2010, Ungaro et al. 2015) that presently provide inconsistent species distributions.

Data accessibility statement. Mitochondrial data: GenBank Accession Numbers for COI, CR and 16S haplotypes KT231929 to KT231977; microsatellite allele data deposited in Dryad (http://dx.doi.org/10.5061/dryad.dh182).

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