

## GENETIC IDENTIFICATION OF THE NORTHEASTERN ATLANTIC SPINY SPIDER CRAB AS *MAJA BRACHYDACTYLA* BALSS, 1922

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### ABSTRACT

The northeastern Atlantic spiny spider crab occurs from the British Islands to Senegal, where it is an important fishery resource. From morphological characters this crab has recently been proposed as a distinct species, *Maja brachydactyla*, although for commercial purposes it is still considered the same species as its Mediterranean congener *M. squinado*. We have studied variation at two mitochondrial genes in several crab populations from the Atlantic (putatively *M. brachydactyla*) and Mediterranean (*M. squinado* and *M. crispata*) basins, in order to clarify the taxonomic status of this crab in the northeastern Atlantic and Mediterranean regions. Phylogenetic analysis revealed that each of these three taxa forms a distinct and well-defined clade. While the divergence within each taxon was 0% for 16S and 0–0.3% for COI, divergence between taxa was 0.6–2.5% for 16S and 5.3–8.7% for COI; values that are in the range of the differences observed between other crustacean species. These results confirm the genetic distinctiveness of each taxa and support their designation as different species. Therefore, the Atlantic spider crab should be referred as *M. brachydactyla*, a fact that should be taken into account for conservation and commercial purposes.

**KEY WORDS:** mtDNA, molecular systematics, *Maja brachydactyla*, spider crabs

### INTRODUCTION

The spiny spider crab occurs in the northeastern Atlantic from the British Islands to Senegal, where it can be an important fishery resource (González-Gurriarán et al., 1993; Freire and García-Allut, 2000). According to FAO statistics, the landings during 2005 were around 5000 tonnes in Europe. This crustacean belongs to the genus *Maja* (Decapoda: Majidae), and it is often considered conspecific with its Mediterranean congener *M. squinado* (Herbst, 1788) (Freire and García-Allut, 2000; González-Gurriarán et al., 2002; Sampedro and González-Gurriarán, 2004). This classification is however controversial: on the basis of some morphological characters, Neumann (1998) suggested that the Atlantic and Mediterranean crabs are in fact different species, *M. brachydactyla* Balss, 1922, and *M. squinado*, respectively. In addition, this genus includes two other morphologically distinct species in the Mediterranean: *M. crispata* Risso, 1827 and *M. goltziana* d'Oliveira, 1888 (see Neumann, 1996).

Indeed, accurate species delimitation is key for an efficient management, commercialization, and preservation of fisheries resources (Brooker et al., 2000; Frankham et al., 2004; Triantafyllidis et al., 2005; Tsoi et al., 2005; Ward et al., 2006). In this regard, molecular studies have already proven very useful in crustaceans, either confirming the species status suggested by morphological studies (*Carcinus*, Geller et al., 1997; *Alpheus*, Mathews et al., 2002) or refuting it (*Brachynotus*, Schubart et al., 2001a; *Chaceon*, Weinberg et al., 2003; *Penaeus*, Tsoi et al., 2005; *Xantho*, Reuschel and Schubart, 2006; *Paratya*, Cook et al., 2006). In particular, sequence variation at the COI barcode region has been recently shown to be very effective for discriminating species of Crustacea (Costa et al., 2007).

In this study, we aimed to clarify the taxonomic status of the Northeast Atlantic spider crab, to which we will

putatively refer as *M. brachydactyla*, using molecular techniques. In particular, we have studied variation at two mitochondrial genes, 16S and COI, in different crab populations from the northeastern Atlantic and Mediterranean regions. Our results clearly corroborate that Atlantic and Mediterranean spiny spider crabs are distinct species.

### MATERIALS AND METHODS

#### Collected Samples

We analyzed 208 spiny spider crabs of three species of *Maja* (Table 1, Fig. 1). One hundred and forty seven individuals of (putative) *M. brachydactyla* were collected in the Eastern Atlantic between 2003 and 2006 from Ireland to the Canary Islands, covering almost its whole distribution range; thirty individuals of *M. squinado* were collected in Capraia Island (Italy) in 2004, and thirty one individuals of *M. crispata* were collected in Mediterranean waters from Spain to Greece between 2004 and 2006. Crabs of both sexes were caught using nets or scuba diving and muscle tissue of one or two walking legs from each crab was preserved in 100% ethanol, as soon as possible after collection.

#### DNA Sequencing

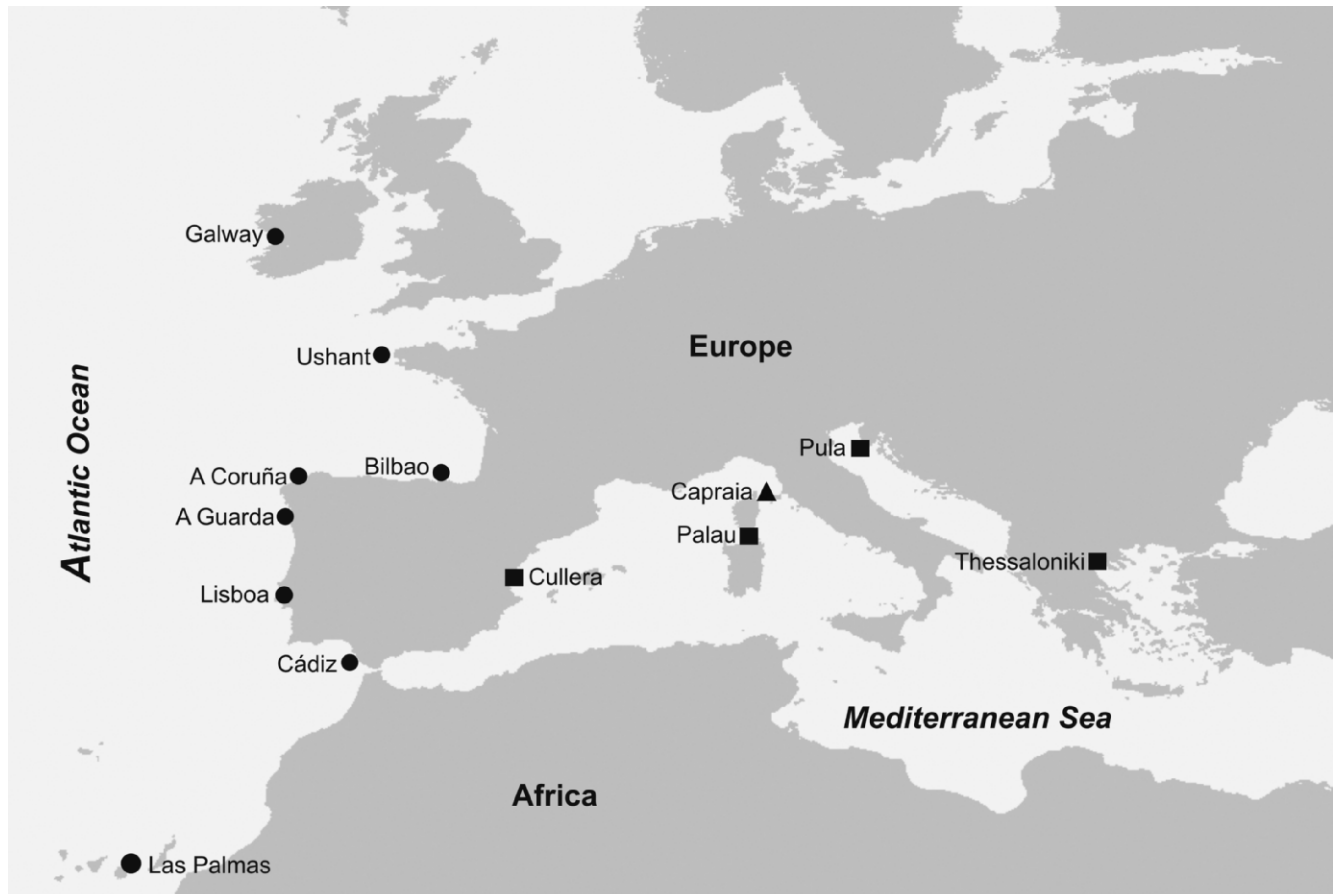
Genomic DNA was extracted using Chelex resin (Estoup et al., 1996), followed by PCR amplification of two fragments of mitochondrial DNA using universal primers: 560 bp of the 16S rRNA gene with 16Sar and 16Sbr (Palumbi et al., 1991) and 710 bp of the COI gene with LCO1490 and HCO2198 (Folmer et al., 1994). Reactions were made in a final volume of 20  $\mu$ l, containing 1  $\mu$ l of DNA extraction, 2  $\mu$ l of 10 $\times$  PCR buffer (160 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 670 mM Tris-HCl pH 8.8, 0.1% Tween 20), 1  $\mu$ l of 50 mM MgCl<sub>2</sub>, 1  $\mu$ l of each primer (20  $\mu$ M), 1  $\mu$ l of 10 mM dNTP Mix (Applied Biosystems), 0.2  $\mu$ l BIOTAQ polymerase (5 U/ $\mu$ l, Boline) and 13  $\mu$ l of sterile bidistilled water. PCR profiles were as follows: 5 min at 95°C, 35 cycles of 20 s at 95°C, 20 s at 55°C, 20 s at 72°C, and 7 min at 72°C for 16S and 5 min at 95°C, 30 cycles of 20 s at 95°C, 20 s at 42°C, 30 s at 72°C, and 7 min at 72°C for COI. For verification, PCR products were run in 2% agarose gels stained with ethidium bromide. Prior to sequencing, SSCP analysis (Single Strand Conformation Polymorphism) was used to survey different haplotypes. This technique consists of electrophoresis of single-stranded DNA fragments of suitable size through a non-denaturing polyacrylamide gel, followed by visualization. Under low temperature and

Table 1. Description of samples, including collection site, geographical coordinates, year of collection and number of individuals (*N*).

Species	Collection site	Coordinates	Year	<i>N</i>
<i>Maja brachydactyla</i>	Galway (Ireland)	53°43'N 9°02'W	2004	25
	Ushant (France)	48°27'N 5°05'W	2004	25
	Bilbao (Spain)	43°15'N 2°58'W	2004	4
	A Coruña (Spain)	43°22'N 8°23'W	2003	24
	A Guarda (Spain)	41°54'N 8°53'W	2004/2005	18
	Lisboa (Portugal)	38°43'N 9°08'W	2005	22
	Cádiz (Spain)	36°32'N 6°17'W	2005	25
	Las Palmas (Canary Islands, Spain)	28°00'N 15°25'W	2006	4
	<i>Maja squinado</i>	Capraia Island (Italy)	43°03'N 9°50'E	2004
Cullera (Spain)		39°10'N 0°16'W	2005	1
<i>Maja crispata</i>	Palau (Sardinia, Italy)	42°25'N 1°57'E	2006	6
	Pula (Croatia)	44°52'N 13°51'E	2004	6
	Thessaloniki (Greece)	40°38'N 22°56'E	2004	18

non-denaturing conditions, DNA strands fold into structures that migrate according to their shape. DNA strands with different sequences generally do not assume the same shape, and so have distinct gel mobilities (Sunnucks et al., 2000). In this way, different migration patterns would be considered different haplotypes and therefore sequenced. We used vertical gels of 23 cm long × 21 cm wide × 1 mm thick containing 8% acrylamide (50:1 acrylamide:bis-acrylamide), 4.5 ml TBE 10× buffer, 200 µl ammonium persulphate 10%, 40 µl TEMED and 33.3 ml distilled water. Five µl of unpurified PCR product were mixed with 5 µl of denaturing loading buffer (950 µl formamide, 10 µl EDTA 0.25 M, 10 µl bromophenol blue 0.05%, 10 µl xylene cyanol 0.05%, 20 µl distilled water for 1 ml of buffer), heated at 95°C for 5 min and then chilled on ice until loaded on the

gels. Gels run in TBE 1× at 8°C and 30 W for 4 and 8 h (16S and COI genes, respectively). Bands were visualized by silver-staining. In order to assess and confirm the sequence variation, all samples with different mobility patterns as well as random samples of the most frequent patterns were sequenced in forward direction with the PCR primers indicated above. Previously, PCR products were purified with GFX PCR DNA and gel band purification kit (Amersham Biosciences) following manufacturer's instructions. Sequencing reactions were performed with dRhodamine terminator chemistry (Applied Biosystems), precipitated with ethanol and ran in an automated sequencer ABI PRISM 310 (Applied Biosystems). The sequences were analyzed with the program DNA Sequencing Analysis 3.7 (Applied Biosystems).

Fig. 1. Sampling sites. Each symbol represents samples of each putative species: circles, *M. brachydactyla*; triangles, *M. squinado*; squares, *M. crispata*.

## Genetic Differentiation and Phylogenetic Analyses

Electropherograms were proofread by eye and sequences were aligned with ClustalW (Thompson et al., 1994). Genetic distances between populations were calculated using the program DnaSP 4.10 (Rozas et al., 2003). We chose the  $D_a$  distance (Nei, 1987), the number of net nucleotide substitutions per site between populations, because it takes into account the intrapopulation variability. With the resulting distance matrix, we constructed a population tree with the neighbor joining (NJ) algorithm (Saitou and Nei, 1987) using PAUP\* 4.0b10 (Swofford, 2002). In these population analyses, we excluded one locality (Cullera) because of its small sampling size (1 individual). In addition, the COI complete data set was collapsed into haplotypes and their phylogenetic relationships were estimated by maximum likelihood (ML) as implemented in PHYML 2.4.4 (Guindon and Gascuel, 2003), using the best-fit substitution model selected by the Akaike Information Criterion (AIC) with MODELTEST 3.06 (Posada and Crandall, 1998) and PAUP\* 4.0b10 (Swofford, 2002). Phylogenetic confidence in the ML tree was assessed with 1000 bootstrap replicates (Felsenstein, 1985).

## RESULTS

## Genetic Differentiation for 16S

The 16S fragment was invariable within each species, and the SSCP approach yielded only three different mobility patterns, one per species. This result was corroborated by sequencing 1-5 individuals from each population at random. The final 16S alignment consisted of 518 bp with one indel due to an extra G in the 246 position of *M. brachydactyla* (GenBank accession numbers 16S: EU000850-EU000852). The model of nucleotide substitution selected by the AIC was HKY85 (Hasegawa et al., 1985) (base frequencies (A, C, G, T) = 0.35, 0.11, 0.18, 0.36; transition/transversion ratio = 5.08). There were 12 substitutions in total, none of them shared between *M. brachydactyla* and *M. squinado*, 3 shared between *M. brachydactyla* and *M. crispata* and the 9 remaining shared between *M. squinado* and *M. crispata* (the percentage of divergence was 2.5, 1.9 and 0.6, respectively).

## Genetic Differentiation for COI

The COI fragment was more variable and rendered very different SSCP profiles. The final length of the alignment was 548 bp, including 73 variable positions (GenBank accession numbers C01: EU000811-EU000849). The model of nucleotide substitution selected by the AIC was TrN + I (Tamura and Nei, 1993, with a proportion of invariable sites) (base frequencies (A, C, G, T) = 0.27, 0.19, 0.16, 0.37; relative rate matrix (AC, AG, AT, CG, CT, GT) = 1, 31.3, 1, 1, 8.5, 1; proportion of invariable sites = 0.80). We observed 39 haplotypes, none of them shared between species: 21

Table 2. Genetic diversity estimates for the COI fragment: net number of haplotypes, haplotype diversity (Hd), average number of nucleotide differences between haplotypes per gene ( $\pi_{\text{gen}}$ ) and per site (nucleotide diversity) ( $\pi_{\text{sit}}$ ).

Species	Haplotypes	Hd	$\pi_{\text{gen}}$	$\pi_{\text{sit}}$
<i>Maja brachydactyla</i>	21	0.87	2.23	0.0041
<i>Maja squinado</i>	4	0.49	0.89	0.0016
<i>Maja crispata</i>	14	0.79	3.07	0.0056

from *M. brachydactyla*, 4 from *M. squinado* and 14 from *M. crispata*. The number of fixed nucleotide differences was 28 between *M. brachydactyla* and *M. squinado*, 35 between *M. brachydactyla* and *M. crispata*, and 21 between *M. squinado* and *M. crispata*. The mean number of differences between haplotypes within each species was 2.23 for *M. brachydactyla*, 0.89 for *M. squinado* and 3.07 for *M. crispata* (Table 2).

In general, the percentage of divergence among *M. brachydactyla* populations was less than 0.05%. There were higher values for the comparisons including Bilbao and Las Palmas (0.05-0.3%), but these could be biased because of the small sample size of these localities (4 crabs each). On the other hand, genetic divergence between *M. brachydactyla* and *M. squinado* was 6.7%, and 8.7% between *M. brachydactyla* and *M. crispata*. In general, divergence within *M. brachydactyla* was at least two orders of magnitude smaller than between different taxa. In the case of *M. crispata*, divergence among populations ranged from 0% to 0.1% and it was 5.3% when comparing to *M. squinado* (Table 3). These genetic differences are reflected in the population tree (Fig. 2), where each taxon forms a distinct clade, and in the haplotype tree (Fig. 3), where the haplotypes are structured in three well-supported (bootstrap values > 95%) monophyletic groups.

## DISCUSSION

According to their genetic differentiation, the three taxa sampled in this study correspond with an interpretation as three distinct species. Both 16S and COI fragments show diagnostic (fixed) differences, and divergence within each taxon was much smaller than between them. The interspecific divergences observed were similar to those reported for other crustacean species (1-5% for 16S and

Table 3. Pairwise genetic distances ( $D_a$ ) between populations based on COI sequences. Boxes enclose intraspecific values.

	Galway	Ushant	Bilbao	A Coruña	A Guarda	Lisboa	Cádiz	Las Palmas	Capraia	Palau	Pula
Ushant	-0.00008										
Bilbao	0.00182	0.00208									
A Coruña	0.00014	0.00027	0.00077								
A Guarda	-0.00001	0.00007	0.00224	0.00027							
Lisboa	-0.00006	0.00002	0.00184	0.00011	-0.00006						
Cádiz	0.00045	0.00051	0.00259	0.00046	0.00038	0.00041					
Las Palmas	0.00071	0.00083	0.00335	0.00085	0.00031	0.00050	0.00024				
Capraia	0.06680	0.06662	0.07102	0.06777	0.06707	0.06636	0.06850	0.06707			
Palau	0.08145	0.08150	0.08248	0.08130	0.08169	0.08081	0.08282	0.08127	0.04997		
Pula	0.08148	0.08153	0.08248	0.08133	0.08173	0.08085	0.08284	0.08127	0.04906	-0.00074	
Thessaloniki	0.08172	0.08176	0.08287	0.08162	0.08199	0.08111	0.08307	0.08150	0.05304	0.00138	0.00160

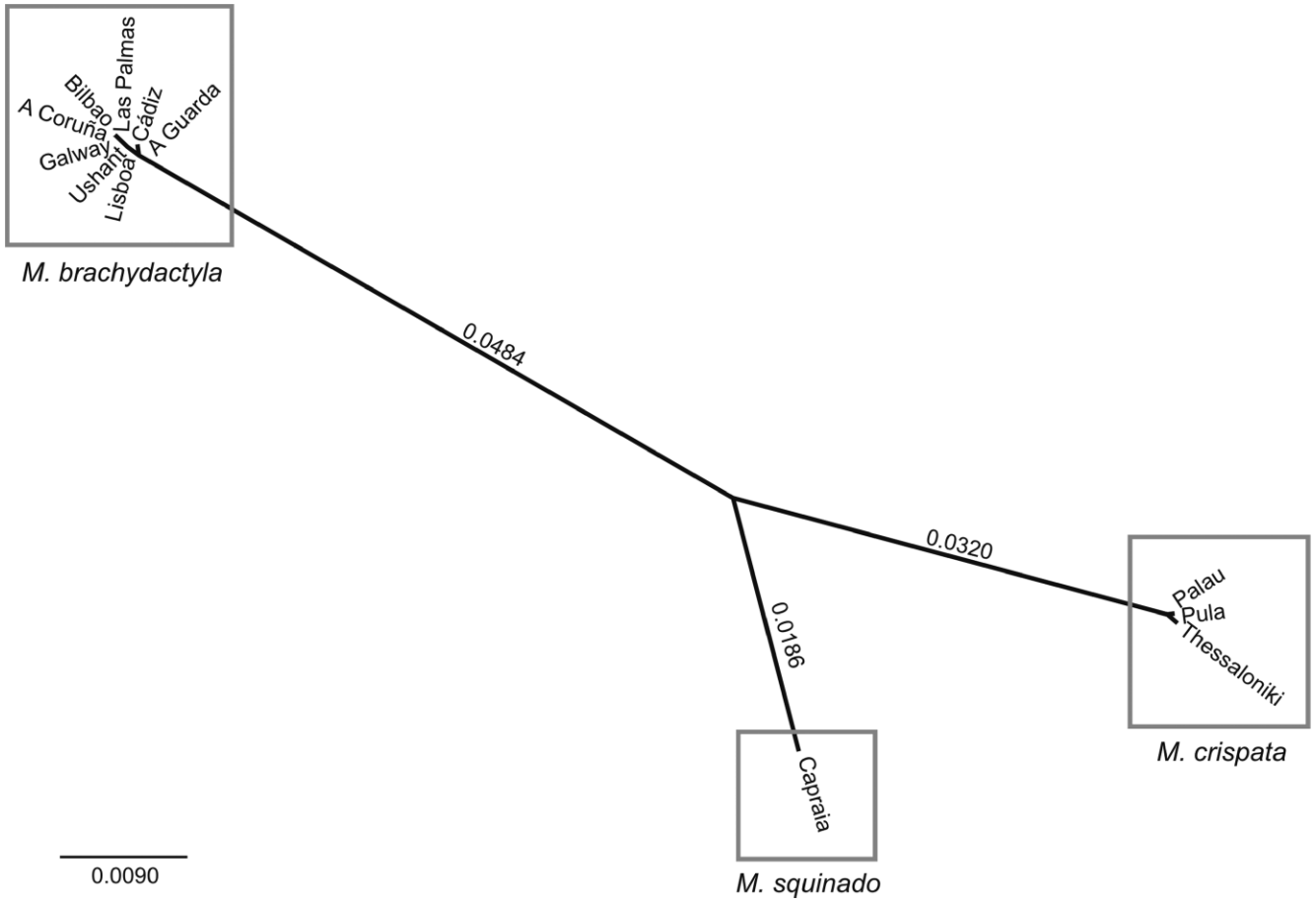


Fig. 2. Unrooted neighbor joining population tree based on COI sequences. Numbers on branches represent  $D_a$  distances.

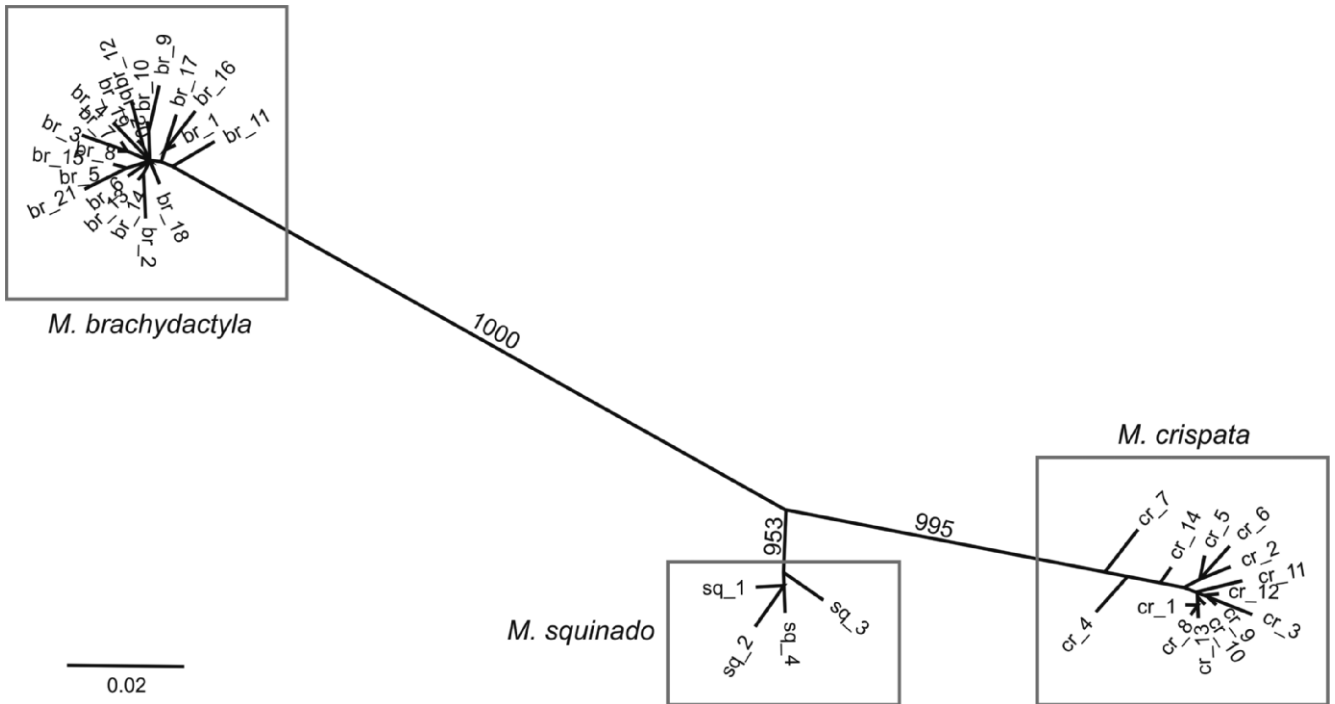


Fig. 3. Unrooted maximum likelihood haplotype tree (COI data set). Haplotype codes: br, *M. brachydactyla*; sq, *M. squinado*; cr, *M. crispata*. Numbers on branches are bootstrap values for 1000 replicates.

2-11% for COI) (Mathews et al., 2002; Schubart and Koller, 2005; Schubart et al., 2001b). For example, the European green crabs *Carcinus maenas* and *C. aestuarii* from the Atlantic and Mediterranean basins, respectively, show divergences around 2.5% for 16S (Geller et al., 1997) and around 11% for COI (Roman and Palumbi, 2004). In addition, the three taxa form three distinct monophyletic groups both in the population and haplotype trees.

As expected, we observed less variability for 16S than for COI. The former was monomorphic within species while the second seems polymorphic enough to be used in further population studies. The unequal sampling scheme in the Atlantic and the Mediterranean does not allow us to compare levels of variation within each taxon, but the distinctiveness and homogeneity of the Atlantic crab populations respect to the Mediterranean samples is clear. If we want to determinate the exact geographic boundary between these crab species, further collection of specimens should focus in the Western Mediterranean.

Our results corroborate the morphological classification previously proposed by Neumann (1996, 1998), and justify the designation of these taxa as different species: *Maja brachydactyla*, *Maja squinado* and *Maja crispata*. In particular, the northeastern Atlantic spider crab should be tagged as *M. brachydactyla* and not as *M. squinado*. Indeed, this species delimitation should be taken into account for the management of these species and for commercial identification. For example, reintroduction of the Spanish Mediterranean spiny spider crabs populations, now in principle extinct, should be implemented using conspecific crabs from the Mediterranean, and never from the Atlantic coasts.

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