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Identification of European species of *Maja* (Decapoda: Brachyura: Majidae): RFLP analyses of COI mtDNA and morphological considerations

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SUMMARY: Four species of crabs of the genus *Maja* have been described along the European coast: *M. brachydactyla*, *M. squinado*, *M. goltziana* and *M. crispata*. The commercially important species *M. brachydactyla* and *M. squinado* achieve the largest body sizes and are the most similar in morphology, and are therefore easily confused. The four species of *Maja* were identified using a novel morphometric index and a polymerase chain reaction followed by restriction fragment length polymorphism analysis (RFLP). The relationship between carapace length and the distance between the tips of antorbital spines was used to distinguish adults of *M. brachydactyla* and *M. squinado*. PCR-RFLP analysis of a partial sequence of the mitochondrial cytochrome oxidase type I (COI) revealed that the four species of the genus *Maja* can be unambiguously discriminated using the combination of restriction endonucleases enzymes *Hpy*CH4V and *Ase* I. The molecular identification may be particularly useful in larvae, juvenile and young crabs, when the morphological differences found in adults are not applicable.

Keywords: *Maja*, *M. squinado*, *M. brachydactyla*, molecular identification, morfometry, COI, RFLP.

RESUMEN: Identificación de las especies europeas del género *Maja* (Decapoda: Brachyura: Majidae): análisis de PCR-RFLP de una región del mtADN COI y consideraciones morfológicas. – Cuatro especies del género *Maja* han sido descritas en las costas europeas: *M. brachydactyla*, *M. squinado*, *M. goltziana* y *M. crispata*. Las especies *M. brachydactyla* y *M. squinado*, que tienen importancia comercial, alcanzan los tamaños más grandes y son morfológicamente muy similares, siendo muy fácil confundirlas. La identificación de las cuatro especies se ha realizado utilizando un nuevo índice morfométrico y un análisis de polimorfismos de fragmentos de restricción (RFLP). La relación entre la longitud del cefalotórax y la distancia entre los extremos distales de las espinas antorbitales se ha utilizado para la diferenciación de los adultos de *M. brachydactyla* y *M. squinado*. El análisis PCR-RFLP de una secuencia parcial de la citocromo oxidasa tipo I mitocondrial (COI) indica que las cuatro especies del género *Maja* pueden ser discriminadas usando una combinación de las endonucleasas *Hpy*CH4V y *Ase* I. La identificación molecular puede ser particularmente útil en las larvas, juveniles y cangrejos jóvenes, cuando las diferencias morfológicas encontradas en los adultos no son aplicables.

Palabras clave: *Maja*, *M. squinado*, *M. brachydactyla*, identificación molecular, morfometría, COI, RFLP.

INTRODUCTION

Four species of spider crabs of the genus *Maja* Lamarck, 1801 (Majoidea, Majidae) have been reported along the European coast: *M. brachydactyla* Balss, 1922; *M. crispata* Risso, 1827; *M. goltziana* D'Oliveira, 1888; and *M. squinado* (Herbst, 1788) (Neumann, 1998; Sotelo *et al.,* 2008, 2009). *Maja brachydactyla* and *M. squinado* are of high commercial value due to their larger size (Števčić, 1974; Le Duff, 1990). While *M. goltziana* adults are easily identified by the presence of a strong dorso-distal

spine on the merus of the pereiopods (Zariquiey-Álvarez, 1968; Neumann, 1998), discrimination among the other three species is more difficult. *M. crispata,* the smallest species, shows considerable ontogenetic variation and has characters that overlap with *M. brachydactyla* and *M. squinado* (Neumann, 1996). Balss (1922) erected *M. squinado* var. *brachydactyla* on three specimens from Canary Islands with short walking legs. For some years this taxon has been considered a synonym of *M. squinado* (Zariquiey-Álvarez, 1968; Neumann, 1996; Ng *et al.,* 2008). However, Neumann (1998) pointed out that Atlantic populations of "*M. squinado"* are separable from those of the Mediterranean based on morphological and biometrical characters, which justifies the recognition of two different species. However, species identification using the key morphological characters for adult Majidae of Neumann (1998) may be difficult for non-specialists to apply. Results of recent genetic analyses support the recognition of *M. brachydactyla* and *M. squinado* as different species (Sotelo *et al.,* 2008, 2009). *M. brachydactyla* has been reported in the eastern Atlantic, while *M. squinado* is restricted to the Mediterranean Sea (Neumann, 1998; d'Udekem d'Acoz, 1999; Sotelo *et al.,* 2008, 2009).

The larval stages of the Atlanto-Mediterranean species of *Maja* are morphologically very similar (Clark, 1986; Paula, 1988; Rodríguez, 2002; Guerao *et al.,* 2008), with the zoea stages being virtually indistinguishable. The morphology of juveniles is also very similar; at least for *M. brachydactyla* (Guerao and Rotllant, 2009) and *M. squinado* (Guerao and Rotllant unpublished data). The possibility of correctly identifying all development stages (larvae, juveniles, adults) of a species is a prerequisite for all studies on population dynamics aimed at developing proper management of fisheries resources and their commercialization.

Among the different polymerase chain reaction (PCR) methods of genotype analysis, restriction fragment length polymorphism (RFLP) of PCR-amplified mitochondrial DNA (mtDNA) fragments has been recently used to identify different crustacean species (e.g. Bossier *et al.,* 2004; Khamnamtong *et al.,* 2005; Van Stappen *et al.,* 2007). Sequence variation at the COI mtDNA barcode region has been shown to be effective for discriminating crustacean species (Costa *et al.,* 2007).

The aim of this paper is to provide a useful molecular technique for identifying all the European species of the genus *Maja* at all stages of development, and also to provide new morphological and morphometric characters that allow easy differentiation between *M. brachydactyla* and *M. squinado* specimens.

MATERIALS AND METHODS

A total of 149 adult specimens of the four species of genus *Maja* collected between 2007 and 2010 in the Atlantic and Mediterranean Sea were used in morphological and/or molecular analyses (Table 1). One pereiopod of each individual was preserved in 94-100% ethanol for posterior DNA extraction. For the morphological study, additional individuals (carapaces) conserved in the laboratory were also measured (n=13 *M. brachydactyla*; n=8 *M. squinado*). The following measurements were taken: carapace length (CL) as the distance between the rostral margin (without rostral spines) and the posterior margin of the carapace (without intestinal spines); and antorbital spine length (ANSL), measured as the distance between the tips of the antorbital spines (the spine at the posterolateral corner of the supraorbital eave; see Figs. 1 and 2). The relationship between CL and ANSL was studied using least squares analysis of linear regression (log ANSL = log a + b log CL). Regression was performed using a SigmaStat 3 (Systat Software Inc., USA) software package. The significance of the correlation was tested with a t-test (H_0: $\rho=0$, H_1: $\rho\neq0$), and the regression equations were compared with the F-test (H_0: $a_1=a_2$, $b_1=b_2$) (Cuadras, 1991).

TABLE 1. – Sampling locations and number of individuals for molecular and morphological studies of *Maja*. Abbreviations: GB, GenBank accession number; MO, morphological studies; n, number of crabs; RF, RFLP studies; T, total studied individuals.

Species	Collection sites	Coordinates	Year	MO n	RF n	T n	COI sequences GB
Maja squinado	W Mediterranean (Catalonia, Spain)	43.9ºN 13.8ºE	2007-09	22	7	25	GU902709
	Central Mediterranean (Adriatic Sea, Italy)	42.0ºN 9.0ºE	2009	17	20	20	HM572324, HM572326
	W Mediterranean (Corsica, France)	41.1ºN 1.2ºE	2010	5	8	8	-
	Previous studies	–		–	–	–	EU000832-35, GQ153551
Maja brachydactyla	E Atlantic (Galicia, Spain)	42.2ºN 8.7ºW	2007-08	30	10	36	-
	E Atlantic (Grand Casablanca, Morocco)	33.6ºN 7.6ºW	2009	10	20	20	-
	SW Mediterranean (Ceuta, Spain, Africa)	35.9ºN 5.3ºW	2009	9	6	9	GU902706-08, GU902711-13
	Previous studies						EU000811-31, GQ153550
Maja crispata	W Mediterranean (Catalonia, Spain)	41.1ºN 1.2ºE	2008-09	4	5	5	GU902710
	Central Mediterraean (Adriatic Sea, Italy)	43.6ºN 13.5ºE	2009	16	20	20	-
	Previous studies						EU000836-49, GQ153554
Maja goltziana	E Mediterranean (Antalya, Turkey)	36.8ºN 30.7ºE	2009	4	5	5	GU939594
	Central Mediterranean (Malta Archipelago)	35.7ºN 14.2ºE	2009		1	1	HM572325
	Previous studies						GQ153555

Total genomic DNA extraction was performed with muscle tissues using the QIAamp DNA Mini Kit (QIAGEN Inc). The concentration of DNA was estimated by spectrophotometry, using a GeneQuant *pro*. The 710 bp region of the mitochondrial cytochrome oxidase subunit I gene was amplified using universal primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G -3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAA TCA-3′) described by Folmer *et al.* (1994). Amplification was carried out with 80-100 ng of genomic DNA in a reaction containing 1U of Taq polymerase (Invitrogen), 1X buffer (Invitrogen), 2 μM of each primer and 800 μM dNTPs. The PCR thermal profile used was 95°C for 5 min for the initial denaturation, and 39 cycles at 94°C (20 s), 42°C (20 s), 72°C (30 s) with a final extension at 70°C for 7 min. In order to estimate the size of restriction fragments precisely, the mitochondrial COI gene was sequenced from 2-10 individuals for each restriction pattern obtained (Table 1). Sequencing reactions were performed with the ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing kit, v3.1 (Applied Biosystems); the products were analyzed in an automated sequencer ABI PRISM 310 (Applied Biosystems). Sequencing reactions were carried out by an external laboratory (Sistemas Genómicos®, Valencia, Spain). In addition, sequences of all four species of *Maja* from GenBank were analyzed (Table 1). To identify conserved nucleotide residues useful for an RFLP analysis, sequence alignment was performed using the BioEdit software (Hall, 1999). The amplification products were then incubated with two restriction enzymes, *Hpy*CH4V (recognition site: 5′- TG′CA -3′/3′- AC′GT -5′) and *Ase* I (recognition site: 5′- AT´TAAT -3′/3′- TAAT′TA -5′) (New England Biolabs). Digestion was performed in a 20 μL mixture that contained 10 μL of PCR product, 25 U of *Hpy*CH4V, 50 U *Ase* I and NEB digestion Buffer 2. Restriction enzyme digestions were incubated for 3.5 h at 37°C. Restriction fragments were electrophoretically separated on 1.5% agarose in 1X TBE buffer, stained with ethidium bromide and visualized under UV light. The number of individuals analyzed is shown in Table 1.

RESULTS

The CL/ANSL ratio is different in *M. squinado* in relation to other European species of the genus *Maja*. Thus, the CL/ANSL ratio is < 3 in *M. brachydactyla* (CL/ANSL=2.52±0.1), *M. crispata* (CL/ANSL=2.01±0.1) and *M. goltziana* (L/ANSL = 2.5±0.06), but > 3 in *M. squinado* (CL/ANSL=3.24±0.12). The correlation between ANSL and CL is highly significant in *M. brachydactyla* (t=53.9; P<0.001), *M. squinado* (t=55.8; P<0.001) and *M. crispata* (t=23.1; P<0.001) (Fig. 1). The equations of the regression of ANSL on CL for *M. brachydactyla* and *M. squinado* are significantly different between the two species (F=682.2; P<0.005; Fig. 1). In addition, the regression of ANSL on CL in

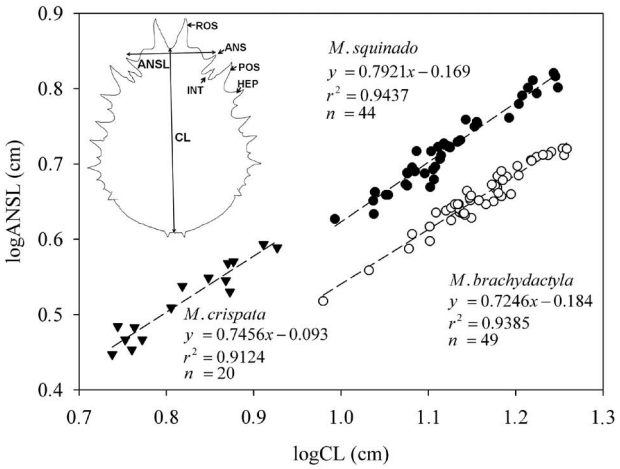

FIG. 1. – Relationship between carapace length (CL) and the distance between the tip of the antorbital spines (ANSL) in *Maja brachydactyla*, *M. squinado* and *M. crispata*. ANS, antorbital spine; INT, intercalated spine; HEP, hepatic spine; POS, postorbital spine; ROS, rostral spine.

M. crispata is significantly different from *M. squinado* (F=6.10; P<0.005; Fig. 1) and *M. brachydactyla* (F=72.01; P<0.005). The small number of *M. goltziana* available made a regression analysis impossible. In addition to the CL/ANSL ratio, the shape and orientation of the antorbital spines have been shown to be a useful tools for easy identification of adult individuals of *M. brachydactyla* and *M. squinado*. Thus, while the antorbital spine in *M. brachydactyla* is curved and directed upward vertically, it is slightly curved, thinner, more pointed and slightly oblique from vertical in *M. squinado* (Fig. 2).

PCR-RFLP analyses

The size of the COI mtDNA fragment amplified in the four species was 710 bp. Restriction fragment length polymorphism analysis revealed that the four species can be unambiguously discriminated using the restriction enzymes *Hpy*CH4V and *Ase* I (Fig. 3). As expected, digestion with both enzymes produced a specific pattern for *M. crispata* (9, 99, 227 and 375 bp), *M. squinado* (333 and 377 bp), *M. goltziana* (15, 122, 198 and 375 bp), and *M. brachydactyla*, which exhibited an alternative specific restriction pattern (the

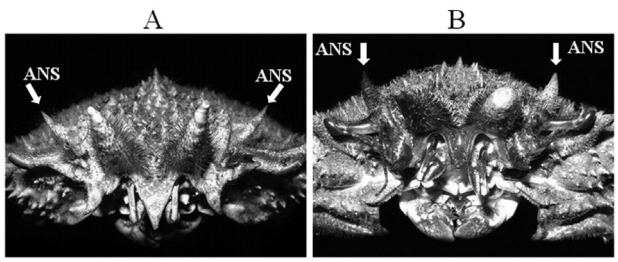

FIG. 2. – Shape and orientation of the antorbital spines (frontal view). *Maja squinado* (A); *M. brachydactyla* (B). Antorbital spines (ANS).

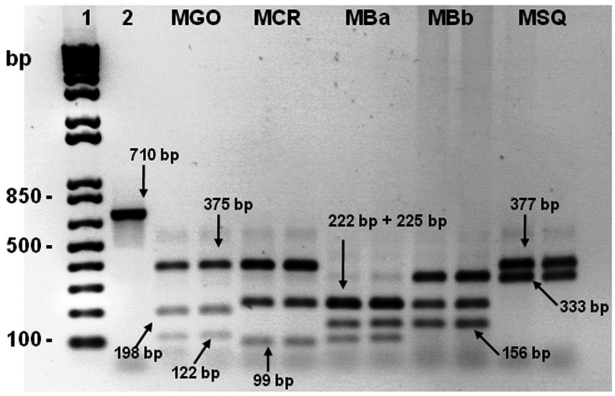

FIG. 3. – RFLP analysis of the COI mtDNA segments after digestion with *Hpy*CH4V and *Ase* I. Molecular weight standard (1); undigested fragment (2); *Maja goltziana* (MGO); *M. crispata* (MCR); *M. brachydactyla*, pattern a (MBa); *M. brachydactyla*, pattern b (MBb); *M. squinado* (MSQ).

most frequent MBa, 108, 154, 222 and 226 bp versus MBb 156, 221 and 333 bp, the least frequent). These alternative patterns (b) occur due to a point mutation at the enzyme recognition site for *Ase* I (Fig. 4) observed in 11% of *M. brachydactyla* individuals from all collection sites (Galicia (n=1), Morocco (n=2) and Ceuta (n=1); see Table 1). In addition, the mutation at the enzyme recognition site related to pattern b can be observed in 14.3% of *M. brachydactyla* sequences from GenBank. None of the studied individuals of *M. squinado* and *M. goltziana* contained the recognition site for the *Ase* I enzyme (Fig. 4). It should be noted that *M. squinado* individuals may show one or two enzyme recognition sites for *Hpy*CH4V; however, as both sites are very close (5 bp, see Fig. 4) the restriction patterns are indistinguishable.

DISCUSSION

The morphological differentiation of the European species in the genus *Maja* is sometime problematic due to a considerable amount of character overlap and ontogenetic variation. *Maja brachydactyla* and *M. squinado* reach the largest body sizes (exceeding 20 cm in carapace length) and they are the most similar species. Neumann (1998) reported that the different morphology of the male first gonopod is particularly useful for identifying the two species. Thus, the proximal margin of the distal region of the gonopod is rounded with gradual transition to the terminal process in *M. brachydactyla*, while it shows an angled outline and abrupt transition into a terminal process in *M. squinado*. However, the distinctive characters of the male first gonopod are subject to ontogenetic changes, making the identification of juvenile individuals very difficult or impossible.

	117 126	222 233
M. brachydactyla a	TAATTGCTCCGG	GTATATTAATCACG
M. brachydactyla b	GAATTGCTCCGG	GTATGTTAATCACG
M. crispata	TAATAGCTCCAG	GCATATTAATTACA
M. squinado	TAATCGCTCCAG	GTATGTTAATCACA
M. squinado	TAATCGCTCCAG	GTATGTTAATCACA
M. goltziana	TAATTGCACCAG	GTATGTTGATTACA

	315 338	538 547
M. brachydactyla a	CCCCTGCGTGCGCAATGGCTGCAGCTA	CGTGTGCAGTAA
M. brachydactyla b	CCCCTGCGTGCGCAATGGCTGCAGCTA	CGTGTGCAGTAA
M. crispata	CTCCTGCGTGTGCAATGGCTGCAGCAA	CGTGGGCAGTAA
M. squinado[1]	CTCCTGCGTGCGCAATGGCTGCAGCGA	CGTGGGCAGTAA
M. squinado[2]	CTCCTGCGTGTGCAATGGCTGCAGCGA	CGTGGGCAGTAA
M. goltziana	CTCCTGCATGAGCAATAGCTGCAGCTA	CGTGGGCAGTAA

FIG. 4. – Position of *Hpy*CH4V and *Ase* I restriction sites on the COI segment. *M. brachydactyla* a, GU902708; *M. brachydactyla* b, GU902707; *M. crispata*, GU902710; *M. squinado*[1], GU902709; *M. squinado*[2], HM572326; *M. goltziana*, HM572325.

Moreover, the study of gonopods requires dissection and detailed microscope observations. In the present study, it was demonstrated that a simple relationship between CL and ANSL is a good morphometric index to differentiate adult individuals of both sexes (CL larger than 9.8 cm) of *M. brachydactyla* and *M. squinado* (see Figs. 1 and 2). However, early juveniles of the two species do not differ in this character and are nearly indistinguishable (Guerao and Rotllant, 2009, 2010). The great similarity between the juveniles of related brachyuran species has been previously documented; species distinction is very difficult at the early postlarval stages, and phenotypic divergence increases with juvenile ontogeny (Ingle and Rice 1984; Martin *et al.,* 1984; Felder *et al.,* 1985; Neumann 1996). The adult individuals of *M. crispata* are smaller than adults of *M. brachydactyla* and *M. squinado*. This species can only be confused with juveniles of the two larger European species of *Maja*.

Identification of crustacean species using PCR-RFLP analysis has been well documented (Power *et al.,* 1999; Bossier *et al.,* 2004; Khamnamtong *et al.,* 2005; Hisar *et al.,* 2008; Pascoal *et al.,* 2008; Dharani *et al.,* 2009). The PCR-RFLP technique, to be diagnostic, must be applied to DNA regions that are highly conserved within species but sufficiently variable between species. The COI mtDNA region has been shown to be a good marker for crustacean species (Costa *et al.,* 2007). Importantly, mtCOI is sufficiently variable to be useful in identifying and discriminating even the most closely related species (Lefébure *et al.,* 2006; Bucklin *et al.,* 2007, 2009; Darling and Tepolt, 2008). Sotelo *et al*. (2008) reported that the COI region displays diagnostic differences between species of the genus *Maja*; divergence between species was much higher than within them. The low level of intraspecific variation observed among the three species indicates that the COI gene fragment amplified utilizing the primers of Folmer *et al*. (1994) is a suitable marker for identifying Majidae species in the NE Atlantic and Mediterranean Sea. Using a combination of restriction enzymes (*Hpy*CH4V and *Ase* I) proved useful for discriminating all four *Maja* European species.

Analysis of *M. brachydactyla* samples, from the eastern Atlantic (Spain, Galicia; and Morocco, Grand Casablanca) to SW Mediterranean (Spain, Ceuta), and *M. squinado* and *M. crispata* samples from the western (Spain, Catalonia) and central (Italy, Adriatic Sea) Mediterranean revealed identical restriction patterns among individuals of the same species. It should be noted that the specimens of *Maja* originating from Ceuta, on the Mediterranean coast (near the Strait of Gibraltar), have been unambiguously assigned to the species *M. brachydactyla* (Table 1). The presence of strictly eastern Atlantic coastal species in the Alboran Sea has been reported in several instances and related to the inflow of the less dense Atlantic surface water into the Mediterranean Sea through the Strait of Gibraltar (García Raso, 1984; García Muñoz *et al.,* 2008; Lasram *et al.,* 2008).

The molecular identification technique might be especially useful when dealing with larvae or juvenile individuals, which do not have the discriminating morphological characters of adults (Chow *et al.,* 2006; Tang *et al.,* 2009). The PCR-RFLP technique, which allows the four *Maja* species to be identified at any development stage, would contribute to the understanding of their life histories and hence to the management of their exploitation. It may also have forensic applications when fraud is suspected in the commerce of the two species (*M. brachydactyla* and *M. squinado*), as they are subject to different capture and marketing regulations.

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