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Exploring molecular variation in the cosmopolitan *Caprella penantis* (Crustacea: Amphipoda): results from RAPD analysis

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Eight populations of Caprella penantis*, three of* Caprella dilatata *and two of* Caprella andreae*, collected from different sites all over the world, were selected for genetic study. Thirteen primers were tested, and the phenogram, based on the similarity coefficient of Nei & Li and the UPGMA method, separated clearly* C. dilatata *and* C. andreae *from the populations of* C. penantis*, supporting the validity of these three species, traditionally considered altogether under the old 'acutifrons' complex. Populations of* C. penantis *(including, at least, forms* simulatrix, testudo *and* lusitanica*) from Spain, Portugal, Morocco, Japan and Brazil were clustered together in the RAPD analysis, indicating that, probably, all the specimens of* C. penantis *could belong to the same species, in spite of morphological variations in the pleura, gills, robustness and presence/absence of proximal projection in adult male gnathopod 2 propodus. The only population which showed genetic differentiation within the* C. penantis *complex was the form* gibbosa *from Coquimbo, Chile. Future analysis based on different molecular approaches (mtDNA, 18S rRNA, ISSR) and additional material from other world areas, should be conducted to confirm these results.*

Keywords: *Caprella penantis*, *C. dilatata*, *C. andreae*, Caprellidae, Amphipoda, random amplified polymorphic DNA (RAPD), genetic variability

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INTRODUCTION

Besides the morphological variation with age and sex, some caprellid species show also considerable intraspecific variation. This is the case for *Caprella penantis* Leach, 1814, a world-wide distributed species of the Caprellidae, which could be a complex of different species in which it is difficult to understand if the morphological variation is intra- or interspecific (Guerra-García *et al.*, 2006). Although there has been traditionally a gap in molecular studies on the Caprellidea, recently, different molecular approaches have been applied also for this group of amphipods. In this sense, Guerra-García *et al.* (2006) showed, for the first time in caprellids, the validity of the RAPD technique as a tool for helping to solve taxonomic problems. Ito *et al.* (2008) conducted the first molecular study to investigate the phylogenetic relationships among the Caprellidea based on the 18S rRNA. Ashton *et al.* (2008), using mitochondrial DNA, revealed multiple northern hemisphere introductions of the invader *Caprella mutica* Schurin, 1935. To our knowledge these are the only three works dealing with molecular tools and caprellids.

In the study of Guerra-García *et al.* (2006), seven populations of *Caprella penantis* from the Strait of Gibraltar were morphologically and genetically compared among them and with other populations of the closest species *Caprella dilatata* Krøyer, 1843, to explore the intraspecific and interspecific genetic differentiation. Their results showed a clear separation between *Caprella penantis* and *C. dilatata* populations (only 8% similarity between them), supporting the morphological differences that indicate that both species are really different and valid species. However, all the populations of *C. penantis* from the Strait of Gibraltar were clustered together (85% similarity) indicating that they probably belong to the same species in spite of morphological variations among populations. Taking into account that in this previous study all the material was coming from the Strait of Gibraltar and that all the studied populations of *C. penantis* belonged to the form *simulatrix*, we have increased significantly the number of samples for the present study and we have considered populations from different areas of the world, not only from the Strait of Gibraltar, including also additional populations of *C. dilatata* and *Caprella andreae* Mayer, 1890.

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MATERIALS AND METHODS

Specimens of eight populations of *C. penantis*, three populations of *C. dilatata* and two populations of *C. andreae*, were collected from different sites of the world (Table 1). For the genetic analysis, the species *Caprella scaura*

Templeton, 1836, separate from the 'acutifrons' complex was also included as an 'outgroup'. The caprellids were fixed in 95% ethanol. DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN). Pooled individuals were used because relatively small quantities of DNA were available per individual and the aim of this study was to compare populations (Thomas *et al.*, 1997). Determination of the concentration and purity of the DNA, RAPD protocol and amplification conditions were the same as those of Guerra-García *et al.* (2006). Amplification products were analysed by electrophoresis in 2.0% agarose (Seakem ® LE Agarose, Lonza) gels run at 90 V for 2.5 hours, stained with ethidium bromide and visualized by illumination with UV light. For band size determination, a 123 bp DNA ladder (Sigma-Aldrich) was loaded in lanes flanking groups of about 7 samples in each gel. All amplifications were repeated at least twice to check the stability of amplification products. Bands were scored as present (1) or absent (0) (Tansley & Brown, 2000; Costa *et al.*, 2004a, b) by eye and only unequivocal bands were scored, with weak bands not being included. To ensure data accuracy, all samples were scored twice by the same individual, and the second round of scoring was conducted without reference to data from the initial round (Star *et al.*, 2003). The coefficient of Nei & Li (1979), recommended to be employed in RAPD analysis (Lamboy, 1994), and used most often in amphipods to estimate the degree of genetic differentiation between populations or species (Stewart, 1993; Culver *et al.*, 1995), was applied to calculate a similarity matrix (NTSYS-pc computer package, version 1.8 (Rohlf, 1993)). The rest of the statistical treatment (UPGMA, SHAN, construction of the phenogram and the cophenetic value matrix) was conducted according to Guerra-García *et al.* (2006).

RESULTS AND DISCUSSION

Thirteen primers yielded a total of 154 consistently well-amplified DNA fragments with an average of 11.85 bands per primer (Table 2). Overall, 23 of these bands were monomorphic (15%) and 131 were polymorphic (85%). Primers 5 and 10 had the highest percentage of polymorphic fragments between samples (both 100%). Cophenetic correlation analysis strongly supported the reliability of the phenogram based on the original distance matrix ($r = 0.845$). The phenogram (Figure 1) separated clearly *C. dilatata* and *C. andreae* from the populations of *C. penantis*, supporting the validity of these species, traditionally considered altogether under the old 'acutifrons' complex. Populations of *C. penantis* from Spain, Portugal, Morocco, Japan and Brazil were clustered together in the RAPD analysis, indicating that, probably, all the specimens of *C. penantis* could belong to the same species. From the eight populations of this species included in the analysis, only the population of Punta Carnero (Cp-Car) and Ceuta (Cp-Ceu) belonged to the form *simulatrix* (without proximal projection on male gnathopod 2 propodus). The remaining populations were all provided with a proximal projection belonging, at least, to the forms *testudo* and *lusitanica*. Interestingly, the cluster did not show the populations with projection within the same group. Population Cp-Ceu was more similar to the Japanese and Brazilian specimens, that were provided with proximal projection, and Cp-Car was more similar to the population of Mindelo (Cp-Min) and to the population of Torreguadiaro (Cp-Tor), also provided with projection in male gnathopod 2 propodus. According to these results, it seems that the presence/absence of this proximal projection should not be strong enough as morphological difference for separating species, and that the different forms of *C. penantis* could be explained by intraspecific variation. The only population which showed higher genetic differentiation within the *C. penantis* complex was the form *gibbosa* from Coquimbo, Chile (Mayer, 1890, p. 52). This form was referred as *Caprella verrucosa* Boeck, 1871, in Guerra-García & Thiel (2001) and Thiel *et al.* (2003) due to the presence of abundant dorsal tubercles on pereonites, especially on pereonites 5, 6 and 7, and the robustness of antenna 1 (figure 4 in Guerra-García & Thiel, 2001). However, the morphology of the specimens changes considerably along the coast of Chile (Thiel *et al.*, 2003). Further molecular and morphological phylogenetic studies are necessary to elucidate if the material from Coquimbo is really belonging to *Caprella penantis*, *C. verrucosa* or to a different undescribed species. One of the limitations of the RAPD molecular approach is that we cannot conclude which genetic difference as measured by the Nei & Li coefficient is necessary to establish specific differences. Consequently, this method should be used with care to make definite statements about the limits considered to distinguish species, and future studies based on mtDNA, 18S rRNA, ISSR should be necessary to consider if *Caprella penantis* f. *gibbosa* should be erected as a new species differing from *C. penantis* and *C. verrucosa*. Unfortunately, material of the real *C. verrucosa* from California or Japan were not available in the present study to be compared with the '*C. verrucosa*' (*C. penantis* f. *gibbosa*) from Coquimbo.

Caprella penantis is regarded as one of the most problematic caprellids throughout the world, since this species has been recorded under several species or subspecies names from the temperate regions of the world and the need for genetic studies to determine its nomenclatural status at each locality has been pointed out in most of the taxonomic caprellids studies (McCain, 1968; Laubitz, 1972). In Mayer's monographs (1890, 1903) he described nineteen forms of the 'acutifrons' group (forms *typica*, *minor*, *tabida*, *tibada*, *neglecta*, *gibbosa*, *andreae*, *carolinensis*, *virginia*, *lusitanica*, *natalensis*, *porcellio*, *simulatrix*, *testudo*, *angusta*, *incisa*, *verrucosa*, *borealis* and *cristibrachium*). Several of these forms have already been given specific rank. Forms *typica* and *minor* have been assigned to *Caprella dilatata* (McCain, 1968) and probably forms *tabida* and *tibada* also belong to *C. dilatata*, although its taxonomic status under *Caprella penantis* is still under discussion (Guerra-García *et al.*, 2006). *Caprella neglecta* Mayer, 1890, was also considered as a valid species (Vassilenko, 1967; Laubitz, 1972), form *andreae* was assigned to *Caprella andreae* (McCain, 1968), forms *natalensis* and *porcellio* to *Caprella natalensis* Mayer, 1903 (Laubitz, 1972), forms *incisa*, *verrucosa*, *borealis* and *cristibrachium* to *Caprella incisa* Mayer, 1903, *C. verrucosa*, *C. borealis* Mayer, 1903, and *C. cristibrachium* Mayer, 1903, respectively (Utinomi, 1943; Dougherty & Steinberg, 1953; McCain, 1968; Laubitz, 1972). The form *angusta* was considered as *Caprella angusta* Mayer, 1903, by Dougherty & Steinberg (1953) and Laubitz (1970); but Laubitz (1972) questioned the validity of this species. Consequently, its position is still unclear. The remaining forms *gibbosa*, *carolinensis*, *virginia*, *lusitanica*, *testudo* (all these forms with proximal projection in the propodus of male ganthopod 2) and *simulatrix* (without

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Table 1. List of species used for the molecular study using RAPD.

Species	Code	Coordinates	Collection data	Substrate	Locality	Notes
Caprella andreae Mayer, 1890	Ca-Cha	28°23.244N 80°32,646′W	4 November 2006	*Caretta caretta*	Charleston, USA	
	Ca-Col	39°51.32′N 00°40.07′E	19 July 1996	Intertidal algae	Columbretes Island, Castellón, Spain	
Caprella dilatata Kroyer, 1843	Cd-Alg	36°10′N 5°24′W	September 2004	On buoys	Algeciras Bay, Spain	Population G in Guerra-García *et al.* (2006)
	Cd-Ali	38°05′N 0°38′W	5 December 2006	Fish farm	Guardamar, Alicante, Spain	
	Cd-Cat	26°58′S 48°35′W	19 October 2006	Mussel farm	Itapocoroi Bay, municipality of Penha, Santa Catarina, Brazil	
Caprella penantis Leach, 1814	Cp-Car	36°04′N 5°25′W	23 July 2006	Intertidal alga *Asparagopsis armata*	Punta Carnero, Algeciras Bay, Spain	Without proximal projection on male gnathopod 2 propodus
	Cp-Ceu	35°53′N 5°20′W	31 August 2004	Intertidal alga *Asparagopsis armata*	Calamocarro, Ceuta, Spain	Population F in Guerra-García *et al.* (2006), without projection on male gnathopod 2 propodus
	Cp-Min	41°20′ 13.02″N 8°44′43.67″W	October 2006	Intertidal alga *Cystoseira*	Mindelo, Portugal	With proximal projection on male gnathopod 2 propodus
	Cp-Par	26°51′S 48°32′W	7 October 2006	Intertidal alga *Pterocladia capillacea*	Praia de Caiobá, Matinhos, Paraná, Brazil	With proximal projection on male gnathopod 2 propodus
	Cp-Saf	32°18′N 9°15′W	21 July 2006	Intertidal alga *Gelidium* sp.	Safi, Morocco	With proximal projection on male gnathopod 2 propodus
	Cp-Tor	36°18′N 5°15′W	1 October 2006	Intertidal alga *Gelidium* sp.	Torreguadiaro, Cádiz, Spain	With proximal projection on male gnathopod 2 propodus
	Cp-Och	33°56.20′N 132°24.06′E	April 2007	Alga *Sargassum muticum*	Ochima, Japan	With proximal projection on male gnathopod 2 propodus
	Cv-Coq	29°58′S 71°21′W	30 October 2006	Floating buoys	Coquimbo, Chile	*Caprella 'verrucosa'* in Guerra-García & Thiel (2001)
Caprella scaura Templeton, 1836	Cs-Coq		30 October 2006	Floating buoys	Coquimbo, Chile	

Table 2. Number of amplification products (bands) obtained per primer in the random amplified polymorphic DNA (RAPD) analysis of the cosmopolitan *Caprella penantis* and related species.

Primer no.	Sequence of bases	No. of bands scored			% Polymorphic
		Total	Monomorphic	Polymorphic	
1	5′–TAGCAGCTTAG	6	4	2	33
2	5′–TCGTACTATGC	8	5	3	38
3	5′–TAGCAGGATCA	2	1	1	50
4	5′–TAAGACGCCTA	6	2	4	67
5	5′–GTTGCGCGAC	13	0	13	100
6	5′–CGGACGCTGG	3	1	2	67
7	5′–CAGTCCCTGG	19	1	18	95
8	5′–TGCTGCAGGT	20	2	18	90
9	5′–GGTGATCAGG	15	1	14	93
10	5′–ACCCGGTCAC	23	0	23	100
11	5′–CAGCTCACGA	5	4	1	20
12	5′–ACGCGCATGT	21	1	20	95
13	5′–CGGTCACTGT	13	1	12	92
Total		154	23	131	

projection) are, consequently, still classified under the species *Caprella penantis* (McCain, 1968; Laubitz, 1970, 1972; Krapp-Schickel, 1993). The present study shows that, using RAPD, all *C. penantis* forms seem to be very similar from the molecular point of view, suggesting that there are no clear evidences to separate them as different species. The presence/absence of the projection seems not to be related with a species differentiation but with phenotypical intraspecific variation. The morphological variation among populations of *C. penantis* could be mainly influenced by the ecological characteristics of the habitat. In this sense, Bynum (1980) conducted a morphometric study using *C. penantis* from coastal and estuarine sites in North Carolina and found a gradient of forms related to the degree of exposure to turbulence. Body parts and appendages associated with grasping the substrate of the same caprellid species are capable of modification depending on the degree of wave exposure, thus exhibiting ecological plasticity or 'ecotopic variation' (Caine, 1989; Guerra-García, 2001). Caprellid species

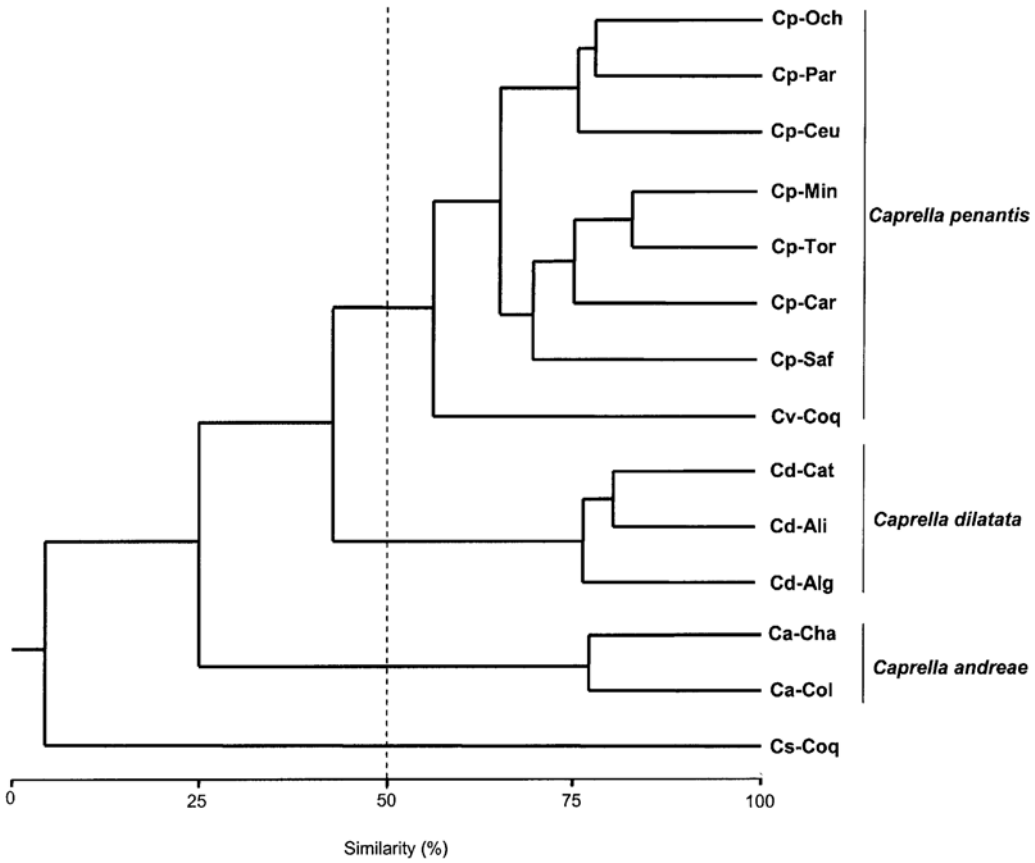

Fig. 1. Phenogram of the fourteen populations studied, based on similarities generated by UPGMA clustering of RAPD band scores.

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in environments with strong wave action tend to have a more robust body than those living in calmer places (Hirayama & Kikuchi, 1980).

The RAPD analysis (Welsh & McClelland, 1990; Williams *et al.*, 1990) requires no prior knowledge of DNA sequence (Hadrys *et al.*, 1992) and is faster and cheaper than many other molecular techniques (Costa *et al.*, 2004b), but this method also has some limitations and low reproducibility (Williams *et al.*, 1990; Pérez *et al.*, 1998). In fact, in spite of using the same methodology and similar laboratory conditions, the number of bands obtained per primer in the study carried out by Guerra-García *et al.* (2006, p. 102; Table 2) differs from the number of the present study (see Table 2). However, although RAPD analysis must be interpreted with caution, the results of the present study based on a wide range of samples throughout the world support the preliminary observations made by Guerra-García *et al.* (2006) based exclusively on material from the Strait of Gibraltar. Additional sampling and future studies based on a larger set of molecular markers and techniques (e.g. mtDNA, 18S rRNA, ISSR, etc.) are necessary to confirm the patterns obtained with RAPD analyses.

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