

Determination of evolutionary units in European representatives of the crab genus *Pilumnus*

Research Article

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Abstract: Bristle crabs of the genus *Pilumnus* (Brachyura: Heterotremata: Pilumnidae) are common inhabitants of European waters. They are easily identifiable as a genus, but with the exception of *P. inermis*, intrageneric classification turns out to be quite complex. There is no general agreement on the number and distinction of species. Therefore, this genus is well-suited for comparative molecular studies. Specimens of the *Pilumnus hirtellus* complex, here defined as including *Pilumnus hirtellus*, *P. villosissimus*, *P. spinifer*, *P. aestuarii*, and an undescribed species, were gathered from throughout the Mediterranean Sea and the eastern Atlantic Ocean. DNA sequence data were obtained from the barcoding region of the cytochrome oxidase 1 mitochondrial gene and used for reconstruction of a phylogenetic tree and a haplotype network. The morphology of the gastric ossicles was compared in the search of separating characters. Our results give evidence for five genetic clusters within the *P. hirtellus* complex. There is negligible geographic variation within these clusters. Unambiguous mtDNA sequences within morphologically variable local populations argue against possible hybridization. The here encountered evolutionary units are relatively young and possibly allow to study ongoing processes of morphological, genetic, and ecological differentiation, leading to speciation and radiations in the coastal marine environment.

Keywords: Molecular diversity • Genetic differentiation • Barcoding • Cytochrome oxidase 1 • Haplotype network • Morphology • Gastric ossicles • Crustacea • Decapoda • Pilumnoidea

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1. Introduction

The taxonomy of bristle crabs of the genus *Pilumnus* (Brachyura: Heterotremata: Pilumnidae) is in constant flux since more than 250 years and not satisfactorily resolved until today [1,2]. In Europe, these crabs are common inhabitants of shallow waters of most marine coastlines. They are easily identifiable as a genus, but with the exception of *P. inermis*, intrageneric classification turns out to be quite complex and is mostly based on the presence of pereopodal spines and/or the shape of characteristic bristles [see keys in 3,4]. Overall, there is no general agreement on the number, nomenclature, and distinction of species that should be recognized for European waters. There are similar problems of interspecific morphological differentiation among American representatives of *Pilumnus* [5]. Therefore, this genus is destined for additional morphological as well as comparative molecular phylogenetic studies.

The genus *Pilumnus* was created by Leach in 1815 [6] and initially comprised the two European species *Pilumnus hirtellus* (Linnaeus, 1761), *P. villosissimus* (Rafinesque, 1814), and the Asian representative *P. vespertilio* (Fabricius, 1793). Since then, 164 species belonging to this genus have been described as new. Currently, 143 species are recognized as valid, of which some must be considered questionable [1]. Bristle crabs have been included either in the family Xanthidae Mac Leay, 1838 [e.g. 7] or in their own family, Pilumnidae Samouelle, 1819. Until recently, they have been considered to belong to the superfamily Xanthoidea [e.g. 8,9], but currently they have been recognized to constitute a separate lineage and are placed into the superfamily Pilumnoidea [1,10].

At the species level, the taxonomic history is even more complex. A. Milne-Edwards and Bouvier [11] considered all the species of *Pilumnus* up to then described as variations of the stem form *Pilumnus hirtellus* (Linnaeus, 1761), as

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the differences in setation and spinulation do not appear to be fixed and constant, but rather show morphological gradients. Pesta [7] concurs with that opinion and recognizes *Pilumnus hirtellus* in only two variations from the Adriatic Sea: *P. h. hirtellus* (Linnaeus, 1761) and *P. h. villosus* Risso, 1827. He considers the latter a senior synonym of *Pilumnus spinulosus* Kessler, 1861 and *P. aestuarii* Nardo, 1869, but does not mention the older *P. villosissimus* (Rafinesque, 1814). Zariquiey Álvarez [3] distinguished five separable species for the Iberian Peninsula, providing short morphological descriptions and an identification key for *Pilumnus hirtellus*, *P. villosissimus*, *P. spinifer* H. Milne Edwards, 1834, *P. aestuarii*, and *Pilumnus inermis* A. Milne-Edwards & Bouvier, 1894. Türkay *et al.* [12] recognized the same set of species for the Northern Sporades (Aegean Sea), providing a plate with photographs (dorsal and frontal) of four of the more similar species (*P. inermis* is easy to distinguish with only four anterolateral spines). D'Udekem d'Acoz [13] expressed doubts on the status of *P. aestuarii*, and in 1999 he proposed the synonymy of *P. aestuarii* sensu Nardo (1869) with *P. hirtellus*. Nevertheless, d'Udekem d'Acoz [13] felt that the animals described as *P. aestuarii* by Zariquiey Álvarez [3] from the Mediterranean coast of Spain were indeed different and should deserve species status, which he tentatively referred to as *Pilumnus* sp. 1, providing a brief description. In the 1999 monograph, d'Udekem d'Acoz [14] put into question the validity of the species *P. spinifer* and suggested it to be merely a form of *P. hirtellus*. Türkay [15] left *P. aestuarii* in the synonymy with *P. hirtellus*, but considered *P. spinifer* a good species. Thus, most recent authors reduce the number of valid endemic European species (not counting the introduced species, e.g. *P. hirsutus* Stimpson, 1858) to four. This has been adopted by Mavdis *et al.* [4] and Oliveira-Biener *et al.* [2].

Some doubts remain though as for the status of *Pilumnus aestuarii*. Müller and Schubart [16] report specimens of *Pilumnus* from the Croatian Adriatic coast, which do not conform to *Pilumnus hirtellus*, *P. spinifer*, nor *P. villosissimus*. Ng *et al.* [1] maintain *Pilumnus aestuarii* in their species list, despite the above mentioned synonymization. Schubart *et al.* [17] mention *Pilumnus hirtellus* forma *aestuarii* from the Lagoon of Venice.

Oliveira-Biener *et al.* [2] provide the first published molecular study on intrageneric relationships within European representatives of the genus *Pilumnus*. They adopt the taxonomy by Mavdis *et al.* [4] and recognize four endemic European species. Their phylogenetic tree based on 544 basepairs of the Palumbi region of the mitochondrial gene cytochrome oxidase subunit 1 shows six genetic clades with European species of *Pilumnus*: *P. inermis* and other outgroups (clade VI), *P. spinifer* (clade I), *P. villosissimus* (clade II), and three clades

including specimens which they refer to as *P. hirtellus* (clades III–V). Some of the DNA samples included had been provided by us, based on preliminary results from unpublished student practices and theses [e.g. 18]. Interestingly, the three specimens that we refer to as *Pilumnus aestuarii* form a clade together with most of the Mediterranean *P. hirtellus* from Oliveira-Biener *et al.* [2], i.e. their clade IV termed “Mediterranean *hirtellus* group”. We also provided two DNA samples of what we refer to as *Pilumnus* sp. 1, one of them identified as such by Cédric d'Udekem d'Acoz. They again form a separate clade (as sister clade to *P. villosissimus*) in Oliveira-Biener *et al.*'s analysis, clustering with two *P. hirtellus* from Croatia as clade III, their “*hirtellus* group”. The third *hirtellus* group from Oliveira-Biener *et al.* [2] is clade V and almost exclusively consists of specimens from the Atlantic Ocean, their “Atlantic *hirtellus* group”.

The aim of the current study is to contribute to the ongoing discussion concerning the number of species (or at least evolutionary units or lineages) and the possibilities to distinguish them. For this purpose, we analyze unstudied morphological characters and provide for the first time molecular data from the mtDNA barcoding region, based on an independent dataset from the one of Oliveira-Biener *et al.* [2], which only overlaps in few taxa for which DNA samples had been exchanged. The results are discussed in their relevance of reflecting the real biological diversity of the European *Pilumnus* and of marine coastal life in the Mediterranean Sea and adjacent Atlantic Ocean.

2. Experimental Procedures

Specimens of all autochthonous European species of *Pilumnus* were gathered from as many localities as possible, ranging from different Atlantic islands in the west to the Black Sea in the east. The selection included the only eastern Atlantic species from deeper waters, *Pilumnus inermis* A. Milne-Edwards and Bouvier, 1894, which can readily be distinguished by only having four anterolateral teeth, and all the presumed European species that have been discussed in the literature over the past half century: *Pilumnus hirtellus* (Linnaeus, 1761), *P. villosissimus* (Rafinesque, 1814), *P. spinifer* H. Milne Edwards, 1834, *P. aestuarii* Nardo, 1869, and *Pilumnus* sp. 1 [13,14]. The latter five species are more typical for shallower coastal waters and will be here referred to as the *Pilumnus hirtellus* complex [see also 2]. They are morphologically relatively similar and morphologically intermediate forms have been observed (C. d'Udekem d'Acoz, personal communication). Table 1 lists all the individuals and localities for which DNA sequences have been obtained for this study.

Species	Location	Date	Collector	Extraction #
<i>Pilumnus aestuarii</i>	Morocco, Nador Lagoon	27.11.2005	C.D. Schubart	E5-01
	Italy, Sardina, Palau	07.06.2006	C.D. Schubart	R553 - 2
	Croatia, Istra, Pula	07.09.2004	C. Müller	E2-01
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	GrA-01
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	GrA-02
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	GrA-03
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	R543-1
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	R543-2
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	R543-3
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	R543-6
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	R543-7
	Greece, Amvrakikos, Mihalitsi	20.09.2003	C.D. Schubart, R. Rubner	R543-8
	Greece, Amvrakikos, West of Sparta	07.06.2012	C.D. Schubart, B.E. Aichinger	R694-5
	Greece, Amvrakikos, West of Sparta	07.06.2012	C.D. Schubart, B.E. Aichinger	R694-6
	Greece, Amvrakikos, West of Sparta	07.06.2012	C.D. Schubart, B.E. Aichinger	R694-9
	Greece, Amvrakikos, West of Sparta	07.06.2012	C.D. Schubart, B.E. Aichinger	R694-8
	Greece, Amvrakikos, West of Sparta	07.06.2012	C.D. Schubart, B.E. Aichinger	R694-14
	Greece, Lesbos, Agios Stefanos	11.07.1992	C. d'Udekem d'Acoz	R553-10
	Bulgaria, Kavarna	04.07.2010	S. Uzunova	R675-5
<i>Pilumnus hiirellus</i>	Italy, Sicily, Messina	2010	C. lo Presti	R543 - 10
	Greece, Lesbos, Agios Stefano	11.07.1992	C. d'Udekem d'Acoz	R553 - 10
	Spain, Cádiz, Rota	20.04.2012	J.A. Cuesta	R693-5
	Spain, Cádiz, Rota	20.04.2012	J.A. Cuesta	R693-7
	France, Roscoff	Apr 1961	R. Zariquiey Álvarez	E5-05
	Helgoland	Aug. 2003	SMF	Rubner
	Spain, Ibiza	2001	C.D. Schubart, C. Müller	Rubner
	Greece, Parga (in <i>Geodia</i> sponge)	2003	C.D. Schubart, R. Rubner	Rubner
	Portugal, Azores, SW Santa Maria	30.05.1981	C. d'Udekem d'Acoz	E6-02
	Portugal, Azores	No data	C. d'Udekem d'Acoz	Rubner
<i>Pilumnus sp1</i>	Spain, Cádiz, Rota	20.04.2012	J. A. Cuesta	R693-4
	Spain, Cádiz, Rota	20.04.2012	J. A. Cuesta	R693-10
	Spain, Cádiz, Rota	23.05.2012	J. A. Cuesta	R693-12
	Spain, Barcelona	09.05.1956	R. Zariquiey Álvarez	E5-03
	Italy, Sicily, Palermo, I. delle femmine	Jan 90	Via C. d'Udekem d'Acoz	SR10-9
<i>Pilumnus spinifer</i>	Italy, Sicily, Faro	20.06.2010	C. lo Presti	R675-4
	Greece, Lesbos, Kratigos	14.07.1992	C. d'Udekem d'Acoz	SR3-02
	Croatia, Rovinj	Sep. 2003	D. Brandis	E1-03
	Greece, Amvrakikos,	01.07.1993	C. d'Udekem d'Acoz	R553 - 3
	Spain, Almería, Isla Alborán	10.05.2004	P. Abelló	E5-04
<i>Pilumnus villosissimus</i>	Corsica, Calvi Bay	15.05.1988	C. d'Udekem d'Acoz	E35-01
	Spain, Alicante, El Portet	25.05.2000	C.D. Schubart	E1-05
	Spain, Alicante, El Portet	09.04.2012	C.D. Schubart	R670-3
	Spain, Alicante, El Portet	15.04.2012	C.D. Schubart	R670-1
	Italy, Sardinia	09.06.2006	C.D. Schubart	R536-10
<i>Pilumnus inermis</i>	Italy, Sicily, Faro	26.04.2010	Carlo lo Presto	R675-12
	Cape Verde Islands, SW Branco	05.09.1986	CANCAP 7.156	E6-4
	Azores, W Pico	07.06.1981	Not C. d'Udekem d'Acoz	E6-1

Table 1. Specimens of *Pilumnus* used for DNA amplification of the mitochondrial cytochrome oxidase subunit 1.

Morphological identification and species assignment was carried out by ourselves or by Cédric d'Udekem d'Acoz based on the available keys [3,4,13]. Muscle tissue was extracted from a merus of one of the walking legs and DNA isolation was performed using a modified Puregene method from Gentra Systems. DNA pellets were re-suspended in 20 µl TE buffer and the concentration was estimated on agarose gels. From the corresponding dilutions of the genomic DNA solution, 1 µl was used for polymerase chain reactions. Two different primer combinations were used for amplifying mitochondrial DNA (mtDNA) of the 5' end of the cytochrome oxidase subunit 1 gene (Cox1). This same mtDNA region is currently widely applied for genetic characterization and identification of animals within the Barcode of Life project [19]. As forward primer COL6 (5'-TYTCHACAAAYCATAAAGAYATYGG-3') was consistently used. It was combined with COH6 (5'-TADACTTCDGGRTGDCCAAARAAYCA-3') amplifying the so-called Folmer region of 658 basepairs (not counting the primers) or with COH1b (5'-TGTATARGCRTCTGGRTARTC-3'), which renders an amplicon of 1276 basepairs (not counting the primers) and includes the Folmer as well as the so-called Palumbi region. These primers sequences have been published by Schubart [20], who showed that they fit the sequences of decapods with known complete mitochondrial genomes better than the universal and widely used Cox1 primers by Folmer *et al.* [21] and Palumbi *et al.* [22].

For PCR, a standard 25 µl reaction was prepared containing 2.5 µl of 10x buffer, 2.5 µl of 1.25 mmol L⁻¹ dNTPs, 0.5 µl of both primers (20 mmol L⁻¹), 1-2 µl of 25 mmol L⁻¹ MgCl₂, 1 µl of 0.5 U ml⁻¹ TAQ and 15-16 µl of double-distilled water in addition of 1 µl DNA. 40 cycles were applied at an annealing temperature of 48 to 50°C. PCR products were cleaned using QuickClean (Bioline) and sequenced with an ABI-PRISM 310 (Applied Biosystems) or outsourced for sequencing to LGC Genomics, Berlin. Sequences were proofread for possible errors made by the computerized analysis with ChromasLite (<http://technelysium.com.au/>). Sequences of one representative sequence for all haplogroups were submitted to the European Nucleotide Archive, directly linked to GenBank under accession numbers (HG328357-HG328362). Due to the lack of indels, the corrected sequences were assembled manually using Bioedit [23] and converted into a nexus file. This was used to calculate a phylogenetic tree with Bayesian inference algorithms implemented in the software MrBayes v2.1.2 [24]. Previously, the best fitting substitution model of the dataset was established with the software MrModeltest v2.3 [25]. As outgroup we

selected a GenBank sequence from the western Atlantic species *Pilumnus floridanus* (HM638057). MrBayes was run for 3 million generations and four chains, saving trees every 5,000 generations. A burnin of 1,250,000 generations was later discarded from the analyses. All sequences were so closely related that a statistical parsimony network was constructed using the algorithm outlined in Templeton *et al.* [26] and implemented in the TCS software package version 1.21 [27]. In order to connect all haplogroups of the *Pilumnus hirtellus* complex in a single network, it was necessary to reduce the parsimony criterion in TCS to 85%.

The possible role of hybridization was tested within one population from *Pilumnus aestuarii*. This species is common in the Gulf of Amvrakikos (western Greece, Ionian Sea). However, within this large marine embayment, also *P. spinifer* has been found and several individuals could not identified with certainty, because they showed intermediate characters between *P. spinifer* and the more common *P. aestuarii* (or *P. hirtellus* according to C. d'Udekem d'Acoz, personal communication). One possible explanation would be hybridization between these species. Therefore, a maximum of Cox1 sequences were obtained from specimens identified as *P. aestuarii* or showing mixed characters from this gulf. In case of hybridization, this population should present a mix of mtDNA sequences of both species, since this molecule is exclusively maternally inherited and can only be encountered as one of the two versions.

On the search for additional morphological characters that may allow distinction among the European species of *Pilumnus*, the gastric tract was extracted from 12 individuals of European *Pilumnus* corresponding to six putative species (Table 2), by carefully detaching the carapace from its posterior end. Under a dissecting microscope, surrounding tissue was removed from the urocardiac ossicle and the latter transferred to 70% ethanol. In an ultrasound shaker the ossicles were cleaned from small obstructing particles. Photo-documentation was carried out with a Canon EOS D60 camera system through a microscope with a 32-fold magnification.

3. Results

According to MrModeltest, the best suited substitution model for the data is the most complex GTR+I+G model. This was implemented in the Bayesian reconstruction of a phylogenetic tree run over 3 million generations. The corresponding phylogenetic relationships based on up to 631 basepairs (bp) of the barcoding region

Species	Genus	Carapace width (mm)	Carapace length (mm)	Collection details
<i>Pilumnus aestuarii</i>	M	27.83	18.79	Greece, Amvrakikos, Mihalitsi, 20.09.2003, C. D. Schubart
	W	22.12	15.04	Greece, Amvrakikos, Mihalitsi, 20.09.2003, C. D. Schubart
<i>Pilumnus villosissimus</i>	M	23.88	18.62	Spain, Cádiz, Cala Olivera, 31.03.2003, C. D. Schubart
	W	22.59	17.04	Spain, Cádiz, Cala Olivera, 31.03.2003, C. D. Schubart
<i>Pilumnus spinifer</i>	W	14.82	11.69	Croatia, Rovinj, 1999, SMF
	M	16.57	11.37	Croatia, Rovinj, 1999, SMF
<i>Pilumnus sp1</i>	M	13.48	10.02	Spain, Cádiz, Rota, 20.04.2004, C. D. Schubart, J. A. Cuesta
	W	15.48	11.81	Spain, Cádiz, Rota, 20.04.2004, C. D. Schubart, J. A. Cuesta
<i>Pilumnus hirtellus</i>	M	31.77	22.68	Greece, Lesbos, 17.07.1992, C. d'Udekem d' Acoz
	M	6.60	4.81	Spain, Cádiz, Rota, 20.04.2012, J. A. Cuesta
	W	10.80	8.86	Franca, Normandy, August 2012, C. D. Schubart
<i>Pilumnus inermis</i>	M	21.34	16.21	Meteor Expedition, SMF, 25.02.1970

Table 2. Specimens of *Pilumnus* used for the study of the gastric ossicles.

of Cox1 are shown as a star-shaped radiation tree (Figure 1), which is an adequate representation, when not all separation events are clearly dichotomous. The longest branch, and thus largest genetic distance, clearly corresponds to the American outgroup species *Pilumnus floridanus*. The first species to split off from this outgroup lineage is *P. inermis*. This was to be expected, because it is the only European species clearly differing in preferred water depth and morphology. The rest of the sequences cluster closely together as a monophylum (posterior probability (pp) of 0.97) and correspond to all the specimens from the so-called *P. hirtellus* complex. Despite having relatively short branches, these branches lead to clearly separable groups of haplotypes (haplogroups) that correspond to the above listed and previously identified species *Pilumnus aestuarii*, *P. hirtellus*, *P. spinifer*, *P. villosissimus*, and the putative species *Pilumnus* sp. 1 [see 13,14]. The branch connecting the ingroup (*P. hirtellus* complex) with *P. inermis* and the outgroup species splits off from the basis of the largest haplogroup, which is the one of *P. aestuarii* (including animals from Morocco, Spain, Italy, Greece and Bulgaria). Next closest to the basis of the ingroup is *P. spinifer* (monophyletic with pp=1.0), before *P. hirtellus* (pp=0.98) branches off, whereas *P. villosissimus* and *P. sp 1* share a common branch (pp=1.0), before also being cleanly segregated into their respective haplogroups (*P. villosissimus* (pp=0.95) and *P. sp 1* (pp=1.0)).

The network analysis does not require inclusion of an outgroup taxon and focuses on relative distances between haplotypes and evolutionary units [26]. It requires sequences of the same length and without

ambiguities. Therefore it became necessary to trim the Cox1 alignment to a length of 554 bp, which all lie within the barcoding Folmer-region. The corresponding network confirms the five mitochondrial haplogroups within the *P. hirtellus* complex (see Figure 2). The haplogroups correspond to the here referred species *Pilumnus hirtellus*, *P. villosissimus*, *P. spinifer*, *P. aestuarii*, and *Pilumnus* sp. 1 [see 13,14]. All haplogroups are separated by at least 14 mutational steps from each other. This least difference can be found between *P. villosissimus* and *P. sp. 1*, whereas within these two species, the maximal intraspecific difference encountered was seven mutational steps, despite vast geographic differences and inclusion of animals from the Atlantic as well as from the Mediterranean Sea. Within haplogroups, there is thus no clear indication of geographic variation, suggesting high levels of gene flow within the presumed species. The most distant haplogroups are the ones of *Pilumnus hirtellus* and *P. sp. 1* that differ by at least 55 mutational steps, which corresponds to roughly 10% genetic divergence.

Sequencing of 14 individuals of *Pilumnus aestuarii* from the Gulf of Amvrakikos did not reveal any indication of hybridization. All animals were characterized by haplotypes that clearly belong within the *P. aestuarii* haplogroup. In contrast, the single individual identified as *P. spinifer* from the Gulf of Amvrakikos (by C. d'Udekem d'Acoz and ourselves) is correctly placed in the *P. spinifer* haplogroup with two conspecific representatives (three in the phylogenetic tree) (Figures 1-2). This confirms our initial morphological identifications. Even if this result is not sufficient to exclude the possibility of hybridization taking place, at least it makes it less likely.

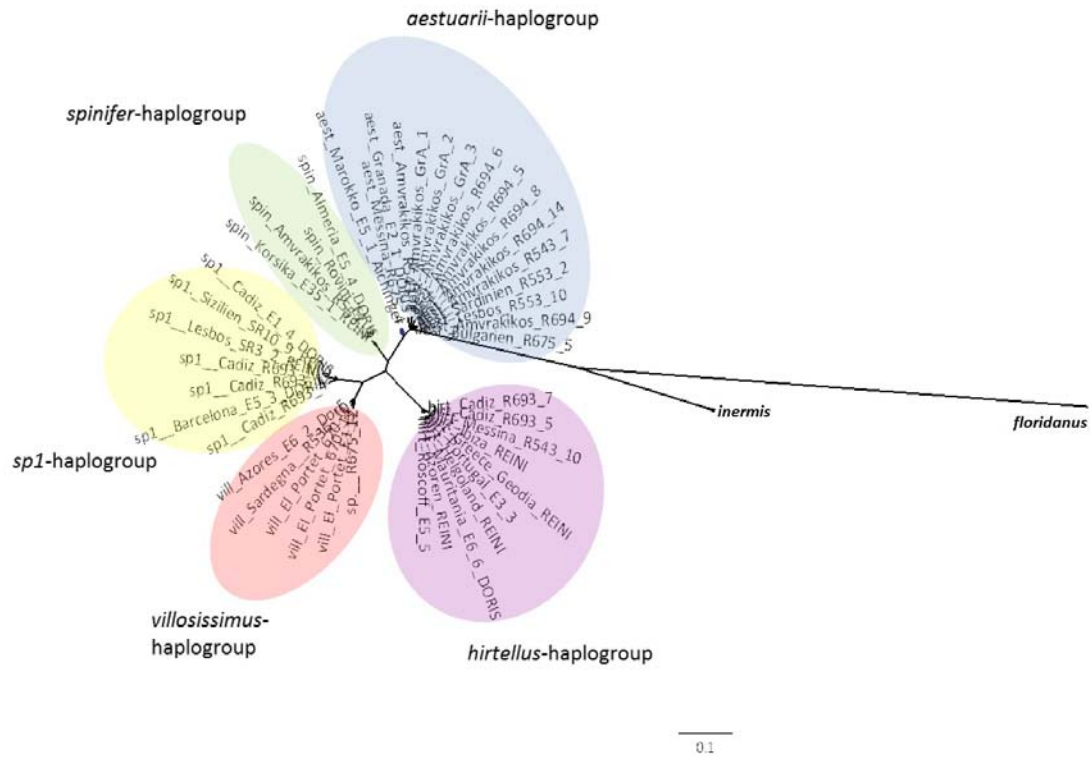


Figure 1. Radial MrBayes-tree of European representatives of the genus *Pilumnus*, GTR+I+G evolution model, 3 million generations (burnin = 1,250,000), 4 Markov Chains, 45 sequences with up to 631 basepairs mtDNA of the Folmer-region of the Cox1 gene.

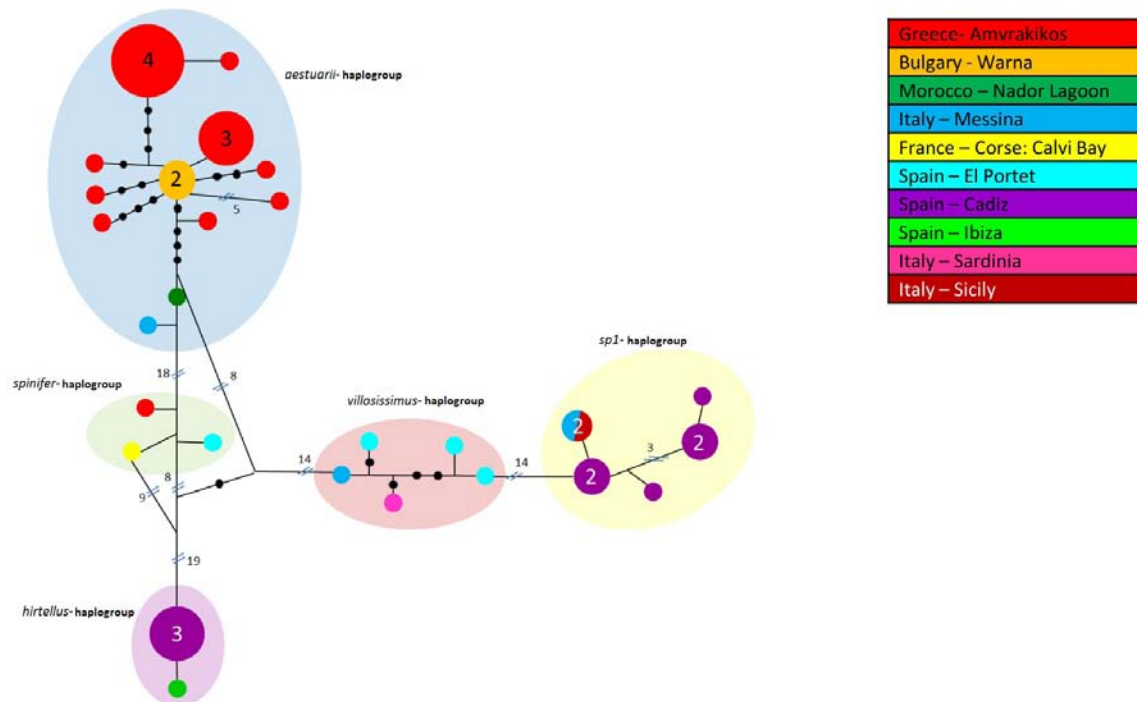


Figure 2. Maximum parsimony haplotype network of European representatives of the *Pilumnus hirtellus* complex constructed with TCS 1.21 based on 554 basepairs of the mitochondrial Cox1 gene.

The morphological comparisons of the gastric ossicles of all European species focused on the shape of the urocardiac ossicle and its dorso-median tooth, as well as on the mesocardiac ossicle. The photographs taken through a dissecting microscope reveal no noticeable differences among all the presumed species of *Pilumnus* from Europe (Figure 3). The dorso-median tooth (“a” in Figure 3A) has a cubic shape with rounded corners (arrows in Figure 3A) and has probably a grinding function according to its molar shape. It is located at the posterior end of the urocardiac ossicles, which in turn is characterized by a pair of anteriorly directed pointed teeth (“b” in Figure 3A). Therefore, the morphology of the gastric ossicles does not seem to provide new characters for the distinction of the closely related species.

4. Discussion

European crabs of the genus *Pilumnus* are known to constitute a relative complex taxonomic system, which gives room to very different interpretations as for the number and validity of species [4]. This apparent difficulty from a morphologist’s and taxonomist’s point of view may represent a good starting point for evolutionary biologists, as it provides an interesting model system to investigate the recent or ongoing differentiation of evolutionary units. The aim of this study is thus not to propose a new taxonomic system. This should be done sometime in the future by gathering all available information and redefining species. Instead, in this study we have uncritically adopted all available names of described and undescribed species and treat them as evolutionary units, or lineages, that we compare in terms of their internal morphology and mitochondrial DNA. The recognition of relatively young evolutionary units may possibly allow a better perception and more accurate description of ongoing processes of morphological, genetic, and ecological differentiation.

The most important result of the current study is to recognize six evolutionary units among the European representatives of *Pilumnus*. The most divergent is the species *Pilumnus inermis*, which is easily distinguishable by its morphology. Five other evolutionary units are much closer related according to genetic distances. Nevertheless, they are clearly separable in terms of mtDNA (monophyletic clades) at a level which is not influenced by their geographic origin (see Figure 2). These five lineages are here referred to as the *Pilumnus hirtellus* complex, since *P. hirtellus* is the oldest available name from the involved taxa. It would be premature to decide, whether these lineages are distinct species

or not, because of the apparent lack of characters (*P. aestuarii*), presence of morphologically intermediate forms (C. d’Udekem d’Acoz, personal information), and the uncertainty, whether hybridization occurs. In the present study, we could not demonstrate the occurrence of hybridization, but it shall be further investigated in future studies with ITS sequences and AFLPs (preliminary results in Rubner [18] and Aichinger [28]). The fact that we could not find distinguishing characters in the cardiac ossicles of the involved species, confirms that they are relatively closely related and difficult to separate morphologically. Cardiac ossicles have been used in other studies to separate closely related taxa [29] and to reconstruct crab phylogenies [30].

Also Oliveira-Biener *et al.* [2] found six evolutionary units with European representatives of *Pilumnus*, which they termed clades I – VI, by comparing sequences of a different region of the same mitochondrial gene. However, as these authors adhere closely to the newest taxonomic revision by Mavidis *et al.* [4] and their morphological classification was done according to the corresponding keys, they refrain from naming the apparent underlying diversity. Instead they report that their “*Pilumnus hirtellus*” appears to be polyphyletic, occurring in three different clades and interpret this as cryptic lineages or species (p. 190).

In our analysis we used the barcoding region of the mitochondrial cytochrome oxidase subunit 1 gene and all six taxa proposed for European species of *Pilumnus* throughout the past 50 years. Depending on the authors (and the taxa) dealing with genetic species delimitation [31], distances at the level of 2 or 3 % in the Cox1 gene are necessary to recognize distinct species. According to these values, all our evolutionary units could be considered species, because they have a minimum of 14 consistent differences out of 554 basepairs, corresponding to 2.53%, separating *P. villosissimus* from *P. sp. 1*. All of the other lineages are separated by higher values of 3.25% up to 9.93% (largest between *P. hirtellus* and *P. sp. 1*). However, there is not a clean barcoding gap between intraspecific and interspecific differentiation, because haplotypes within the *P. aestuarii* haplogroup differ by as many as 15 mutational steps, which expressed in percent (2.73%) overlaps with the above mentioned minimal distance between *P. villosissimus* and *P. sp. 1*. Strict barcoding rules would thus not allow delimiting and identifying the number of species involved.

The mechanisms and origin of the documented recent genetic isolation of five genetic lineages within the European *Pilumnus hirtellus* complex remain unclear. There is definitely not enough information available concerning the ecology of the respective

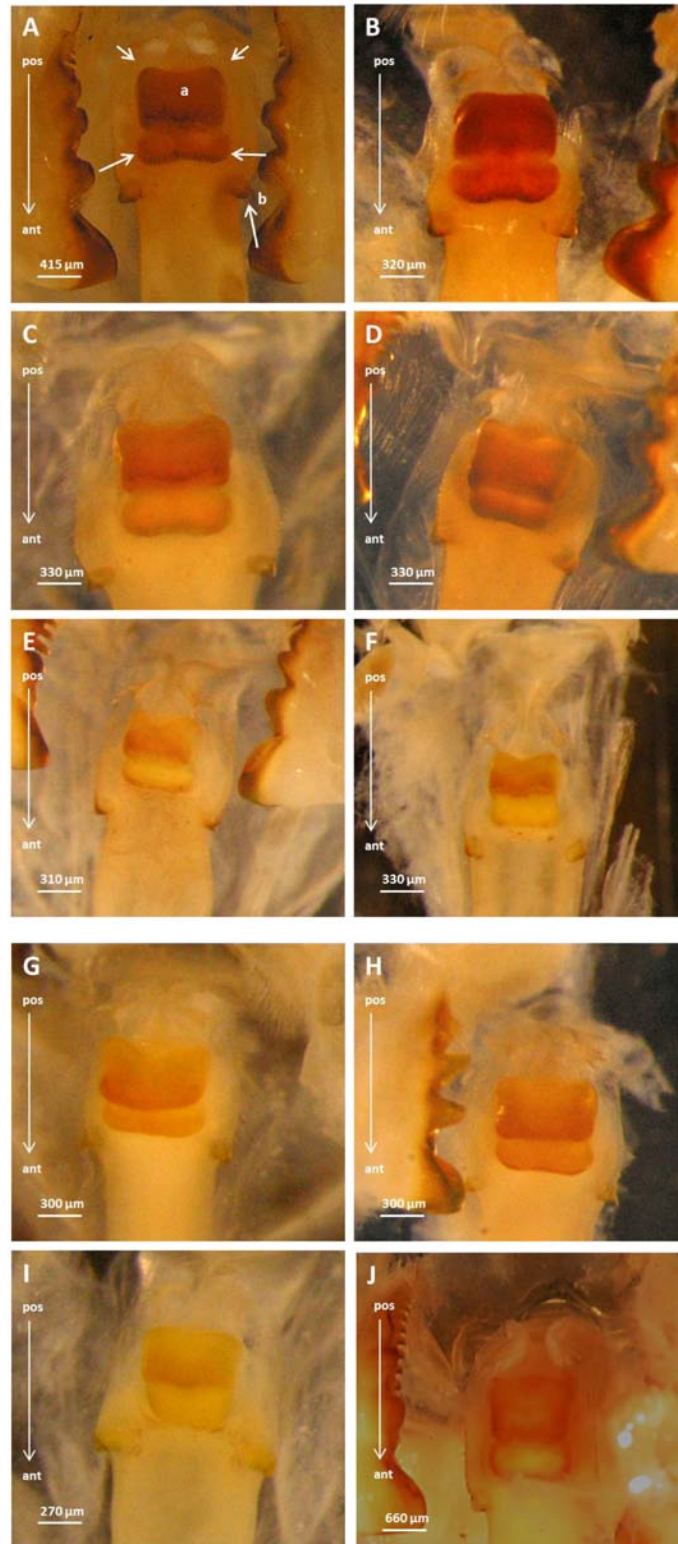


Figure 3. Urocardiac region of the gastric ossicles of six European representatives of the genus *Pilumnus*. A - B. *P. aestuarii*, C - D. *P. villosissimus*, E - F. *P. spinifer*, G - H. *P. sp 1.*, I. *P. inermis*, J. *P. hirtellus*; ant: anterior, pos: posterior, a: dorso-median tooth, b: small anterior projection, VII: urocardiac ossicle, arrows: rounded borders of dorso-median tooth or structures described in text.

evolutionary units. This is not only true for the European taxa, but for all representatives of the genus, which has a worldwide distribution with far more than one hundred species. We thus consider these crabs as good model organisms, having a great potential to study speciation and radiations in the coastal marine environment.

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