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Contrasting morphological and DNA barcode-suggested species boundaries among shallow-water amphipod fauna from the southern European Atlantic coast

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Abstract

In this study we compared DNA barcode-suggested species boundaries with morphology-based species identifications in the amphipod fauna of the southern European Atlantic coast. DNA sequences of the cytochrome *c* oxidase subunit I barcode region (COI-5P) were generated for 43 morphospecies (178 specimens) collected along the Portuguese coast which, together with publicly available COI-5P sequences, produced a final dataset comprising 68 morphospecies and 295 sequences. Seventy-five BINs (Barcode Index Numbers) were assigned to these morphospecies, of which 48 were concordant (i.e., 1 BIN=1 species), 8 were taxonomically discordant, and 19 were singletons. Twelve species had matching sequences (< 2% distance) with conspecifics from distant locations (e.g. North Sea). Seven

morphospecies were assigned to multiple, and highly divergent, BINs, including specimens of *Corophium multisetosum* (18% divergence) and *Dexamine spiniventris* (16% divergence), which originated from sampling locations on the west coast of Portugal (only about 36 km to 250 km apart, respectively). We also found deep divergence (4-22%) among specimens of seven species from Portugal compared to those from the North Sea and Italy. The detection of evolutionarily meaningful divergence among populations of several amphipod species from southern Europe reinforces the need for a comprehensive re-assessment of the diversity of this faunal group.

Key words: Amphipoda, cryptic species, cytochrome c oxidase subunit I, DNA barcode reference library.

Introduction

The growing application of standardized DNA barcodes (Hebert et al. 2003) to investigate taxonomic diversity has been exposing unforeseen layers of hidden diversity in numerous faunal groups, especially in less-studied taxa or geographic regions (Hajibabaei et al. 2006; Burns et al. 2008; Moura et al. 2008). The hidden diversity is typically exposed by comparing morphology-based and DNA barcode-suggested species boundaries (Costa and Carvalho 2010). In some cases, this has led to the detection of numerous putative cryptic species, prompting a new appreciation of the diversity of entire faunal groups (e.g. Gomez et al. 2007; Mutanen et al. 2013). DNA barcodes are therefore increasingly applied to probe and revise the taxonomic diversity of faunal groups, providing a quick screening method for highlighting mismatching morphological and molecular data, detection of putative cryptic species and taxonomic complexes, and also inaccurate or misleading identifications (e.g. Borges et al. 2012; Khaksar et al. 2015; Decaëns et al. 2016).

Amphipods are one of the most diverse and widely distributed groups of crustaceans, often constituting dominant, key elements of marine and estuarine/coastal communities, deep sea, and polar regions (Sousa et al. 2006; d'Udekem d'Acoz and Robert 2008; Lörz et al. 2009; Ilarri et al. 2012; Knox et al. 2012; Novais et al. 2015). The ecological relevance of amphipods in the management and conservation of aquatic systems is reflected by their extensive use as biological indicators and environmental test organisms (Costa et al. 2005; Löf et al. 2016) and their relevance in trophic interactions (Sousa et al.

2007, 2008; Ilarri et al. 2014). As with many other marine invertebrate taxa, knowledge of amphipod diversity is constrained by the taxonomic impediment (Coleman 2015). Specific features of amphipods—such as sexual dimorphism, ontogenic variations, and morphological uniformity among closely related species—further limit our ability to discriminate species based on morphological characters (e.g. Costa et al. 2004). By June 2015, the World Register of Marine Species (WoRMS; <http://www.marinespecies.org>) database listed 9628 accepted amphipod species, including marine, freshwater and terrestrial species, but there is no doubt that numerous species await formal description (Coleman 2015). The amphipod fauna of southern Atlantic Europe is particularly rich and unique, consisting of a mixture of species from adjacent biogeographic regions such as the Mediterranean Sea and the warm and cold NE Atlantic. Being a biogeographic crossroad where many amphipod (and other) species have their northern or southern limits, this region is particularly relevant for monitoring alterations in distribution driven by climate change or by the potential introduction of alien species due to intense offshore maritime traffic and port activities (Chainho et al. 2015).

Despite the growing number of articles reporting cryptic amphipod species, detected through the examination of molecular data, a DNA barcode reference library is required as a starting point for making comparisons with morphology-based identifications (Witt et al. 2006; Costa et al. 2009; Bradford et al. 2010; Knox et al. 2011; Richards et al. 2012). In fact, comprehensive and carefully curated reference libraries of DNA barcodes, where each established species is linked to an array of diagnostic DNA sequences, can overcome many of the challenges posed by routine morphology-based taxonomic identifications (Costa and Antunes, 2012). Therefore, in this study we aimed to review and probe the taxonomic diversity of the shallow-water amphipod fauna of the southern European Atlantic coast, by comparing morphologically identified specimens with molecular operational taxonomic units (MOTUs) derived from DNA barcodes of these species. The DNA barcodes here generated were compared with publicly available sequences of the same species from other regions to determine: i) close matches between fairly distant populations, ii) cases of deep divergence between distant populations, and iii) cases of deep divergence between relatively proximate populations.

Materials and methods

Specimens were collected along the coast and estuaries of continental Portugal (Fig. 1) from sediment samples and macroalgae. A corer sampler (110 mm diameter, 495 mm height) was used to

collect sediment samples, which were then immediately sieved through a 0.5 mm screen. Samples were transported refrigerated to the laboratory, where specimens were individually separated and stored in absolute ethanol. The individuals were photographed and morphologically identified to the lowest possible taxonomic level using a stereomicroscope and identification keys provided in Chevreux and Fage (1925), Hayward and Ryland (1995), Lincoln (1997), and Ruffo (1998). The online databases WoRMS and Integrated Taxonomic Information System (ITIS; www.itis.gov) were used as a cross-reference for currently accepted species' names (accessed October 2015).

DNA extraction, amplification, and sequencing were carried out in three institutions: CBMA – Centre of Molecular and Environmental Biology, University of Minho (Portugal); MARE – Marine and Environmental Science Centre, Lisbon New University (Portugal); and BIO – Biodiversity Institute of Ontario, University of Guelph (Canada). At CBMA and MARE, a small piece of tissue (approximately 1 mm³) was used to extract the DNA (elution volume = 50 µL) from each individual using the E.Z.N.A Mollusc DNA Kit (Omega Biotek), following the manufacturer's instructions. An iCycler (Bio-Rad) thermal cycler was used to amplify the cytochrome *c* oxidase subunit I barcode region (COI-5P) employing three different primer pairs depending on amplification success. Each PCR reaction consisted of 2.5 µL 10× *Taq* polymerase buffer, 2.5 µL of 1.5 mM MgCl₂, 0.5 µL of 0.2 mM dNTP mixture, 0.2 µL of 5U/µL of DNA *Taq* polymerase plus 10 µM of each primer (1.5 µL for LoboF1/LoboR1; 0.5 µL for LCO1490/HCO2198 and CrustDF1/CrustDR1), 4 µL of DNA template and filled up with sterile milliQ-grade water to make up a total volume of 25 µL. The primer sequences, combinations, and PCR thermal conditions are listed in Table 1. PCR products were separated by electrophoresis in 1.5% agarose gel in TAE buffer and subsequently stained with Greensafe (NZYTech, Lisbon, Portugal) for visualization. Successfully amplified products were then cleaned using a mix of 10 U exonuclease I and 1 U of shrimp alkaline phosphatase and sequenced bidirectionally using the BigDye Terminator 3 kit, and run on an ABI 3730XL DNA analyser (all from Applied Biosystems™). At BIO, a small piece of tissue was initially lysed in 45 µL cetyltrimethylammonium bromide (CTAB) and 5 µL proteinase K. The samples were then incubated at 56 °C for 12–18 h, followed by DNA extraction with a 3-µm glass fibre plate and re-suspended in 50 µL of ddH₂O. PCR reactions were carried out in a total volume of 12.5 µL, containing 6.25 µL of 10% trehalose, 2 µL of ddH₂O, 1.25 µL of 10 X PCR buffer, 0.625 µL of MgCl₂ (50 mM), 0.125 µL of

each primer (10 M), 0.0625 μ L dNTPs (10 mM), 0.06 μ L platinum *Taq* polymerase, and 2 μ L of DNA template (10–50 ng).

Sequence trace files were carefully checked for the presence of ambiguous base calls and edited to remove poor quality terminal sections. After multiple alignment using Clustal W (Thompson et al. 1994) implemented in MEGA v 6.0 (Tamura et al. 2013), translation into amino acids was performed to check for the eventual presence of indels, stop codons, or unusual amino acid sequence patterns, and detect possible nuclear mitochondrial pseudogenes (numts) (see Song et al. 2008). We used GenBank BLASTn search (Altschul et al. 1990) and BOLD Identification System tool (BOLD-IDS) using all Barcode Records (Ratnasingham and Hebert 2007) to search for matching sequences in these public data repositories. When publicly available, sequences belonging to the same species (with and without sequence similarity) or genus were added to our alignment for subsequent analyses. In order to visualize the clustering pattern for all aligned sequences, a Maximum Likelihood (ML) tree was built in MEGA v 6.0, using the best-fit model TN93 (Tamura and Nei 1993) indicated by the same software according to the BIC (Bayesian Information Criteria) score. To allow a broad comparison of our results with published DNA barcoding data, we used the Kimura-2-parameter model (K2P, Kimura 1980) to determine intra-specific, congeneric, and confamilial distances using pairwise deletion of missing data.

To assign sequences to molecular operational taxonomic units (MOTUs), we used the Barcode Index Numbers (BINs, Ratnasingham and Hebert 2013) implemented in BOLD. The BIN algorithm assigns all compliant sequences lodged in BOLD to a BIN (i.e. MOTU) with a unique identifier and webpage with information on the BIN intra-specific distances and nearest-neighbour BIN. This provides a straightforward way to contrast morphology-based identifications with molecular-based boundaries among specimens. We examined BIN composition in two ways: using the BIN discordance report tool, which flags BINs comprised of specimens from more than one taxon, and checking in the number of BINs for each morpho-species in our dataset.

Images, collection, and sequence metadata of all new specimens here examined are publicly available in the project titled “Amphipoda of Portugal [AMPPT]” in BOLD and the GenBank accession numbers for sequences are included between KX223965 and KX224099. The global dataset examined, including also the sequences mined from public repositories, was compiled in the data set ID DS-BIBLIO also available in BOLD (dx.doi.org/10.5883/DS-BIBLIO).

Results

From the specimens collected along the coast and estuaries of continental Portugal, 178 were processed and assigned to 42 morphospecies (see Table 2) in 21 families. Publicly available COI-5P sequences of the same species or genus were added, producing a final BOLD dataset comprising 68 morphospecies and 295 sequences (see data set ID DS-BIBLIO in BOLDSystems). The number of specimens per species varied from 1 (several species) to 17 (*Talitrus saltator* (Montagu, 1808)). The most represented families were Caprellidae, Gammaridae, and Ischyroceridae, the first two with five species and the latter with four. Forty-two percent of the COI-5P sequences were generated using the primer pair CrustDF1/CrustDR1, 36% using the primer pair LCO1490/ HCO2198, and 21% using the primer pair LoboF1/LoboR1.

No indications of the presence of pseudogenes were detected after detailed inspection of the sequences. The chromatograms of the sequences were mostly of high quality. Stop codons, indels and unusual substitution patterns in more conserved regions of the nucleotide or amino acid sequences were not observed.

Intra-specific, congeneric, and confamilial K2P distances are compiled in Table 3. The mean intra-specific distance was 0.6%, and the mean congeneric and confamilial distances were similar, 24.9% and 25.0%, respectively. The number of taxa used to calculate the intra-specific distances was 26, but only 6 and 5 were used to calculate the congeneric and confamilial analysis, respectively. The species with high genetic divergence values were excluded from the intraspecific analysis (see Table 3). The genera used for the inter-specific distances were *Ampelisca*, *Caprella*, *Corophium*, *Gammarus*, *Jassa*, and *Melita*, whilst the families used for the confamilial distances were Aoridae, Caprellidae, Gammaridae, Maeridae, and Melitidae. The global values were similar when the public sequences were added to the analysis.

The ML phenogram generated with all COI-5P sequences of amphipods used in this study (295 individuals belonging to 68 morphospecies) is shown in Fig. 2. Mostly, species, genera, and even families clustered according to their taxonomic hierarchy, although some exceptions were found. For instance, three species of *Ampelisca* (*A. diadema* (Costa, 1853), *A. ledoyeri* Bellan-Santini & Kaim-Malka, 1977, and *A. tenuicornis* Lilljeborg, 1855) grouped in the same clade with a null genetic

divergence. On the other hand, sequences of specimens of *Ampelisca diadema* also were distributed among three separate branches.

Deep intra-specific divergences were found among specimens of *Ampelisca diadema* (22%), *Corophium multisetosum* (18%), *Dexamine spiniventris* (16%), *Gammarella fucicola* (18%), *Jassa pusilla* (22%), *Phtisica marina* (4%), and *Talitrus saltator* (13%).

Seventy-five BINs were obtained for 295 records belonging to 68 morphospecies, of which only 48 were concordant, 8 were discordant, and 19 were singletons. Conflicting BINs were identified to the species level in *Ampelisca diadema*, *Ampelisca ledoyeri*, *Ampelisca* sp., *Ampelisca tenuicornis*, *Ampithoe helleri* Karaman, 1975, *Jassa* sp., *Melita formosa* Murdoch, 1866, and *Urothoe pulchella*.

Discussion

Global analyses

The global pattern of sequence divergence and clustering obtained in this study confirm the good species discrimination ability observed in previous DNA barcoding studies with these organisms (e.g. Costa et al. 2007, 2009; Radulovici et al. 2009; Raupach et al. 2015). The average within- and between-species distances (0.6% and 24.9%, respectively) were also within the range reported for amphipods and other marine invertebrates (Costa et al. 2007, 2009; Radulovici et al. 2009; Raupach et al. 2015; Borges et al. 2016; Lobo et al. 2016), and resulted, as expected, in a clear “barcoding gap” (see Meyer and Paulay 2005) for nearly all species in the dataset (56 of 68 species; Fig. 3). The ML phenogram with the full dataset (i.e. including published sequences from elsewhere) is also characterized by long branches, clearly separating the species clusters, which in turn display low within-cluster variation.

The inclusion of publicly available sequences, obtained from other geographic regions, in our analyses disclosed interesting patterns of concordance and discordance between species identifications and MOTUs (in this case BINs), as well as between geographic and genetic distances between populations. We may divide these patterns in 3 categories: i) long range matches between geographically distant populations; ii) high genetic distances between distant populations; and iii) deep genetic discontinuities among proximate populations.

DNA barcode matches between distant populations

Among species with sequences from geographically distant populations (n=19), the majority (n=12) displayed closely matching clusters (< 2% maximum within-cluster distance). This includes mostly species with populations sampled from Portugal and the North Sea, therefore at least 2000 km apart. The most extreme case was *Apohyale prevostii* that includes specimens from Canada, the North Sea, and Portugal, with an average intra-specific distance of 0.22%. Because amphipods are direct developers, it may be assumed that their populations are more prone to show genetic structure associated with geographic distance (France et al. 1992) than other marine invertebrates, which have either lecithotrophic or planktotrophic larvae with potential to increase their dispersal capacity. The results shown here for a diverse set of amphipod species (21 families) confirm that the lack of larval dispersive stage does not promote necessarily the genetic differentiation among populations, and suggests the occurrence of other efficient dispersal mechanisms in these organisms. Indeed, long-range matches have been observed among fairly distant populations (e.g. amphi-Atlantic distributions) of various other amphipod species such as populations of *Gammarus oceanicus* Segerstråle, 1947 from Hudson Bay, in the Canadian Arctic, compared to those of Iceland, the North Sea, Svalbard, and the Baltic Sea (Costa et al. 2009). In this particular case, the Canadian Arctic populations are assumed to descend from a relatively recent expansion of the NE Atlantic populations (Costa et al. 2009, Krebs et al. 2011). The observed genetic proximity among populations from Portugal and other distant regions may be also explained by relatively recent patterns of expansion, either across East-West or along South-North Atlantic Europe. While the current dataset is perhaps too restricted for conclusive inferences, the continued growth of the reference library for amphipods archived in BOLD, with broader taxonomic and geographic coverage, will hopefully enable probing such broad multi-species phylogeographic patterns in this group.

High divergence among geographically distant populations

Comparatively high genetic divergences between geographically distant populations were recorded in some species (n=6, or 33% of the species with specimens from distant populations). Four of those cases were found between populations of Portugal and the North Sea, with mean divergences between these populations ranging from 3.7 % (*Phthisica marina*) to 22.3 % (*Jassa pusilla*). *P. marina*

appears to have a very wide distribution (WoRMS, unreviewed) extending from the Atlantic and Mediterranean coasts to South Africa and Mozambique. Guerra-García et al. (2010) argue that this amphipod species can be identified based solely on the presence of developed pereonites 3 and 4, a very distinctive character that makes the morphological recognition of this species relatively simple. However, according to our results, detailed morphological and molecular studies are necessary to assess whether these widespread records belong to only one or more species. *Gammarella fucicola* populations of Portugal and North Sea also display high and uncommon intra-specific distances (18%). Because all these organisms were identified separately in two different studies, a morphological re-examination of the specimens is required to discard possible misidentifications. If morphological identifications are confirmed, 16% of the southern Atlantic European species here analysed will need to be re-assessed. Nevertheless, increasing the number of genera per family in the DNA barcode library can help to discard congeneric misidentifications. Except for *Jassa pusilla* and *Ampelisca diadema*, the remaining four species are unique representatives of their genus in this dataset. For example, DNA barcodes from all specimens of *Jassa pusilla* are clearly distinct from any other of the four *Jassa* species in the NJ phenogram. However, the specimens from Portugal diverge as much as 22.3% from their North Sea conspecifics, suggesting the existence of two taxonomic units within the *Jassa pusilla* morphotype.

The group consisting of the remaining six specimens - *Ampelisca diadema* - is perhaps the most complex case in our dataset. The genus *Ampelisca* is well represented by six morphospecies but displays as many as 11 MOTUs (intra-specific divergence range 0-2.5%). One of the MOTUs closely groups three distinct morphospecies (*A. diadema*, *A. ledoyeri*, and *A. tenuicornis*) and includes only specimens collected in Italy (Marusso et al. 2013). We suspect that some of the specimens in this BIN may have been misidentified, and that they are in fact *A. ledoyeri*, since the morphospecies *A. tenuicornis* and *A. diadema* occur solely in other distant BINs; however, due to the low number of specimens analysed no definitive conclusions can be drawn. Hence, if we exclude this BIN, *A. diadema* occurs in three other BINs: one from the North Sea, one from Portugal, and one from Italy. Considering the high genetic distances between these BINs (range 17.56-25.3%), the fact that they do not match with any of the five other *Ampelisca* morphospecies, and that they correspond to geographically distant populations, this also suggests the existence of hidden diversity within the *A.*

diadema morphotype. Once again, and due to the high number of species in this genus, morphological identification also requires a good level of expertise.

The beach hopper *Talitrus saltator* displays an interesting and unique pattern of divergence within this dataset. Deep genetic divergences between relatively close populations within the Mediterranean (e.g. Tyrrhenian and Adriatic Sea) have been reported early on, based on allozymes (De Matthaeis et al. 1995) and later supported by COI sequence data (Ketmaier et al. 2010, Wildish et al. 2012). Additionally, a population from the Baltic Sea (Poland) was found to diverge deeply from the Mediterranean ones (Ketmaier et al. 2005, 2010). Our results reinforce this pattern, indicating that the Atlantic populations from the North Sea and Portugal form a unique cohesive cluster (0.45% divergence), but are deeply divergent from both Mediterranean populations (14.0% from Tyrrhenian and 11.2% from Adriatic). Curiously, specimens from the Adriatic or Tyrrhenian Seas appear to be genetically closer to the Atlantic populations than to one another. Independently of the evolutionary history that led to this pattern, the high degree of divergence between these spatially segregated populations warrants their consideration as probable independent taxonomic units. *T. saltator* is a widespread and ecologically relevant species that is common in sandy beaches across Europe and is also an important environmental indicator (Rainbow et al. 1998; Nardi et al. 2003; Fanini et al. 2005). Therefore, a re-appreciation of its taxonomic (both morphological and genetic) diversity across Europe is important.

Sharp genetic discontinuities among proximate populations

Within the dataset of specimens from Portugal, we have detected two cases of deep divergence among relatively proximate populations (*Corophium multisetosum* and *Dexamine spiniventris*). In these cases we have found two BINs for each morphospecies, with approximate geographic distances among specimens in each BIN pair ranging from 36 km in *D. spiniventris* to 250 km in *C. multisetosum*. The deep genetic divergences among BIN pairs (18% *C. multisetosum* and 16% *D. spiniventris*) are well beyond the reported intra-specific distances of COI-5P in crustaceans, and in amphipods in particular (Costa et al. 2009; Radulovici et al. 2009; Raupach et al. 2015), and strongly suggest that they belong to separate taxonomic units. Due to the availability of numerous specimens for examination, we were able to confirm that all specimens were correctly assigned to the *C. multisetosum* morphotype. This species is a dominant macrobenthic species and an important contributor to the

secondary benthic production in several coastal and estuarine ecosystems in Portugal, where high densities have been recorded: 200×10^3 individuals m^{-2} in “Ria de Aveiro” (Cunha et al. 2000), 656 ind. m^{-2} in Lima estuary (Sousa et al. 2006), and 551 ind. m^{-2} in Minho estuary (Sousa et al. 2008). *C. multisetosum* has also been used in ecotoxicological testing (e.g. Ré et al. 2009), which, together with its ecological role (e.g. important prey for many species), calls for a deeper investigation of the cryptic diversity here detected. Detailed morphological examination was not possible for *D. spiniventris* due to the low number of specimens and populations investigated. Within the genus *Dexamine*, the available data also suggest the existence of hidden diversity within *D. spinosa*, namely between France and North Sea specimens, which have 24% genetic divergence. Curiously, some *D. spiniventris* and *D. spinosa* BINs are nearest neighbours to each other instead of their conspecifics BINs. Both species require deeper examination of their taxonomic status.

Concluding remarks

In this study we have found a high degree of conformity in DNA barcodes of amphipod species from the Southern European Atlantic coast with those obtained independently from distant populations, particularly those from the North Sea (Raupach et al. 2015; 11 BIN matches). However, we have also detected a sizeable number of morphospecies ($n=7$) displaying deep genetic divergences between populations from Portugal and elsewhere, or even among Portuguese populations. These patterns of divergence were exposed by the examination of a relatively low number of specimens and populations. More definitive conclusions about the species status and levels of hidden diversity requires not only a much wider spatial sampling, but also the focused collection of further morphological, molecular, and ecological evidence from the target species. Still, most of the levels of COI-5P intra-specific divergence reported here are much higher than what has been recorded in numerous studies with crustacean (including amphipod) species (e.g. Raupach et al. 2015), and, at the very least, they indicate ecologically and evolutionary significant patterns of genetic divergence between populations. If future data and studies confirm the divergent lineages as independent taxonomic units, the comparison here performed between Portuguese specimens and other publically available data would have contributed toward the early detection of up to seven new taxonomic units among shallow water marine amphipod species of the Southern European Atlantic, about 16% of the species examined from Portugal.

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Table 1. Primer pairs and thermal cycling conditions used for amplification of COI-5P from amphipods in this study

Reference	Primer	Direction (5´ - 3´)	PCR thermal cycling conditions
Folmer et al. (1994)	LCO1490	(F) GGTCAACAAATCATAAAGATATTGG	1) 94°C/60s; 2) 5 cycles: 94°C/30s, 45°C/90s, 72°C/60s; 3) 35 cycles: 94°C/30s, 51°C/90s, 72°C/60s; 4) 72°C/5 min
	HCO2198	(R) TAAACTTCAGGGTGACCAAAAAATCA	
Lobo et al. (2013)	LoboF1	(F) KBTCHACAAAYCAYAARGAYATHGG	1) 94°C/5min; 2) 5 cycles: 94°C/30s, 45°C/90s, 72°C/60s; 3) 45 cycles: 94°C/30s, 54°C/90s, 72°C/60s; 4) 72°C/5 min
	LoboR1	(R) TAAACYTCWGGRTGWCCRAARAAYCA	
Steinke et al. (2016)	CrustDF1	(F) GGTCWACAAAYCATAAAGAYATTGG	1) 94°C/60s; 2) 5 cycles: 94°C/30s, 45°C/90s, 72°C/60s; 3) 35 cycles: 94°C/30s, 50–56°C/90s, 72°C/60s; 4) 72 °C (5 min)
	CrustDR1	(R) TAAACYTCAGGRTGACCRAARAAYCA	

Table 2. Intra-specific distances and Barcode Index Number (BIN) assignments of amphipod species collected from the Portuguese coast and estuaries.

Species	N	Mean intra-specific distance between BINs (%)	BIN (Country)
Species with high intra-specific distance			
<i>Ampelisca diadema</i> (Costa, 1853)	6	21.93	BOLD:AAZ2175 (Portugal); BOLD:ABW2163 (North Sea); BOLD:ACH8190 (Italy); BOLD:ACH8191 (Italy)
<i>Corophium multisetosum</i> Stock, 1952	13	17.60	BOLD:AAZ5635 (Portugal); BOLD:ACG5268 (Portugal)
<i>Dexamine spiniventris</i> (Costa, 1853)	4	15.79	BOLD:AAO1909 (Portugal); BOLD:AAZ9698 (Portugal)
<i>Gammarella fucicola</i> (Leach, 1814)	12	17.97	BOLD:AAJ2617 (Portugal); BOLD:ACP7515 (North Sea)
<i>Jassa pusilla</i> (Sars, 1894)	4	22.32	BOLD:AAZ5420 (Portugal); BOLD:ACP9511 (North Sea)
<i>Phthisica marina</i> Slabber, 1769	5	3.71	BOLD:AAF6487 (Portugal); BOLD:ABW2045 (North Sea)
<i>Talitrus saltator</i> (Montagu, 1808)	17	13.05	BOLD:AAC4712 (Portugal; North Sea) *

Species with low intra-specific distance		Mean intra-specific distance (%)	
<i>Abludomelita obtusata</i> (Montagu, 1813)	10	0.03	BOLD:AAJ4244 (Portugal)
<i>Ampelisca</i> sp.1	2	0.00	BOLD:ACO1992 (Portugal)
<i>Ampelisca</i> sp.2	2	0.62	BOLD:AAJ2286 (Portugal)
<i>Ampithoe helleri</i> Karaman, 1975	11	0.39	BOLD:AAZ3071 (Portugal)
<i>Aora typica</i> Krøyer, 1845	4	0.18	BOLD:AAV5438 (Portugal)
<i>Apherusa jurinei</i> Milne Edwards, 1830	8	0.92	BOLD:AAV5341 (Portugal; North Sea)
<i>Apohyale prevostii</i> (Milne Edwards, 1830)	7	0.19	BOLD:AAB5862 (Portugal; Canada; North Sea)
<i>Caprella acanthifera</i> Leach, 1814	3	0.21	BOLD:AAV5434 (Portugal)
<i>Caprella penantis</i> Leach, 1814	13	0.86	BOLD:ACA3998 (Portugal)
Corophiidae ni 3	4	0.00	BOLD:AAU1797 (Portugal; North Sea)
Corophiidae ni 4	4	0.24	BOLD:AAU1637 (Portugal, North Sea)
<i>Echinogammarus marinus</i> (Leach, 1815)	7	0.43	BOLD:AAC5398 (Portugal; North Sea)
<i>Elasmopus rapax</i> Costa, 1853	6	0.10	BOLD:AAZ3047 (Portugal)
<i>Gammarus chevreuxi</i> Sexton, 1913	5	1.31	BOLD:AAE6169 (Portugal; France)
<i>Gammarus crinicornis</i> Stock, 1966	5	1.10	BOLD:AAF5354 (Portugal; Belgium; North Sea)
<i>Gammarus insensibilis</i> Stock, 1966	4	0.31	BOLD:AAE6201 (Portugal)
<i>Gammarus locusta</i> (Linnaeus, 1758)	8	0.90	BOLD:AAC0402 (Portugal; North Sea)
<i>Haustorius arenarius</i> (Slabber, 1769)	6	0.05	BOLD:ACG9283 (Portugal; North Sea)
<i>Jassa falcata</i> (Montagu, 1808)	9	0.44	BOLD:AAU2334 (Portugal; North Sea)
<i>Jassa</i> sp.	5	1.73	BOLD:AAX8442 (Portugal; North Sea)
<i>Melita hergensis</i> Reid, 1939	7	0.34	BOLD:AAZ3082 (Portugal)
<i>Melita palmata</i> (Montagu, 1804)	15	0.82	BOLD:AAV5475 (Portugal; North Sea)
<i>Microdeutopus</i> sp.	6	1.99	BOLD:AAV5364 (Portugal); BOLD: ABZ5208 (Portugal)
<i>Parajassa pelagica</i> (Leach, 1814)	3	1.18	BOLD:AAV5675 (Portugal)
<i>Quadrimaera inaequipes</i> (A. Costa, 1851)	10	0.15	BOLD:AAV8382 (Portugal)

<i>Stenula</i> sp.	2	0.31	BOLD:AAI0077 (Portugal)
Singletons			
<i>Caprella danilevskii</i> Czerniavskii, 1868	1	-	BOLD:AAW8076 (Portugal)
Corophiidae ni 1	1	-	BOLD:ACO1403 (Portugal)
Corophiidae ni 2	1	-	BOLD:ACO1845 (Portugal)
<i>Gammaropsis</i> sp.	1	-	BOLD:AAW5931 (Portugal)
<i>Harpinia</i> sp.	1	-	BOLD:AAZ3024 (Portugal)
<i>Leucothoe incisa</i> (Robertson, 1892)	1	-	BOLD:ACO2198 (Portugal)
<i>Microdeutopus chelifer</i> (Bate, 1862)	1	-	BOLD:ACG4978 (Portugal)
<i>Parapleustes bicuspis</i> (Krøyer, 1838)	1	-	BOLD:AAX1074 (Portugal)
<i>Pseudoprotella phasma</i> (Montagu, 1804)	1	-	BOLD:AAL1889 (Portugal)
<i>Urothoe grimaldii</i> Chevreux, 1895	1	-	BOLD:AAZ2735 (Portugal)

N number of specimens per species; * lack of BINs of the specimens from Italy.

Table 3. Intra-specific, congeneric, and confamilial genetic distances (K2P) among amphipod taxa analyzed in this study.

		Taxa	Mean of the minimum distances (%)	Mean of the average distances (%)	Mean of the maximum distances (%)
Within-species*	This study	26	0.0	0.6	2.0
	This study plus public sequences	38	0.0	0.7	2.0
Within-genus	This study	6	19.6	24.9	30.5
	This study plus public sequences	14	16.5	25.0	37.2
Within-family	This study	5	21.0	25.2	29.6
	This study plus public sequences	8	21.0	26.4	31.4

**Ampelisca diadema*, *Corophium multisetosum*, *Dexamine spiniventris*, *Gammarella fucicola*, *Jassa pusilla*, *Phtisica marina* and *Talitrus saltator* were excluded from the intraspecific analysis due to the high divergence values.

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Figure captions

Figure 1. Map showing the collection sites of amphipods along the Portuguese continental coast and estuaries (black stars).

Figure 2. Maximum Likelihood tree generated from 295 COI-5P sequences assigned to 68 amphipod morphospecies. Node support based on 1000 bootstrap iterations (only values ≥ 90 are shown). Scale bar represents 0.1 substitutions per site. Colored dots indicate the species with deep divergences.

Figure 3. Scatterplot for 56 of 68 species in the dataset. The minimum distance to Nearest Neighbour (NN) is plotted against the maximum intra-specific distance.

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Atlantic
Ocean

Spain

Portugal

Lisbon

0 100 200 300 400 km

<https://mc06.manuscriptcentral.com/genome-pubs>

Scatterplot representing for nearly all species in the dataset (56 of 68 species) the minimum distance to Nearest Neighbour (NN) plotted against the maximum intra-specific distance.

312x236mm (72 x 72 DPI)