Multigene Molecular Systematics Confirm Species Status of Morally Convergent Pagurus Hermit Crabs

Joana Matzen da Silva¹,², Antonina dos Santos³, Marina R. Cunha², Filipe O. Costa⁴, Simon Creer¹, Gary R. Carvalho¹*

¹ Molecular Ecology and Fisheries Genetics Laboratory, School of Biological Sciences, Environment Centre for Wales, Bangor University, Bangor, Wales, United Kingdom, ² Departamento de Biologia, Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro, Aveiro, Portugal, ³ Instituto Nacional de Recursos Biológicos, L-IPIMAR, Lisboa, Portugal, ⁴ Departamento de Biologia, Centro de Biologia Molecular e Ambiental (CBMA), Universidade do Minho, Braga, Portugal

Abstract

Introduction: In spite of contemporary morphological taxonomy appraisals, apparent high morphological similarity raises uncertainty about the species status of certain Pagurus hermit crabs. This is exemplified between two European species, Pagurus excavatus (Herbst, 1791) and Pagurus alatus (Fabricius 1775), whose species status is still difficult to resolve using morphological criteria alone.

Methodology/Principal Findings: To address such ambiguities, we used combinations of Maximum Likelihood (ML) and Bayesian inference (BI) methods to delineate species boundaries of P. alatus and P. excavatus and formulate an intermediate Pagurus phylogenetic hypothesis, based upon single and concatenated mitochondrial (cytochrome oxidase I [COI]) and nuclear (16S and 28S ribosomal RNA) gene partitions. The molecular data supported the species status of P. excavatus and P. alatus and also clearly resolved two divergent clades within hermit crabs from the Northeast Atlantic Ocean and the Mediterranean Sea.

Conclusions/Significance: Despite the abundance and prominent ecological role of hermit crabs, Pagurus, in North East Atlantic Ocean and Mediterranean Sea ecosystems, many important aspects of their taxonomy, biology, systematics and evolution remain poorly explored. The topologies presented here should be regarded as hypotheses that can be incorporated into the robust and integrated understanding of the systematic relationships within and between species of the genus Pagurus inhabiting the Northeast Atlantic Ocean and the Mediterranean Sea.

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* E-mail: joanamatzen@yahoo.com (JMdS); g.r.carvalho@bangor.ac.uk (GRC)

Introduction

Although hermit crabs are one of the most morphologically and ecologically diverse group of decapod crustaceans, their evolutionary history at many taxonomic levels is far from being resolved [1–4]. More than 1000 species, 127 genera and 6 families are currently reported for the superfamily Paguroidea [5], that inhabit diverse biotopes from intertidal to deep seas [6]. However, the true taxonomic and ecological heterogeneity associated with hermit crabs is likely to be underestimated because many species and life-histories appear to be undescribed [4]. One of the most diverse groups belong to the family Paguridae, with species distributed widely through all oceans [5].

The genus Pagurus Fabricius, 1775 is considered to be ancient, with fossils being assigned to the genus as early as the lower Cretaceous [7] and from the Cenozoic [8]. Pagurus is the least complex of all paguroids, sharing a reduced or virtually nonexistent rostrum, no sexually modified appendages other than the regular female egg-bearing pleopods, and having no penis (or sexual tubes) [3]. Despite its comparatively morphological conservatism, Pagurus exhibits a high degree of species proliferation. Recently, 172 species (of which five are fossil records) are recognised [9], that possess specialized adaptations for housing stability, relying upon gastropod shells for protection. Such commensalism has constrained morphological evolution over 150 million years [7,8] by requiring a decalcified asymmetrical abdomen capable of looping into gastropod shells. Despite the abundance of hermit crabs in the North East Atlantic and Mediterranean Sea [6] many important aspects of their taxonomy [10], biology [11–18], ecology [17,19–22], systematics and evolution [3,8,23] are poorly documented.

Systematic problems remain among the Paguridae, such as the polyphyletic genus Pagurus Fabricius, 1775 [4]. To date, most systematic studies on hermit crabs have been based on morphology with relatively few studies utilising molecular tools to resolve species status [4,24–26] or to determine phylogenetic relationships among major taxa [2,4,23]. Within the region of the Northeast Atlantic Ocean and Mediterranean Sea Pagurus is
represented by 23 species [6] and high morphological similarity among some species has resulted in the recognition of two groups characteristic of the North Atlantic and Mediterranean Sea [10]. In some cases, morphology suggests very close relationships between congeners, raising uncertainty about their independent species status. Such a situation exists between the “alatus” group, Pagurus excavatus (Herbst, 1791) and Pagurus alatus (Fabricius, 1775) that is still difficult to resolve using morphology alone (Table 1) [6,10]. In spite of the prevailing taxonomic challenges, Ingle [10] has recognized both species, based mainly from the differences observed in the dorsal aspects of shield, antennular peduncle, the shape of the right cheliped, the length of larger pereiopod and male pleopods. Furthermore, due to a lack of life history studies there has always been considerable confusion regarding ongoing synonymies that are assigned among P. alatus, P. excavatus and P. variabilis (A. Milne-Edwards & Bouvier, 1892) [10,27]. As currently recognised, P. excavatus is distributed southwards from the southern part of the Bay of Biscay and into the Mediterranean Sea. Pagurus alatus extends northwards into Iceland waters, but remains sympatric with P. excavatus in many southern regions of the area [10]. Here, we use molecular phylogenetic analyses of mitochondrial cytochrome oxidase I (COI), mitochondrial 16S ribosomal RNA (16S), and nuclear 28S ribosomal RNA (28S) DNA partitions to reconstruct the systematics of selected Pagurus species, in order to make inferences on taxonomic status of P. alatus and P. excavatus.

Results

Pagurus diversity

The variation in COI diversity was examined among 11 species (Table 2 and 3). For each species, one to six representative individuals were analysed (Table 4), and where possible, from different geographical areas, yielding a total of 46 sequences. No insertions, deletions, stop codons or sequences indicative of pseudogenes [28,29] were observed, and BLAST searches confirmed that the sequences corresponded to decapod mtDNA COI. There was also evidence for base composition bias in the sequences, notably a pronounced underrepresentation of guanine at the third codon positions (35.3% T; 18.1% C; 28.5% A; 18.1% G) a phenomenon commonly observed in metazoan mitochondrial [30]. The COI alignment contained a total of 513 bp with 177 variable characters, of which 170 were parsimony informative (33.13%). The high observed percentage of parsimony-informative character suggests that COI is sufficiently diverse for intragenic phylogeny and clearly resolved all eleven Pagurus species examined in the present study (Figure 1) [31,32]. The 11 species comprise a monophyletic clade (Figure 1) with an average between genetic species distance of 17.01% (Table 2). Among the Pagurus species, P. acadianus exhibits the least genetic divergence to P. bernhardus (6.80%) and in contrast, P. urinatus and P. excavatus exhibited the highest genetic distances (23.10%) (Table 3). P. pubescens exhibited the highest average distance values (Table 3) with a range of 11.50–21.70% (see also Figure 1).

Phylogenetic relationships

Overall, the phylogenetic algorithms (ML, BI) resulted in congruent topologies, delimiting the designated true Pagurus species (Figure 1). The resulting molecular phylogeny agrees in several respects with the current morphologically based classification of all species. All analyses support the basal placement of P. longicarpus and identify two main clades (Figure 1). In contrast, the relationships among inner clades of Pagurus were poorly resolved. Clade I is represented by four the Northeast Atlantic Ocean and Mediterranean Sea species and clade II by six species of the North Atlantic Ocean (East and West coasts) and Bering Sea specimens.

Molecular systematic assignments

The three independent genes revealed concordant phylogenetic differences between P. alatus and P. excavatus. Pagurus alatus is substantially divergent from P. excavatus, with a mean divergence of 14.9% and 5.1% for COI and 16S sequences respectively (Table 5). The 16S alignment was more conserved than the COI partition yielding 84/462 variable characters, of which 75/462 were parsimony informative. The 16S sequences are A+T-rich (71.86%), indicating a moderate compositional bias. The pattern of nucleotide substitution was also biased in favour of transitions over transversions, yielding a τc:τv = 1.1 and for 28S a τc:τv = 3.2.

In the BI analysis, systematic positions of five species were not stable (Figure 2 A), but the trees underpinned by the 16S, 28S and the concatenated data partitions were broadly congruent with the COI hypothesis (Figure 1). The combined molecular analysis,

Table 1. Selected morphological characters by Ingle (1985) to distinguish Pagurus alatus (Fabricius 1775) vs Pagurus excavatus (Herbst 1791).

| Morphological selected characters by Ingle (1985) | Pagurus alatus (Fabricius 1775) | Pagurus excavatus (Herbst 1791) |
|-------------------------------------------------|---------------------------------|---------------------------------|
| Dorsal aspect of shield and associated appendages | Segment 1 of antennular peduncle, outer distal margin with one, two or sometimes three spines. | Segment 1 of antennular peduncle, outer distal margin without or with small obtuse spines at the most. |
|                                                  | Outer dorso-lateral process of antenna (of large circa 80 mm SL, specimens) reaching just beyond distal margin of segment 4 and acicle reaching well beyond distal extremity of cornea; breadth of cornea slightly exceeding 1/2 length of eye. | Outer dorso-lateral process of antenna (of large specimens circa 80 mm SL) not reaching to distal margin of segment 4 and acicle reaching only to extremity of cornea; breadth of cornea slightly less than 1/2 length of eye. |
|                                                  | Outer (particularly upper) surface of right cheliped palm not strikingly concave. | Outer upper (and sometimes lower) surface of right cheliped palm strikingly concave. |
|                                                  | Larger pereiopod 3, dactyl (of large specimens, circa 10 mm SL) as long as combined lengths of propodus + carpus and noticeably curved; males with 3 unpaired pleopods. | Larger pereiopod 3, dactyl (of large specimens, circa 9–10 mm SL) slightly longer than combined lengths of propodus + carpus and noticeably curved; males with 4 unpaired pleopods. |

Words in bold are highlighting the solely differences between species and “SL” is the abbreviation of shield length.

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based on strong posterior probabilities values (100%; in Figure 2, CON), clearly reveals the two independent lineages here discussed.

**Discussion**

*Pagurus* diversity

Systematics work to date in *Pagurus* has primarily been in the area of morphological taxonomy, with few hypotheses presented regarding species relationships. Generating such inferences has been difficult because of generally remarkable similarities in morphology among members of the genus. These similarities are perhaps surprising given the degree of the genetic divergence observed in this study between the most phenotypically closest species, *P. alatus* and *P. excavatus* (14.8%). A similar pattern has been observed in penaeid shrimps [33], porcellanids crabs [34], diogenid hermit crabs [35] and has been attributed to stabilizing selection acting on morphological differentiation has been associated with life as a commensal of thallassinidean shrimps, accompanying by neutral molecular divergence [36].

Likewise, strong stabilizing selection acting on morphological/ecological characters, or that they are associated with life as a commensal of thallassinidean shrimps, accompanying by neutral molecular divergence [36].

The smallest mean intraspecific divergence values observed (Table 3) are possibly underestimated, because samples were obtained from a single locality. Global-scale phylogeography surveys of COI sequence diversity have estimated average intraspecific diversity values of less than 1% within crustaceans, whereas interspecific values typically are greater than 4% among congeneric species [37–39] and especially among decapods that can exhibit congeneric divergence values greater than 15% [40].

Elsewhere, five species of the genus *Pagurus* from Sea of Japan exhibited lower levels of genetic identity when compared with the genera *Metapenaeus* and *Penaeus* [25]. Here, the high genetic diversity observed among the pagurid species is in line with the observed morphological variability in informal morphological groups among adults and larvae described by Ingle (1985) and McLaughlin and Gore (1988), respectively.

**Phylogenetic relationships**

Ingle [10] delineated two main groups of Northeast Atlantic Ocean and Mediterranean Sea *Pagurus* based on three adults, and additional larval morphological characters [10] that are fully congruent with the current molecular systematic analysis. Furthermore, *P. armatus*, *P. ochotensis* and *P. bernhardus* that were assumed to be most related, based on morphology [10], share the most basal position in clade II. In addition, all species from clade I and II agree with two distinct larval groups described by McLaughlin and Gore [41] based on the characteristics and species assigned. The inconsistent phylogenetic position of *P. bernhardus*, observed in Mantelatto et al. [4] was still unresolved here (Figure 1), represented by the weak support of the two focal nodes (<50% bootstrap support) within clade II. However, the observed COI molecular divergence does support a sister group relationship between *P. acadianus* and *P. bernhardus*, a taxonomic relationship derived also from morphological comparisons [7,42]. In summary, the phylogenetic patterns observed for *Pagurus* are consistent with morphological groups established by Ingle [10] and McLaughlin and Gore [41] but further analyses would be required to establish the precise cladistic relationships within the genus as a whole.

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**Table 2.** Pairwise COI nucleotide divergences for *Pagurus* spp using K2P distances (%).

| Pairwise divergences comparisons | n  | Taxa | Min Dist(%) | Mean Dist(%) | Max Dist(%) | SE Dist(%) |
|---------------------------------|----|------|-------------|--------------|-------------|-----------|
| *Pagurus* (11 species)          |    |      |             |              |             |           |
| Within a species*               | 44 | 9    | 0           | 0.632        | 2.002       | 0.055     |
| Between species                 | 46 | 1    | 6.432       | 17.019       | 23.086      | 0.106     |

*Number of specimens with more than 1 sequences analysed.

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**Table 3.** Pairwise COI nucleotide divergences for each selected *Pagurus* spp using K2P distances (%).

| Species                         | Distances (%) |
|---------------------------------|---------------|
|                                 | Within species| Between species |
|                                 | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |   |
| 1 Pagurus acadianus (Benedict, 1910) | 0.2 |    |    |    |    |    |    |    |    |    |    |   |
| 2 Pagurus alatus (Fabricius, 1775)   | 0.5 | 17.70 |    |    |    |    |    |    |    |    |    |   |
| 3 Pagurus arcuatus (Squires, 1964)  | 1.2 | 13.80 | 17.50 |    |    |    |    |    |    |    |    |   |
| 4 Pagurus bernhardus (Linnaeus, 1758) | 0.7 | 6.80 | 16.70 | 14.00 |    |    |    |    |    |    |    |   |
| 5 Pagurus cuanensis (Bell, 1845)    | 0  | 17.80 | 13.40 | 19.50 | 18.00 |    |    |    |    |    |    |   |
| 6 Pagurus excavatus (Herbst, 1791)  | 0.1 | 19.50 | 14.80 | 23.10 | 20.00 | 13.00 |    |    |    |    |    |   |
| 7 Pagurus prideauxii (Leach, 1815)  | 0.4 | 18.20 | 13.70 | 20.50 | 17.30 | 15.00 | 15.60 |    |    |    |    |   |
| 8 Pagurus pubescens (Kraye, 1838)   | 1.6 | 11.50 | 17.70 | 13.90 | 12.70 | 18.90 | 21.70 | 21.20 |    |    |    |   |
| 9 Pagurus longicarpus (Say, 1817)   | 0.6 | 19.30 | 16.20 | 19.80 | 18.80 | 20.50 | 21.30 | 18.70 | 19.80 |    |    |   |
| 10 Pagurus armatus (Dana, 1851)     | -  | 12.20 | 21.40 | 19.80 | 12.90 | 21.10 | 22.80 | 21.30 | 17.80 | 21.70 |    |   |
| 11 Pagurus ochotensis (Brandt, 1851) | -  | 13.50 | 20.00 | 16.90 | 14.90 | 20.90 | 21.20 | 21.50 | 19.30 | 21.10 | 9.20 |   |

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*Number of specimens with more than 1 sequences analysed.
**Table 4. Pagurus and outgroup specimens used for the COI phylogenetic reconstructions.**

| Species                  | Collection site        | GenBank accession No. |
|--------------------------|------------------------|-----------------------|
| *Pagurus acadianus*      | Maine (United States)  | AF483156              |
| *Pagurus acadianus*      | Prince Edward Island (Canada) | FJ581812          |
| *Pagurus acadianus*      | Quebec (Canada)        | FJ581815              |
| *Pagurus acadianus*      | Quebec (Canada)        | FJ581814              |
| *Pagurus acadianus*      | Quebec (Canada)        | FJ581813              |
| *Pagurus alatus*         | Costa Algarvia (Portugal) | JN107574            |
| *Pagurus alatus*         | Costa Algarvia (Portugal) | JN107575            |
| *Pagurus alatus*         | Costa Algarvia (Portugal) | JN107576            |
| *Pagurus arcuatus*       | Quebec (Canada)        | FJ581818              |
| *Pagurus arcuatus*       | Quebec (Canada)        | FJ581817              |
| *Pagurus armatus*        | Nova Scotia (Canada)   | AF483159              |
| *Pagurus bernhardus*     | Wales (United Kingdom) | JN107580              |
| *Pagurus bernhardus*     | Wales (United Kingdom) | JN107581              |
| *Pagurus bernhardus*     | Wales (United Kingdom) | JN107582              |
| *Pagurus bernhardus*     | Costa de Prata (Portugal) | JN107583          |
| *Pagurus bernhardus*     | England (United Kingdom) | JN107578          |
| *Pagurus cuenensis*      | Azores (Portugal)      | JN107584              |
| *Pagurus cuenensis*      | Azores (Portugal)      | JN107585              |
| *Pagurus excavatus*      | Costa de Prata (Portugal) | JN107586          |
| *Pagurus excavatus*      | Costa de Prata (Portugal) | JN107587          |
| *Pagurus excavatus*      | Costa de Prata (Portugal) | JN107589          |
| *Pagurus excavatus*      | Costa de Prata (Portugal) | JN107591          |
| *Pagurus excavatus*      | Sicily (Italy)         | JN107588              |
| *Pagurus excavatus*      | Sicily (Italy)         | JN107589              |
| *Pagurus longicarpus*    | New Brunswick (Canada) | FJ581825              |
| *Pagurus longicarpus*    | New Brunswick (Canada) | FJ581824              |
| *Pagurus longicarpus*    | New Brunswick (Canada) | FJ581823              |
| *Pagurus longicarpus*    | New Brunswick (Canada) | FJ581822              |
| *Pagurus longicarpus*    | New Brunswick (Canada) | FJ581826              |
| *Pagurus longicarpus*    | Nova Scotia (Canada)   | FJ581820              |
| *Pagurus ochotensis*     | Alaska (United States) | AF483158              |
| *Pagurus prideauxii*     | England (United Kingdom) | JN107597         |
| *Pagurus prideauxii*     | Costa de Prata (Portugal) | JN107595          |
| *Pagurus prideauxii*     | Costa de Prata (Portugal) | JN107596          |
| *Pagurus prideauxii*     | Sicily (Italy)         | JN107592              |
| *Pagurus prideauxii*     | Sicily (Italy)         | JN107593              |
| *Pagurus pubescens*      | Bear Island Slide (Norway) | JN107594          |
| *Pagurus pubescens*      | Quebec (Canada)        | FJ581829              |
| *Pagurus pubescens*      | Bear Island Slide (Norway) | JN107598          |
| *Pagurus pubescens*      | Bear Island Slide (Norway) | JN107599          |
| *Pagurus pubescens*      | Svalbard (Norway)      | JN107600              |
| *Pagurus pubescens*      | Svalbard (Norway)      | JN107601              |
| *Pagurus pubescens*      | Svalbard (Norway)      | JN107602              |
| *Dardanus arrosor*       | Sicily (Italy)         | JN107572              |
| *Macropodia longipes*    | Costa de Prata (Portugal) | JN107573          |

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Figure 1. BI phylogram of the 46 COI sequences of 11 Pagurus species selected. The numbers on branches are ML bootstrap values and posterior probabilities of BI <50% (ML/BI percentages values, respectively). Each oceanographic region/specimens are defined: Northeast Atlantic Ocean (NEA), Northwest Atlantic Ocean (NWA), North Atlantic Ocean (NA), Mediterranean Sea (MED) and Bering Sea (BER) (see Table 4 for complement information). Two major clades have been roman number-coded, I and II: represent two groups defined previously by Ingel (1985) based on adult and larval morphological characters.

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Table 5. Sequence identity matrix estimated from 16S with TVM+G model (above diagonal) and COI (below diagonal) with TIM2+I+G model between selected Pagurus species of Northeast Atlantic Ocean and Mediterranean Sea.

| Species                  | Collection site          | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     | 13     | 14     |
|--------------------------|--------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Pagurus alatus (Fabricius, 1775) Costa Algarvia (Portugal) | 0.02  | 0.02  | 0.04  | 1.81  | 0.61  | 0.57  | 0.57  | 0.5   | 0.5   | 0.5   | 1.92  | 1.83  |
| Pagurus alatus (Fabricius, 1775) Costa Algarvia (Portugal) | 0.1   | 0     | 0.02  | 1.77  | 0.59  | 0.55  | 0.55  | 0.47  | 0.5   | 0.47  | 0.47  | 1.87  | 1.79  |
| Pagurus alatus (Fabricius, 1775) Costa Algarvia (Portugal) | 0     | 0.1   | 0.02  | 1.77  | 0.59  | 0.55  | 0.55  | 0.47  | 0.5   | 0.47  | 0.47  | 1.87  | 1.79  |
| Pagurus alatus (Fabricius, 1775) Costa Algarvia (Portugal) | 0     | 0     | 1.81  | 0.61  | 0.57  | 0.57  | 0.53  | 0.5   | 0.5   | 0.5   | 1.83  | 1.83  |
| Pagurus bernhardus (Linnaeus, 1758) Wales (United Kingdom) | 5.35  | 4.69  | 5.35  | 5.35  | 1.81  | 1.74  | 1.74  | 1.9   | 1.89  | 1.9   | 1.9   | 0.56  | 0.51  |
| Pagurus excavatus (Herbst, 1791) Sicily (Italy) | 3.82  | 3.59  | 3.82  | 3.82  | 7.04  | 0.02  | 0.02  | 0.8   | 0.77  | 0.8   | 0.8   | 2.05  | 1.97  |
| Pagurus excavatus (Herbst, 1791) Sicily (Italy) | 3.82  | 3.59  | 3.82  | 3.82  | 7.04  | 0     | 0.76  | 0.73  | 0.76  | 0.76  | 1.98  | 1.9   |
| Pagurus excavatus (Herbst, 1791) Costa de Prata (Portugal) | 3.82  | 3.59  | 3.82  | 3.82  | 7.04  | 0     | 0.76  | 0.73  | 0.76  | 0.76  | 1.98  | 1.9   |
| Pagurus excavatus (Herbst, 1791) Bear Island Slide (Norway) | 3.56  | 3.39  | 3.56  | 4.99  | 4.99  | 4.05  | 4.05  | 0.02  | 0     | 0     | 2.01  | 1.93  |
| Pagurus prideauxii (Leach, 1815) Wales (United Kingdom) | 3.65  | 3.48  | 3.65  | 5.1   | 4.15  | 4.15  | 4.15  | 0.02  | 0.02  | 2     | 1.92  |
| Pagurus prideauxii (Leach, 1815) Costa Algarvia (Portugal) | 3.65  | 3.48  | 3.65  | 5.03  | 4.15  | 4.15  | 4.15  | 0.04  | 0.02  | 0     | 2.01  | 1.93  |
| Pagurus prideauxii (Leach, 1815) Sicily (Italy) | 3.65  | 3.48  | 3.65  | 5.1   | 4.15  | 4.15  | 4.15  | 0.02  | 0     | 0.02  | 2.01  | 1.93  |
| Pagurus pubescens (Kroyer, 1838) Svalbard (Norway) | 5.33  | 5.02  | 5.33  | 5.33  | 2.7   | 7.12  | 7.12  | 7.12  | 7.29  | 7.44  | 7.44  | 0.04  |
| Pagurus pubescens (Kroyer, 1838) Svalbard (Norway) | 5.64  | 5.26  | 5.64  | 5.64  | 2.85  | 7.07  | 7.07  | 7.07  | 7.17  | 7.32  | 7.32  | 0.18  |

All values are expressed as percentage.
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Molecular systematic assignments

Concordance across molecular and morphological characters provides a reliable indicator of longstanding evolutionary independence and consequently provides an operational criterion for species recognition [43]. In the present study, morphological differences between *P. alatus* and *P. excavatus* were evident when two morphological variants were compared directly (Table 1), supporting molecular findings that justify separation at the species level. It is important to mention that a restricted collection from one biogeographical region of a species with a wide distribution cannot represent the complete range of morphological variation that is often found in decapods [4,27,44]. We also know that phenotypic plasticity in morphological traits (especially organisms with commensal behaviours) might be strongly influenced by environmental factors between different areas [45–47]. The precise cladistic relationships among the five species were not stable (Figure 2, A), most likely due to lower taxon sampling and also the inability of COI to accurately resolve deep nodes. However, the combined molecular analysis (Figure 2, CON) clearly reveals the evidence for long-standing evolutionary independence between *P. excavatus* and *P. alatus*.

Conclusion

Molecular data have not been used before to investigate systematic relationships among *Pagurus* of the Northeast Atlantic Ocean and Mediterranean Sea. Despite the perceived limitations regarding the use of morphological characters for inferring evolutionary relationships among commensal species, our molecular data support the morphological taxonomy. Our data may indicate the possible existence of two monophyletic groups (clade I and II), supporting previous assertions based on larval and adult morphological criteria. However, the current data confirm the complexity of the relationships within *Pagurus*, highlighting the absence of complete and integrated morphological descriptions for the diverse and heterogeneous members of the genus. Since the present taxonomic and geographic coverage is incomplete, the topologies presented here should be regarded as working hypotheses. *Pagurus* have diversified into a wide variety of marine habitats and exemplify classic commensal, anti-predator evolutionary traits. Thus, the group provides an excellent model for studying the interplay between speciation, neutral molecular divergence and potential stabilising selection on body form.

Materials and Methods

Sampling

Twenty nine hermit crabs of six species, *Pagurus alatus* (Fabricius, 1775), *P. bernhardus* (Linnaeus, 1758), *P. cuanensis* (Bell, 1845), *P. excavatus* (Herbest, 1791), *P. prideaux* (Leach, 1815), and *P. pubescens* (Kroyer, 1838), were collected from the Portugal (Costa Algarvia,
Costa de Prata, and Azores), United Kingdom (North Wales), Norway (Bear Island Slide and Svalbard) and Italy (Sicily) between 2006 and 2008. Specimens were harvested as by-catch from rough ground bottom trawls from INRB-IPIMAR, Sicilian fisheries survey and collected by physical searches. Species were identified (Ingle, 1985) prior to the excision and preservation of muscle tissue in 95% ethanol (stored at −20°C) and whole body storage at −20°C. In order to accurately assign specimens to currently accepted (female) species of P. alatus and P. excavatus we used morphological criteria based upon four main groups of characters: the dorsal aspects of shield (Table 1), antennular peduncle, the shape of the right cheliped, length of larger pereiopod and male pleopods (Ingle 1985).

DNA isolation, amplification and sequencing

Total genomic DNA was extracted from approximately 1 mm³ of muscle tissue or whole legs for small specimens using the Chelex dry release method [48]. The COI gene was amplified with alternative sets of primers depending on PCR reaction success following by the protocol develop by Costa et al. [34]. All PCRs were performed in a 25 μl final volume containing 1× PCR buffer, 3–4 mM MgCl₂, 0.1–0.3 mM dNTP, 1 U Taq polymerase, 5–10 pmol of each primer, and 2–10 ng of DNA template (Table 6). The thermal cycling conditions are listed in Table 6 for each set of primers. Following amplification, PCR products were cleaned by incubation with 10 U Exonuclease I (New England Biolabs®) and 1 U Shrimp Alkaline Phosphatase (Promega®) at 37°C for 1 hour, followed by heating at 80°C for 5 min. Samples were sequenced by Macrogen Inc. (South Korea) using an Applied Biosystems® 3730 sequencer.

All empirically derived sequences were manually checked for ambiguities in CodonCode Aligner version 1.3.0, aligned using the ClustalX plugin embedded within Mega 4 [49], prior to manual quality control and megablast annotation.

Phylogenetic relationships

The most popular barcode marker COI is generally used to study close to moderately deep interspecific taxon relationships of crustaceans [50–54]. To provide a comprehensive sister-species coverage and assessment of interspecific variation, Pagurus COI sequences from GenBank were merged with our data (Table 4). In that propose we use using Kimura 2-parameters (K2P) genetic distances within and among species implemented in Mega 4.1, and compared to literature data. Amino acid translations of the target genes were examined to ensure that no gaps or stop codons were present in the alignment.

Table 6. Primers sequences and thermocycling conditions for the amplification reactions.

| Locus and primers names | Sequences | Reference | Cycling conditions |
|-------------------------|-----------|-----------|--------------------|
| COI Forward LCO1490     | 5’-GGTCAACAAATCATATAAGATATGG-3’ | Folmer et al., [68] | Denaturation 94°C/60 s; |
| CrustF1 Reverse         | 5’-TTTCTACAAATCATAAAGACATTGG-3’ | Costa et al., [37] | 35–40 cycles at 94°C/30 s, 45°C/90 s, 72°C/60 s; |
| HCO2198                 | 5’-TAACTTTCAAGGGTACCAAAAATCA-3’ | Folmer et al., [68] | Final extension at 72°C/5 min. |
| 16S Forward 16SL2       | 5’-TGCTGTGTTGTATCAAAACAT-3’ | Mathews et al., [69] | Denaturation 94°C/3 min.; |
| 16sar Reverse           | 5’-CGCTGTGTTGTATCAAAACAT-3’ | Palumbi et al., [33] | 35–40 cycles at 94°C/30 s, 45°C/90 s, 72°C/60 s; |
| 16S-1472 Reverse        | 5’-AGATAGAAACCAACTGG-3’ | Schubart et al., [70] | Final extension at 72°C/5 min. |
| 28S Forward 28S-rD1.2a  | 5’-CCGCGGTGTTTCAACTACATG-3’ | Palumbi et al., [33] | Denaturation 94°C/5 min.; |
| 285 Reverse 28S-rD1.2a  | 5’-CCGCGGTGTTTCAACTACATG-3’ | Palumbi et al., [33] | 35 cycles at 95°C/30 s, 1 cycle at 95°C/30 s; |
| 285rd3.2b1              | 5’-TYACGGTTTCACTCATGTGA-3’ | This study | 45°C/45 s and final extension at 72°C/5 min. |

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To identify phylogenetic groups among the resulting eleven putative *Pagurus* species, the 46 COI sequences comprising 513 bp was analyzed using Maximum Likelihood (ML) and Bayesian Inference (BI) phylogenetic reconstruction methods. The crab *Macropodia longipes* (Milne-Edwards & Bouvier, 1899) (Brachyura:Inachidae) and the most phylogenetically closest related species, *Dardanus arrosor* (Herbst, 1796) (Anomura: Diogenidae) were used as outgroups.

Since Bayesian posterior probability support values (bpp) can often be inflated for certain clades, relative to ML bootstrap values [55], we constructed trees using both Bayesian approaches and ML. For these searches, we set the substitution model parameters calculated by jModeltest in RAxML 7.0.4 [56] and in MrBayes 3.1 [57], respectively. Ten independent ML analyses were conducted using GTR+I+G with invariant sites (I) and gamma distributed rates (G) [58] (see below) as the model (using the GTR+CAT setting) with 4 categories of rate variation (500 bootstrap replicates were undertaken for estimation of node support) for each partition on combined data. In order to find the ML tree, 10 independent runs of RAxML 7.0.4 were conducted. ModelTest [59] identified the HKY+I+G model [60] as best indicated by Akaike Information Criterion (AIC) [61], however, since this model is not implemented in the current version of RAxML, the GTR+I+G model was selected as the closest matching alternative. In MrBayes, two independent Markov chain Monte Carlo (MCMC) analyses were run using four chains for 5610^6 generations with the initial 1 million generations (20%) cycles discarded as burn-in. To check that stationarity had been reached, we monitored the fluctuating value of the likelihood graphically with Tracer v1.4 [62]. Once the parameters reach stationarity, a 50% majority rule consensus tree was obtained from the remaining saved trees. The consensus tree was selected from the posterior distribution and visualized using FigTree V.1.0 (http://tree.bio.ed.ac.uk/software/figtree/). Since substitution rates

Table 7. Selected Northeast Atlantic Ocean and Mediterranean Sea *Pagurus* species and outgroup *Dardanus* species for molecular systematic reconstructions with respective date and site of collection, museum catalogue number, and genetic database accession numbers (Genbank).

| Species                  | Collection site       | Catalogue No. | Genbank accession No. |
|--------------------------|-----------------------|---------------|-----------------------|
| *Pagurus alatus* (Fabricius, 1775) | Costa Algarvia (Portugal) | MB89000415 | JN107574 JN107604 JN107621 |
| *Pagurus alatus* (Fabricius, 1775) | Costa Algarvia (Portugal) | MB89000418 | JN107575 JN107606 JN107622 |
| *Pagurus alatus* (Fabricius, 1775) | Costa Algarvia (Portugal) | MB89000450 | JN107576 JN107607 JN107619 |
| *Pagurus alatus* (Fabricius, 1775) | Costa Algarvia (Portugal) | MB89000463 | JN107577 JN107605 JN107620 |
| *Pagurus bernhardus* (Linnaeus, 1758) | Wales (United Kingdom) | MB89000491 | JN107579 JN107608 JN107623 |
| *Pagurus excavatus* (Herbst, 1791) | Costa de Prata (Portugal) | MB89000268 | JN107586 JN107609 JN107626 |
| *Pagurus excavatus* (Herbst, 1791) | Sicily (Italy) | MB89000078 | JN107588 JN107610 JN107627 |
| *Pagurus excavatus* (Herbst, 1791) | Sicily (Italy) | MB89000079 | JN107589 JN107611 JN107628 |
| *Pagurus prideauxi* (Leach, 1815) | Costa de Prata (Portugal) | MB89000311 | JN107596 JN107614 JN107631 |
| *Pagurus prideauxi* (Leach, 1815) | Sicily (Italy) | MB89000086 | JN107593 JN107615 JN107632 |
| *Pagurus prideauxi* (Leach, 1815) | Sicily (Italy) | MB89000492 | JN107597 JN107613 JN107630 |
| *Pagurus prideauxi* (Leach, 1815) | Sicily (Italy) | MB89000493 | JN107594 JN107612 JN107629 |
| *Pagurus pubenscens* (Krøyer, 1838) | Svalbard (Norway) | MB89000489 | JN107601 JN107616 JN107633 |
| *Pagurus pubenscens* (Krøyer, 1838) | Svalbard (Norway) | MB89000490 | JN107602 JN107617 JN107633 |
| *Dardanus arrosor* (Herbst, 1796) | Sicily (Italy) | MB89000494 | JN107572 JN107603 JN107618 |

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Table 8. Substitution models for the molecular systematic analyses of selected *Pagurus* species from Northeast Atlantic Ocean and Mediterranean Sea.

| Gene | Substitution model | Among-site rate variation* | Base frequencies |
|------|-------------------|--------------------------|-----------------|
|      |                   | I | I’ | A | C | G | T |
| COI**| TIM2              | 0.454 0.41 | 0.3104 0.1484 0.1625 0.3787 |
| 1st codon | TrN | 0.629 - | 0.2901 0.1667 0.307 0.2363 |
| 2nd codon | F81 | - - | 0.1306 0.2565 0.1688 0.4441 |
| 3rd codon | TPM3uf | - 0.622 | 0.4405 0.0937 0.0724 0.3935 |
| 16S | TVM | - 0.382 | 0.3532 0.1048 0.1766 0.3654 |
| 28S | TrN | 0.327 0.258 | 0.1889 0.2976 0.2282 0.2852 |

* I proportion of invariable sites; I’ , gamma distribution shape parameter. I and I’ values refer to the AIC.
**COI partition represented by first (1st), second (2nd) and third (3rd) codon positions.
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among the four nucleotides and among different nucleotide sites in mitochondrial protein-coding genes as been reported [63–65], codon models of substitution TrNef+G [66], F81 [67] and HKY+G [68] were implemented in our BI analyses for first, second and third codon positions respectively.

Molecular systematic analyses

Use of nuclear genes in addition to mitochondrial genes adds to the number of independent markers in a dataset, thus increasing the chances to understand the systematic relationships between and within P. alatus and P. excavatus (Table 7). Here we analysed partial sequences of nuclear 28S (385 bp), mitochondrial 16S (462 bp), and the barcode region of COI (540 bp). The three gene regions were partitioned separately according to the previously determined model parameters (Table 6) in subsequent BI analyses. Gaps in 16S and 28S sequences were treated as a fifth character-state. BI analysis was conducted for each gene data set and the concatenated partition (CON) with the three gene regions partitioned separately according to the previously determined model parameters (Table 8) as described before. To evaluate the range of intrageneric sequence identity found among Pagurus species, we compared pairwise distances of COI and 16S (Table 5).

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Author Contributions

Conceived and designed the experiments: JMdS AdS. Performed the experiments: JMdS AdS. Analyzed the data: JMdS AdS. Contributed reagents/materials/analysis tools: JMdS AdS MRC FOC SC GRC. Wrote the paper: JMdS SC GRC.

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