MOLECULAR PHYLOGENY OF THE GENERA PALAEMON AND PALAEMONETES (DECAPODA, CARIDEA, PALAEMONIDAE) FROM A EUROPEAN PERSPECTIVE

BY

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ABSTRACT

A molecular phylogenetic study by Murphy & Austin (2003) showed that Australian representatives of three shrimp genera of the family Palaemonidae (Palaemon, Palaemonetes, and Macrobrachium) do not cluster according to their generic classification. According to their results, the monophyly of these genera is questioned and the generic classification of the subfamily Palaemoninae at stake. An important number of representatives of Palaemon and Palaemonetes inhabit European waters, including the type species of each genus. To clarify the phylogeny of these species, and thus the position of the generic names Palaemon and Palaemonetes on a phylogenetic tree, we obtained DNA sequences of the same genetic markers (16S mtDNA) as used by Murphy & Austin (2003) and re-addressed the question of taxonomy and phylogeny of these two genera within the subfamily Palaemoninae. Our results confirm the paraphyly of Palaemon and Palaemonetes. In contrast, the resulting monophyletic clades reflect the geographic distribution of the species according to their respective continents: Africa-Europe, Asia, Australia, and America. With exception of Palaemon elegans, the Afro-European species cluster in a way that would support monophyly of the two genera, if representatives from other continents were excluded. Possible taxonomic solutions are discussed.

ZUSAMMENFASSUNG

Eine molekularphylogenetische Studie von Murphy & Austin (2003) hat gezeigt, dass die Gruppierung von australischen Vertretern von drei Garnelen-Gattungen der Familie

Palaemonidae (Palaemon, Palaemonetes und Macrobrachium) nicht mit deren taxonomischer Klassifizierung übereinstimmt. Dadurch ist die Monophylie der drei Gattungen, und die derzeitige Taxonomie der Untergattung Palaemoninae, in Frage gestellt. Die Typusarten der Gattungen Palaemon and Palaemonetes finden sich in europäischen Gewässern, die auch von einer wichtigen Anzahl anderer Vertreter beider Gattungen bewohnt werden. Um die Phylogenie dieser Arten, und somit die Position der Gattungsnamen Palaemon and Palaemonetes auf einem entsprechenden Stammbaum, zu klären, generierten wir DNS-Sequenzen des selben genetischen Markers (16S mtDNS) der von Murphy und Austin (2003) verwendet wurde und gehen der Frage nach der Taxonomie und Phylogenie dieser beiden Gattungen aus der Unterfamilie Palaemoninae nach. Unsere Ergebnisse bestätigen die Paraphylie von Palaemon and Palaemonetes. Stattdessen geben die resultierenden monophyletischen Kladen die geographische Verbreitung der jeweiligen Arten in Bezug auf Kontinente wieder: Afrika-Europa, Asien, Australien und Amerika. Mit Ausnahme von Palaemon elegans, gruppieren sich die afro-europäischen Arten so, dass die beiden Gattungen monophyletisch wären, wenn man Vertreter anderer Kontinente nicht in Betracht zieht. Mögliche taxonomische Lösungen werden diskutiert.

INTRODUCTION

The genera Palaemon Weber, 1795 and Palaemonetes Heller, 1869 comprise 41 and 31 species, respectively (De Grave & Fransen, 2011). These species are distributed throughout five continents, inhabiting marine shallow waters, marshes, estuaries, and rivers. Varying degrees of tolerance to waters of different salinities allow coexistence of a high number of these species within a short range of distribution. In European waters (including Turkey), six species of Palaemon (viz. P. adspersus Rathke, 1837, P. elegans Rathke, 1837, P.

longirostris H. Milne-Edwards, 1837, P. serratus (Pennant, 1777), P. xiphias Risso, 1816, and the recently introduced P. macrodactylus Rathbun, 1902 (Ashelby et al., 2004; Cuesta et al., 2004)) and four species of Palaemonetes (viz. P. antennarius (H. Milne-Edwards, 1837), P. turcorum Holthuis, 1961, P. varians (Leach, 1813), and P. zariquieyi Sollaud, 1939) can be found (see González-Ortegón & Cuesta, 2006). Only the presence or absence of a mandibular palp consistently separate Palaemon and Palaemonetes. European representatives can furthermore be distinguished by the denticulation of the rostrum (see González-Ortegón & Cuesta, 2006 for more details). No other morphological characters allow clear distinction between European representatives of these genera.

Murphy & Austin (2003), using mitochondrial DNA (mtDNA) sequences of the 16S rRNA gene (16S), showed that Australian representatives of three shrimp genera of the family Palaemonidae Rafinesque, 1815 (Palaemon, Palaemonetes, and Macrobrachium Spence Bate, 1868) do not cluster in a phylogenetic tree according to their current classification. This result brought up the question about the monophyly of these genera. Considering that the type species of the genera Palaemon and Palaemonetes inhabit European waters, and that also in this area there is an important number of representatives of both genera (a total of ten species), it seemed necessary to address the question of taxonomy and phylogeny of these two genera of the subfamily Palaemoninae Rafinesque, 1815 from a European perspective, also using 16S sequences and incorporating some non-European species for comparative purposes. This should clarify, where on a phylogenetic tree the names Palaemon and Palaemonetes will be encountered and contribute to possible solutions in the reconciliation of phylogenetic relationships and taxonomic classification.

MATERIAL AND METHODS

Material studied

Specimens of Palaemon and Palaemonetes used in this analysis were collected during several field trips, donated by other researchers, or loaned from scientific collections (types of Palaemonetes turcorum from Naturalis Museum Leiden). Details of localities, data of collection, museum catalogue numbers, as well as GenBank sequence accession numbers are listed in Table I. Published mtDNA sequences of nine additional species of Palaemon and Palaemonetes from Asia, Australia, America and Europe, and the outgroups of the genus Macrobrachium were also included in our dataset and the available information listed in Table I.

DNA extraction, amplification, and sequencing

The analysis was based exclusively on a partial sequence of the 16S rDNA gene, which has proven to be useful for phylogenetic studies on decapods (summarized in Schubart et al., 2000; Schubart, 2009). Total genomic DNA was extracted from abdominal muscle tissue, and then ground and incubated for 1–24 hours in 600 µl lysis buffer at 65ºC. Protein was precipitated by addition of 200 µl of 7.5 M ammonium acetate and subsequent centrifugation, and DNA precipitation was obtained by addition of 600 µl isopropanol and posterior centrifugation. The resulting pellet was washed with ethanol (70%), dried, and finally resuspended in 20 µl TE buffer.

Target mitochondrial DNA from the large subunit rRNA (16S) gene was amplified with polymerase chain reaction (PCR) and the following cycling conditions for reactions: 2 min at 95ºC, 30 cycles with 20 s at 95ºC, 20 s at 45-48ºC, 45 s at 72ºC, and final elongation for 5 min at 72ºC. Primers 1472 (5´- AGA TAG AAA CCA ACC TGG -3´) (Crandall &

Fitzpatrick 1996) and 16L2 (5´-TGC CTG TTT ATC AAA AAC AT-3´) (Schubart et al., 2002) were used to amplify approximately 570 basepairs (bp) of 16S. PCR products were purified with Microcon 100* filters (Millipore Corp.) and sequenced with the ABI BigDye terminator mix (Big Dye Terminator® v 3.0 Cycle Sequencing Kit; Applied Biosystems) in an ABI Prism automated sequencer (ABI Prism™ 3100 Genetic Analyzer; Applied Biosystems).

Sequences were edited using the software Chromas version 2.0 and manually aligned with BioEdit (Hall 1999), excluding primer regions. The final alignment consisted of 536 bp.

Phylogenetic analyses

The best-fitting model of nucleotide substitution was selected by testing alternative models of evolution using Modeltest version 3.7 (Posada & Crandall, 1998). This model was implemented in a Bayesian inference analysis (BI), which was run for four generations over four chains (three heated and one cold), sampling every hundred trees with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The so-called burnin (time to reach stable likelihood values) was estimated graphically by plotting the log-likelihood values. Similarly, maximum parsimony (MP) phylogeny reconstruction was performed with PAUP* (Swofford, 2003). Heuristic searches were carried out using random sequence addition with 100 replicates and tree-bisection-reconnection branch swapping. The nodal confidence of obtained topologies was assessed via 10,000 bootstrap pseudoreplicates. Majority-rule consensus trees were built with maximum parsimony and Bayesian approaches. Thereby we analysed both unconstrained trees and those constrained for monophyletic genera. Statistical differences between unconstrained and constrained topologies were ascertained with the implementation of the SH test (Shimodaira & Hasegawa, 1999) in PAUP. Specifically, we used differences of log-likelihoods for statistics and the non parametric bootstrap with re-estimated log-likelihood

(RELL) approximation (Kishino et al., 1990). Moreover, all unconstrained MP trees saved during the bootstrap analysis, as well as BI trees found after stationary, were filtered to find those trees that are consistent with the alternative (monophyletic genera) topology.

RESULTS

The 16S dataset consisted of 28 sequences and an alignment length of 536 bp, excluding the primers and including a few shorter sequences from Murphy & Austin (2003). 219 positions were variable and 172 parsimony-informative. A GTR+I+G substitution model with γ distributed rate heterogeneity ($\underline{\alpha}$=0.52) and a proportion of invariable sites ($\underline{I}$=0.46) was selected by Modeltest and implemented in later analyses. The results of two phylogenetic methods (MP, BI) are combined in a single tree based on the BI topology (Fig 1). Only minor and non-significant differences, related to the position of Palaemonetes sinensis (Sollaud, 1911), were observed among the topologies of the different tree-building methods.

The 16S gene tree topology shows well-supported genetic clustering according to geographical distribution of the species by continents and by genera, with the exception of the Australian Palaemonidae. In a global context, however, a monophyletic assemblage of the genera could not be recovered. Trees constrained to reflect such a monophyly have significantly lower likelihood scores than the best non-monophyletic unconstrained Bayesian tree as revealed by the S-H test ($P \leq 0.01$) (Table II). Furthermore, from all unconstrained trees saved during the MP (35,635 tree) and BI (24,935 tree) analyses, none was found to be consistent with a monophyletic genera topology. This confirms the paraphyly of Palaemon and Palaemonetes, but with repeated cases of regional monophyly, as for example in the case of the four European and the four American species of Palaemonetes.

At the base of the tree we find the European and American representatives of Palaemon in three different clades. With the exception of Palaemon elegans, all analysed Afro-European representatives of the genus Palaemon, including the type species P. adspersus, are consistently supported as a clade with confidence values above 90% (Fig. 1). The three haplotypes of P. elegans constitute a well supported clade (99, 100%) which appears distinct from both European and American Atlantic species of Palaemon.

Among the remaining taxa, the four American species of Palaemonetes represent the next split and are confirmed as monophyletic group with high support in the two reconstruction methods (100, 92%). The next group to branch off are the four European-Turkish species of Palaemonetes again with high confidence values (100, 96%). The position of Palaemonetes sinensis, the only Asian representative of Palaemonetes in this study, is poorly resolved: only the Bayesian approach supports a clustering within the remaining Asian-Australian ingroup clade. What can be said is that this species is well separated from the two Asian representatives of the genus Palaemon, P. macrodactylus and P. serrifer (Stimpson, 1860), which form a strong monophyletic assemblage (100% support). On the other hand, the four Australian taxa are clustered with representatives of the genera being phylogenetically mixed. These species cluster in exactly the same way as in Murphy & Austin´s (2003, 2005) analyses, although in their tree Palaemon intermedius is still considered to be a representative of Macrobrachium.

DISCUSSION

Our results confirm the paraphyly of Palaemon and Palaemonetes, as already pointed out by Murphy & Austin (2003, 2005) based on Australian representatives. The really groundbreaking addition of the present study, and a parallel one by Ashelby et al. (2012), is that this paraphyly of Palaemon and Palaemonetes is now expanded to a world-wide scale and includes the type species of the two genera. The different monophyletic clades obtained in our analyses (Fig. 1) mostly reflect the geographic distribution of these species, but overall increase the taxonomic chaos. When considering only the Turkish-European species, the genus Palaemonetes is a well supported monophyletic taxon, including the type species Pt. varians, whereas the genus Palaemon, with the type species P. adspersus, is only monophyletic with the exclusion of P. elegans, but including the South African Atlantic species Palaemon peringueyi. This is in certain taxonomic agreement with the fact that Palaemon elegans is the type species of the subgenus, Palaeander Holthuis, 1950. A recent molecular study by Reuschel et al. (2010) suggests that P. elegans may actually consist of two species, of which one is originally endemic to the Mediterranean and Black seas and later introduced into the Baltic Sea. The separate position of two other species of the subgenus Palaeander, P. northropi (Rankin, 1898), and P. floridanus Chace, 1942, do not support the monophyly of this subgenus. In contrast, in the analyses by Ashelby et al. (2012), these species cluster together with different representatives in two separate analyses (16S rDNA and histone H3). However, a fourth species of the subgenus Palaeander, P. semmelinkii (De Man, 1881) seems to be unrelated to P. elegans, P. northropi and P. floridanus (see Ashelby et al., 2012). The validity of this taxon, which was based on the number of mandibular palp segments (2 instead of 3) is thereby definitely refuted.

Overall, the phylogenetic results support either lumping all these species into a single genus Palaemon or alternatively taxonomic separation of these geographic clusters of species into different taxa. However, due to the limited number of species included in the present study (20 out of 72 known species, De Grave & Fransen, 2011) and the limited confidence that can be given to a single mitochondrial marker, no taxonomic conclusions can be drawn. Comparison with the study by Ashelby et al. (2012) which included more representatives of both genera from different continents, confirms the occurrence of regional monophyletic units but also shows that most clusters are not stable enough to warrant monophyletic lineages. Nevertheless, it would be premature to carry out taxonomic revisions of these genera at regional scale, with the consequent establishment of new subgenera or genera for these geographic groupings. Furthermore, it is questionable if useful morphological characters will be found to support them. Therefore the more stable current solution would be to lump all these species, as well as representatives of Coutierella and Exopalaemon (see Ashelby et al. 2012), within the genus Palaemon sensu lato. On the other hand, Ashelby et al. (2012) also showed that some species currently included in the genus Palaemon will have to be excluded in order to make the genus monophyletic

The morphological feature used to separate the genera Palaemonetes and Palaemon (presence / absence of the mandibular palp) is phylogenetically unreliable and plastic as mentioned by Murphy & Austin (2003), and also previously indicated by Fujino & Miyake (1968) and Chace (1972). Future studies should not only be based on molecular data, but also include new evidence from morphology, including that of larvae. In larval morphology, until now few constant differences in zoeal morphology allow separation of Palaemon from Palaemonetes (see Fincham & Figueras, 1986; Knowlton & Vargo, 2004). According to Knowlton & Vargo (2004) “the intergeneric similarities and intrageneric ambiguities of larval form that resulted from our analysis call into question the validity of these two genera, as demarcated at present”. Some differences, especially in the number of larval stages, could reflect adaptations to freshwater, brackish or marine waters rather than phylogenetic origin. However, intraspecific plasticity in the number of zoeal stages, which characterizes palaemonid larval development, complicates comparative analysis.

In the case of the American Palaemonetes, Collins (1998) pointed out that freshwater species could have arisen from marine ancestors several times independently in time and space, and thus do not have to form a monophyletic lineage. Our current results, however, support a common ancestry for four of the species.

In contrast, in a multilocus molecular phylogenetic study of species of Macrobrachium from Southeast and East Asia, Wowor et al. (2009) revealed five independent invasion events from marine to freshwater habitats (reflected in the loss of a marine larval phase) as well as at least another two or three independent events to adaptation to cave habitats. However, this does not affect the taxonomy of the genus Macrobrachium and its monophyly is only under question due to the inclusion of the older American genus Cryphiops (see Pileggi & Mantelatto, 2010; Dennenmoser et al., 2010). Also Ashelby et al. (2012) emphasize the repeated and independent colonization of freshwater habitats from within the Palaemon sensu lato complex, postulating physiologically plastic ancestors.

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TABLE I

Species of palaemonid shrimps included in this study with localities and accession numbers of museum collections where voucher specimens are deposited, and genetic database entries of the corresponding 16S mtDNA sequences. Abbreviations: CCDB: Crustacean Collection of the Biology Department of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo; ICM, Instituto de Ciencias del Mar, Barcelona, Spain; RNMH, Naturalis Museum, Leiden, The Netherlands; SMF, Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt a.M., Germany. Symbols: (?), no specified locality; (-), no voucher specimen data.

Species	Locality	Catalogue No.	GenBank accession number
Macrobrachium australiense Holthuis, 1950	Australia: NSW, Murray River	(-)	AF439521
Macrobrachium carcinus (Linnaeus, 1758)	Tropical America (?)	(-)	AY282779
Macrobrachium rosenbergii (De Man, 1879)	Australia: (?)	(-)	AY282774
Palaemon adspersus	Spain: Cádiz: San Pedro Estuary	ICMD-20111108-01	JQ042293
Palaemon elegans (Type I)	Germany: Helgoland	ICMD-20111108-02	JQ042298
Palaemon elegans (Type II)	Spain: Almería	(-)	HE573179
Palaemon elegans (Type III)	Black Sea: Bulgaria	(-)	HE573180
Palaemon floridanus	USA: East Florida, Fort Pierce	UMML 32.9620	GQ227820

Palaemon intermedius	Australia: Victoria, Hopkins River	(-)	AF439515
Palaemon longirostris	Spain: Cádiz: Guadalquivir Estuary	ICMD-20111108-03	JQ042292
Palaemon macrodactylus	Spain: Cádiz: Guadalquivir Estuary	ICMD-20111108-04	JQ042297
Palaemon northropi	Brasil: São Paulo: São Sebastião	CCDB2623	HM352424
Palaemon peringueyi	South Africa: Suider Kust	ICM-312/1991	JQ042296
Palaemon serenus Heller, 1862	Australia: Western Australia, Swan River Estuary	(-)	AF439519
Palaemon serratus	Spain: Cádiz: San Pedro Estuary	ICMD-20111108-05	JQ042291
Palaemon serrifer	Asia: (?)	pending	JQ042295
Palaemon xiphias	Spain: Cádiz: Cádiz Bay	ICMD-115/2004	JQ042294
Palaemonetes antennarius	Italy: Toscana, Arno River	SMF- 40683	JQ042306
Palaemonetes argentinus Nobili, 1901	Argentina: Mar Chiquita Lagoon	SMF-40685	JQ0423305
Palaemonetes atrinubes Bray, 1976	Australia: Western Australia, Swan River	(-)	AF439520
Palaemonetes australis Dakin, 1915	Australia: Western Australia, Swan River	(-)	AF439517
Palaemonetes pugio Holthuis, 1949	USA: South Carolina, Combahee River	ICMD-20111108-06	JQ042304
Palaemonetes sinensis	East Asia:(?)	(-)	DQ194970
Palaemonetes texanus Strenth, 1976	USA: Texas, Riviera, Los Olmos Creek	SMF-40684	JQ042303

Palaemonetes turcorum	Turkey: near Ankara	RMNH-D 13971	JQ042302
Palaemonetes varians	Spain: Cádiz: Guadalquivir Estuary	ICMD-20111108-07	JQ042301
Palaemonetes vulgaris (Say, 1881)	USA: South Carolina, Fort Johnson, Charleston	ICMD-20111108-08	JQ042300
Palaemonetes zariquieyi	Spain: Valencia: Sollana	ICMD-20111108-09	JQ0422299

TABLE II

Results of the Shimodaira-Hasegawa tests, using the difference of log-likelihoods for unconstrained (U) and constrained (C) (monophyletic genera) majority-rule consensus trees obtained using maximum parsimony (MP) and Bayesian (BI) approaches. * P value for the SH test significant at 0.01.

Tree	Likelihood score	P
C-MP	4238.33	0.003*
U-MP	4217.02	0.039
C-BI	4228.20	0.011*
U-BI (best)	4191.65	

FIGURE CAPTION

Fig. 1. Topology of Bayesian Inference tree with confidence values of Bayesian, and maximum parsimony reconstructions based on 536 basepairs of the 16S rRNA gene, showing inferred phylogenetic relationships within selected representatives of the genera Palaemon, Palaemonetes and Macrobrachium (underlined names represent the respective type species of the three genera). Numbers next to nodes indicate Bayesian posterior probabilities ($Pp \geq 50$) and MP bootstrap support (only values above 50% shown).