

## Regional scale speciation reveals multiple invasions of freshwater in Palaemoninae (Decapoda)

CHRISTOPHER W. ASHELBY, TIMOTHY J. PAGE, SAMMY DE GRAVE, JANE M. HUGHES & MAGNUS L. JOHNSON

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The generic level, systematic relationship in Palaemoninae was inferred from analyses based on the mitochondrial 16S rDNA and nuclear Histone (H3) genes, primarily focussed on the genera *Palaemon* and *Palaemonetes*, as previous morphological and molecular studies indicated potential paraphyly in some genera. *Palaemonetes*, *Exopalaemon*, *Coutierella* and certain *Palaemon* recover as a strongly supported monophyletic clade, but with the exception of *Palaemon concinnus*, *P. pandaliformis* and *P. gracilis*. Within this clade, six major clades are identified with geographic relationships appearing stronger than generic relationships. The data strongly suggest that *Palaemon*, *Palaemonetes*, *Exopalaemon* and *Coutierella* are synonymous and that the morphological characters currently used to define these genera require re-evaluation. Freshwater species are not closely related to each other, but instead group with geographically close marine species, suggesting multiple invasions of freshwater by physiologically plastic ancestors rather than a single colonisation event with subsequent speciation.

Corresponding author: Christopher W. Ashelby, CEMS, University of Hull, Scarborough Campus, Filey Road, Scarborough, YO11 3AZ, UK; Thomson Unicomarine, 7 Diamond Centre, Works Road, Letchworth Garden City, SG6 1LW, UK. E-mail: cbrisasbelby@unicomarine.com

Timothy J. Page, Australian Rivers Institute, Nathan Campus, Griffith University, Queensland 4111, Australia. E-mail: penguintim@hotmail.com

Sammy De Grave, Oxford University Museum of Natural History, Parks Road, Oxford, OX1 3PW, UK. E-mail: sammy.degrave@oum.ox.ac.uk

Jane M. Hughes, Australian Rivers Institute, Nathan Campus, Griffith University, Queensland 4111, Australia. E-mail: jane.bughes@griffith.edu.au

Magnus L. Johnson, CEMS, University of Hull, Scarborough Campus, Filey Road, Scarborough, YO11 3AZ, UK. E-mail: m.johnson@hull.ac.uk

### Introduction

The caridean shrimp family Palaemonidae contains two currently recognised subfamilies: the morphologically diverse and strictly marine Pontoniinae Kingsley 1879 and the Palaemoninae Rafinesque, 1815, which are the subject of this study. The Palaemoninae are found in marine, brackish and freshwater in tropical and temperate regions and are all free-living with a relatively conservative gross morphology. The Palaemoninae contains 21 recognised genera (De Grave & Fransen 2011), numerically dominated by *Macrobrachium* Spence Bate, 1868, that are restricted to freshwater and brackish water. *Macrobrachium* have been intensively studied because of their diversity in

easily accessible habitats and have been the subject of many molecular phylogenetic studies (e.g. Murphy & Austin 2003, 2004, 2005; Liu *et al.* 2007; Chen *et al.* 2009; Wowor *et al.* 2009; Pileggi & Mantelatto 2010). The second and third most speciose genera in the subfamily are *Palaemon* Weber, 1795 and *Palaemonetes* Heller, 1869, with 41 and 31 species, respectively, (De Grave & Fransen 2011). These have worldwide distributions in both tropical and temperate regions and have representatives in marine, brackish and freshwaters, with two species of *Palaemon* reported from all three water types. Both *Palaemon* and *Palaemonetes* are well represented in the literature across many scientific disciplines and several molecular studies

have been conducted at the population level within certain species of each genus (e.g. Berglund & Lagercrantz 1983; Fidhiany *et al.* 1988; Teske *et al.* 2007; Reuschel *et al.* 2010; Chaves-Campos *et al.* 2011). However, neither genus has been subjected to the same level of phylogenetic analysis as *Macrobrachium*. The only in-depth phylogenetic study of these genera to date is the morphological cladistic study of Pereira (1997). Despite this limited treatment, previous studies have hinted at probable paraphyly in these genera (Pereira 1997; Murphy & Austin 2004). In contrast, within *Palaemonetes*, Strenth (1976) suggested that the majority of the freshwater species formed a strongly supported monophyletic clade based on the morphology of the larval antennal scale. A thorough molecular treatment is thus long overdue.

As many Palaemoninae are highly conservative in morphological features (Walker & Poore 2003; Short 2004) problems exist in delineating genera. The current genus-level classification of the Palaemoninae relies heavily on a differential combination of a small number of characters, prime amongst which is the presence or absence of the mandibular palp. *Palaemonetes* is currently separated from *Palaemon* solely through the absence of a mandibular palp vs. presence in the latter genus. This character is widely used as a diagnostic character in numerous genera across several caridean families, although invariably in combination with several other characters. Early on, Kemp (1925) suggested that a single negative character may not be seen to support an entire genus and Bruce (1989) concluded that the presence or absence of a mandibular palp as the single difference does not seem to be an adequate character on which to separate genera. Furthermore, variability in the presence or absence of the mandibular palp as well as the number of segments has been demonstrated in species of each genus, most notably by Fujino & Miyake (1968), Chace (1972), Bray (1976) and Carvacho (1979). Despite this inherent variability in the only diagnostic morphological character, *Palaemon* and *Palaemonetes* have been maintained as separate genera in all recent classifications of Decapoda (e.g. De Grave & Fransen 2011).

Owing to the conflicting evidence from these previous studies, this study was conceived to elucidate the relationship between *Palaemon* and *Palaemonetes*, including related Palaemoninae genera, effectively testing their reciprocal monophyly.

## Materials and methods

### *Taxon sampling*

Thirty-one species of *Palaemonetes* (hereafter abbreviated to *Pt.*) and 41 species of *Palaemon* (hereafter abbreviated to *P.*) are currently recognised. Of these, specimens of 11 (35%) species of *Palaemonetes* and 25 (61%) species of

*Palaemon* were sequenced for this study, covering a wide geographical range and a variety of habitats and lifestyles. Additional sequences were obtained from GenBank. In some cases, multiple specimens of a species from different locations were sequenced or additional GenBank sequences obtained, and, where these sequences were clearly divergent, indicating possible cryptic species, we have included more than one exemplar in the final analyses (designated by different numbers).

Other Palaemoninae included in this study comprised two species of *Macrobrachium*, four species of *Exopalaemon* and one species each of *Brachycarpus*, *Coutierella*, *Creaseria*, *Cryphiops*, *Leptocarpus*, *Nematopalaemon*, *Leander* and *Urocaridella*. Two species of two genera belonging to the Kakaducarididae were also included and the tree was rooted with *Anchistiooides antiguensis* (Schmitt, 1924) (Anchistiooididae).

Details of the species used in the analysis can be found in Table 1. All material newly sequenced for this study is accessioned in the Zoological Collections of Oxford Museum of Natural History (OUMNH-ZC). DNA samples were extracted from fifth pleopods, where possible, to avoid damage of any taxonomically informative morphological characters.

### *Molecular data retrieval*

Total genomic DNA was extracted and gene fragments sequenced in both directions as per Page *et al.* (2008). Two genes were sequenced, the mitochondrial, ribosomal gene 16S rDNA (16S) and a nuclear gene, protein coding Histone (H3). These markers were selected primarily because they give resolution at species and generic levels, which is the focus of this study. The 16S rDNA gene has both fast and slowly evolving regions and, therefore, can provide useful information across a broad taxonomic spectrum (Murphy & Austin 2004). As a nuclear gene, H3 is more conserved and is likely to provide resolution at deeper levels than 16S. Forward primers for the 16S polymerase chain reaction were 16S-F-Car (von Rintelen *et al.* 2007) or 16Sar (Palumbi *et al.* 1991), and reverse primers 16S-R-Car or 16S-R-Car1 (von Rintelen *et al.* 2007) or 16Sbr (Palumbi *et al.* 1991). Primers for Histone were H3-F and H3-R (Colgan *et al.* 1998).

### *Phylogenetic analysis*

Both genes were analysed separately and in combined analyses. The datasets were aligned with SEQUENCHER version 4.1.1 b1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) at default settings. The best-fit models of molecular evolution (Akaike's Information Criterion) were selected separately for both gene regions within each dataset and for the combined dataset as a whole, using MODELTEST

Table 1 Palaemonid species used in the analyses, including H3 and 16S GenBank accession numbers and provenance of specimens

Taxon	Museum accession number	Sampling location	Habitat	GenBank accession number		GenBank reference
				16s	H3	
<i>Anchistoides antiguenis</i> (Schmitt, 1924)	–	Caribbean	Marine	EU920911	EU921043	Toon et al. (2009)
<i>Brachycarpus biurguiculatus</i> (Lucas, 1846)	OUMNH-ZC 2006-10-0011	Punto Vargas, Costa Rica	Marine	JN674323	JN674391	Toon et al. (2009)
<i>Coutierella tonkinensis</i> Sollaund, 1914	OUMNH-ZC 2003-07-0001	Mai Po, Hong Kong	Brackish	JN674324	EU921053	Porter et al. (2005)
<i>Creaseria motleyi</i> (Creaser, 1936)	–	Americas	Freshwater	EU449001	DQ079671	Porter et al. (2005)
<i>Cryphiops (Cryphiops) caementarius</i> (Molina, 1782)	–	Americas	Freshwater	DQ079711	DQ079672	Yang & Chen (unpub)
<i>Exopalaemon amandalei</i> (Kemp, 1917)	–	Asia	Freshwater	DQ647670	–	Chen et al. (2009)
<i>Exopalaemon carinicauda</i> (Holthuis, 1950)	OUMNH-ZC 2009-16 0005	Mai Poi, Hong Kong	Brackish	JN674325	JN674393	Chen et al. (2009)
<i>Exopalaemon modestus</i> (Heller, 1862)	–	Taiwan	Freshwater	EU493144	–	Chen et al. (2009)
<i>Exopalaemon orientis</i> (Holthuis, 1950)	OUMNH-ZC 2010-02-0078	Pingtung County, Taiwan	Marine	JN674326	JN674387	Page et al. (2008)
<i>Kakaducaris glebra</i> Bruce, 1993	–	Australia	Freshwater	EF588318	EU249461	Page et al. (2008)
<i>Leander tenuicornis</i> (Say, 1818)	OUMNH-ZC 2009-05-0004	Vaca Key, Florida, USA	Marine	JN674327	JN674388	Page et al. (2008)
<i>Leptocarpus potamiscus</i> (Kemp, 1917)	OUMNH-ZC 2009-03-0006	Sungai Petani river, Malaysia	Fresh & Brackish	JN674328	JN674392	Page et al. (2008)
<i>Leptopalaemon gagadju</i> Bruce, 1993	–	Australia	Freshwater	EF588310	EU249459	Page et al. (2008)
<i>Macrobrachium australiense</i> Holthuis, 1950	–	Australia	Freshwater	EF588317	EU249460	Page et al. (2008)
<i>Macrobrachium lar</i> (Fabricius, 1798)	–	French Polynesia	Freshwater	EF588316	EU249462	Page et al. (2008)
<i>Nematopalaemon tenuipes</i> (Henderson, 1893)	NTOU	Pingtung County, Taiwan	Marine	JN674329	JN674382	Page et al. (2008)
<i>Palaemon adspersus</i> Rathke, 1837	OUMNH-ZC 2004-16-0001	Algarve, Portugal	Marine	JN674330	JN674360	Wowor et al. (2009)
<i>Palaemon affinis</i> H. Milne Edwards, 1837	OUMNH-ZC 2004-12-0002	Mission Bay, New Zealand	Marine	–	JN674379	Wowor et al. (2009)
<i>Palaemon concinnus</i> Dana, 1852	OUMNH-ZC 2010-02-0079	Pingtung County, Taiwan	Marine, Brackish and Freshwater	JN674331	JN674363	Wowor et al. (2009)
<i>Palaemon debilis</i> Dana, 1852	–	Indonesia	Marine, Brackish and Freshwater	FM958134	–	Wowor et al. (2009)
<i>Palaemon debilis</i> Dana, 1852	OUMNH-ZC 2009-21-0020	Wailoa, Hawaii	Marine, Brackish and Freshwater	JN674332	JN674375	Wowor et al. (2009)
<i>Palaemon dolospinus</i> Walker & Poore, 2003	OUMNH-ZC 2009-25-0002	Port Phillip Bay, Victoria, Australia	Marine	JN674333	JN674389	Bracken et al. (2009)
<i>Palaemon elegans</i> Rathke, 1837	OUMNH-ZC 2010-02-0004	The Fleet, England	Marine	EU868696	JN674378	Bracken et al. (2009)
<i>Palaemon floridanus</i> Chace, 1942	–	USA	Marine	GQ227820	–	Baeza (2010)
<i>Palaemon gracilis</i> (Smith, 1871)	OUMNH-ZC 2009-18-0016	Miraflores Locks, Panama	Brackish	JN674334	JN674362	Bracken et al. (2009)
<i>Palaemon guangdongensis</i> Liu, Liang & Yan, 1990	OUMNH-ZC 2009-16-0004	Pearl River mouth, south China	Marine	JN674335	JN674394	Bracken et al. (2009)
<i>Palaemon intermedius</i> (Stimpson, 1860)	OUMNH-ZC 2009-09-0004	Loders Creek, Gold Coast, Australia	Marine	JN674336	JN674361	Bracken et al. (2009)
<i>Palaemon litoreus</i> (McCulloch, 1909)	OUMNH-ZC 2004-14-0019	Rottnest Island, Western Australia	Marine	JN674337	JN674369	Bracken et al. (2009)
<i>Palaemon longirostris</i> H. Milne Edwards, 1837	OUMNH-ZC 2009-07-0003	River Thames, England	Brackish	–	JN674386	Bracken et al. (2009)
<i>Palaemon longirostris</i> H. Milne Edwards, 1837	OUMNH-ZC 2001-09-0052	River Thames, England	Brackish	JN674338	–	Mitsuhashi et al. (2007)
<i>Palaemon macrodactylus</i> Rathbun, 1902	OUMNH-ZC 2001-09-0053	River Thames, England	Brackish	DQ642875	JN674385	Mitsuhashi et al. (2007)
<i>Palaemon northropi</i> (Rankin, 1898)	OUMNH-ZC 2008-14-0129	Bocas Del Toro, Panama	Marine	–	JN674380	Mitsuhashi et al. (2007)
<i>Palaemon ortmanni</i> Rathbun, 1902	OUMNH-ZC 2003-06-0002	Seto Inland Sea, Japan	Marine	JN674339	–	Mitsuhashi et al. (2007)
<i>Palaemon pacificus</i> (Stimpson, 1860)	OUMNH-ZC 2008-18-0024	Keelung Ho-Ping Island Park, Taiwan	Marine	JN674340	–	Wowor et al. (2009)
<i>Palaemon pandalliformis</i> (Stimpson, 1871)	OUMNH-ZC 2008-14-0020	Rio Oeste, Panama	Freshwater	JN674341	JN674364	Wowor et al. (2009)

Table 1 Continued

Taxon	Museum accession number	Sampling location	Habitat	GenBank accession number		GenBank reference
				16s	H3	
<i>Palaemon paucidens</i> De Haan, 1844	OUMNH-ZC 2003-06-0003	Utanogawa River, Japan	Marine, Brackish and Freshwater	JN674342	JN674383	
<i>Palaemon peringueyi</i> (Stebbing, 1915)	OUMNH-ZC 2003-09-0001	Kariega River Estuary, South Africa	Marine	JN674343	JN674365	
<i>Palaemon ritteri</i> Holmes, 1895	OUMNH-ZC 2009-18-0011	Coiba, Panama	Marine	JN674344	JN674367	
<i>Palaemon semmelinki</i> (De Man, 1881)	–	Fly River at Sturt Island (Siolowa), Papua New Guinea	Marine	JN674345	JN674400	
<i>Palaemon serenus</i> Heller, 1862	OUMNH-ZC 2009-09-0011	Wellington Point, Queensland, Australia	Marine	JN674346	JN674368	
<i>Palaemon serratus</i> Pennant, 1777)	OUMNH-ZC 2010-02-0002	River Orwell, England	Marine	–	JN674377	
<i>Palaemon serrifer</i> (Stimpson, 1860)	OUMNH-ZC 2005-04-0001	Busan, South Korea	Marine	JN674347	JN674366	
<i>Palaemon serrifer</i> (Stimpson, 1860)	OUMNH-ZC 2004-13-0004	Labrador Beach, Singapore	Marine	–	JN674370	
<i>Palaemon serrifer</i> (Stimpson, 1860)	OUMNH-ZC 2010-02-0077	Pingtung County, Taiwan	Marine	JN674348	JN674373	
<i>Palaemon xiphias</i> Risso, 1816	OUMNH-ZC 2003-02-0001	Blanes, Spain	Marine	–	JN674376	
<i>Palaemon xiphias</i> Risso, 1816	OUMNH-ZC 2010-02-0001	Ebro Delta, Spain	Marine	JN674349	–	
<i>Palaemonetes antennarius</i> H. Milne Edwards, 1837	OUMNH-ZC 2003-03-0002	Epta Piges River, Rhodos Island, Greece	Freshwater	JN674350	JN674395	
<i>Palaemonetes argentinus</i> Nobili, 1901	OUMNH-ZC 2002-03-0001	Mar del Plata, Argentina	Freshwater	JN674351	JN674397	
<i>Palaemonetes atrinubus</i> Bray, 1976	OUMNH-ZC 2009-21-0012	Couran Cove, South Stradbroke Is., Queensland, Australia	Marine	JN674352	JN674399	Murphy & Austin (2003)
<i>Palaemonetes atrinubus</i> Bray, 1976	–	Australia	Marine	AF439520	–	
<i>Palaemonetes australis</i> Dakin, 1915	OUMNH-ZC 2009-21-0010	Rosa, Blackwood River, Western Australia, Australia	Freshwater	JN674353	JN674401	
<i>Palaemonetes intermedius</i> Holthuis, 1949	OUMNH-ZC 2002-14-0001	Manatee Bay, Florida, USA	Brackish	JN674354	JN674396	
<i>Palaemonetes paludosus</i> (Gibbes, 1850)	OUMNH-ZC 2004-14-0002	Jefferson County, Florida, USA	Freshwater	–	JN674381	
<i>Palaemonetes pugio</i> Holthuis, 1949	OUMNH-ZC 2009-07-0005	Ocean Springs, Mississippi, USA	Brackish	JN674355	JN674371	
<i>Palaemonetes schmitti</i> Holthuis, 1950	OUMNH-ZC 2002-18-0001	Gulf of Nicoya, Pacific coast of Costa Rica	Marine	JN674356	JN674390	
<i>Palaemonetes sinensis</i> (Sollaud, 1911)	OUMNH-ZC 2003-06-0005	Bait store, Japan	Freshwater	–	JN674384	
<i>Palaemonetes varians</i> (Leach, 1813)	OUMNH-ZC 2009-07-0009	River Blackwater, England	Brackish	JN674357	JN674374	
<i>Palaemonetes vulgaris</i> (Say, 1818)	OUMNH-ZC 2009-07-0006	Ocean Springs, Mississippi, USA	Marine	JN674358	JN674372	
<i>Urocaridella antonbruunii</i> (Bruce, 1967)	OUMNH-ZC 2010-02-0073	Kenting, Taiwan	Marine	JN674359	JN674398	

NTOU, National Taiwan Ocean University; OUMNH-ZC, Oxford Museum of Natural History Zoological Collections.

version 3.06 (Posada & Crandall 1998) in PAUP\* version 4.0 b10 (Swofford 2002). We carried out two different forms of phylogenetic inference on the combined dataset; maximum likelihood analysis (PHYML version 2.4.4; Guindon & Gascuel 2003) and parsimony (TNT version 1.1; Goloboff *et al.* 2008) and both were bootstrapped 1000 times.

## Results

### Sequences

We obtained 37 new 16S rDNA sequences and 42 new H3 sequences (Table 1), a further 14 16S and 10 H3 GenBank sequences were included. Five species were only represented by a 16S sequence: *Exopalaemon annandalei*, *E. modestus*, *Palaemon floridanus*, *P. ortmanni* and *Palaemonetes atrinubes* 1, whilst six only by H3: *P. affinis*, *P. northropi*, *P. serratus*, *P. serrifer* 2, *Pt. paludosus* and *Pt. sinensis*.

The H3 sequences were 328 base pairs (bp), and the 16S dataset was 418 bp (aligned) and corresponds to positions 11365 – 11764 of the *Macrobrachium dacqueti* (Sunier, 1925) mitochondrial genome (accession number NC006880, Miller *et al.* 2005).

### Phylogenetic analyses

For the combined and 16S phylogenetic analyses, Modeltest selected a General Time Reversible model of evolution, and a Hasegawa, Kishino and Yano model for the H3 analysis. Chi-square tests of homogeneity of base frequencies across ingroup taxa found no significant differences ( $P > 0.99$ ). PHYML recovered a single phylogenetic tree for each dataset (combined log score = -8917.50; 16S = -6587.95; H3 = -2686.67). TNT recovered 16 equally parsimonious topologies of 1851 steps for the combined dataset.

The maximum likelihood phylograms produced for 16S (Fig. 1), H3 (Fig. 2) and the combined analysis (Fig. 3) are given here. Most clades were recovered with high bootstrap support, but many deeper nodes were poorly supported. Only bootstrap values >50% are shown on the trees.

The well-supported clades obtained in the individual gene trees were very similar (Figs 1 and 2) but some differences were observed in the topology of the poorly supported branches.

### Analyses of the major clades

In all three analyses, *Palaemonetes*, *Exopalaemon*, *Coutierella* and certain *Palaemon* recover as a strongly supported single monophyletic clade (the 'Palaemon' Clade, Fig. 3) with the exception of *Palaemon concinnus*, *P. gracilis* and *P. pandaliformis*. *Palaemon gracilis* and *P. pandaliformis* are closely related to each other and recover with strong support (Fig. 3) whilst *P. concinnus* has no clear affinity to any of the taxa included in our analysis.

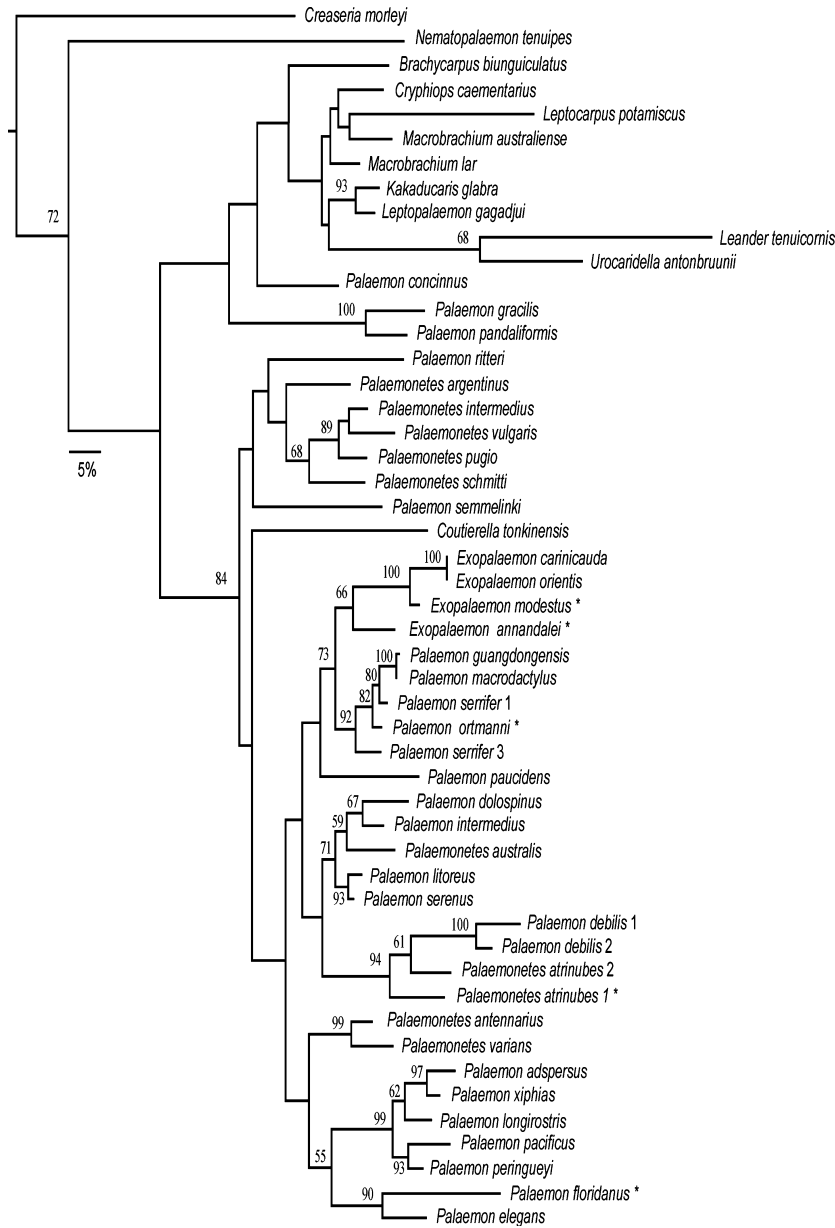
Within the 'Palaemon' clade, six smaller clades can be identified from the combined analysis (Fig. 3), most of which broadly reflect the current geographic distribution of the constituent species. For ease of reference, these clades are referred to by the current geographic region of the majority of their constituent species.

The Asia 1 Clade comprises just two species: *Coutierella tonkinensis* and *Palaemon paucidens* but has low support compared with many of the other clades in this analysis (Fig. 3) and was not retrieved as monophyletic in the 16S analysis (Fig. 1).

The American Clade comprises six species from both North and South America, distributed on both sides of the continent in marine, brackish and freshwater. The relationship between *Pt. intermedius*, *Pt. pugio*, *Pt. vulgaris* and *Pt. schmitti* is well supported, whilst the inclusion of *P. ritteri* and *Pt. argentinus* in this clade is less well supported (Fig. 3). The American Clade does not recover as monophyletic in the H3 analysis (Fig. 2) because of the exclusion of *P. ritteri*. *Palaemonetes paludosus*, a freshwater species from the Americas, has no close affinity to the American Clade based on the H3 analysis (Fig. 2) and is of uncertain placement.

Eight currently recognised species of *Palaemon* and *Exopalaemon* are included in the Asia 2 Clade. However, high levels of genetic divergence were noted amongst material identified as *P. serrifer*, indicating the existence of three probable cryptic species. Conversely, material identified as *Exopalaemon carinicauda* and *E. orientis* had very low genetic diversity suggesting that they are very recently diverged or possibly represent conspecific ecotypes. *Palaemon ortmanni*, *Exopalaemon modestus* and *E. annandalei* were represented by 16S sequences only, whilst *P. serrifer* 2 from Singapore was only represented in the H3 tree but their inclusion in this clade is well supported. Within the Asia 2 Clade, *Exopalaemon* spp. recover as a strongly supported monophyletic clade (Fig. 3). *Palaemon semmelinki*, which is found in south Asia, is of uncertain placement in our analyses but does not have a strong relationship to either Asian clade (Fig. 3). It is the only species of the 'Palaemon' Clade not to have a well-supported relationship to any of the six clades identified in the combined analysis. Likewise, *Palaemonetes sinensis* does not appear closely related to either the Asia 1 or Asia 2 Clade based on the H3 tree (Fig. 2). These two species may represent further Asian lineages.

The five constituent species of the Australian Clade (*P. dolospinus*, *P. intermedius*, *P. litoreus*, *P. serenus*, and *Pt. australis*) form a well-supported group (Fig. 3). Another Australian species, *Pt. atrinubes*, does not form part of the Australian clade, as already speculated in the original description of the species (Bray 1976) and *Palaemon affinis* from New Zealand, represented by a H3 sequence only,



**Fig. 1** Maximum likelihood phylogram of 16S analysis. Maximum likelihood bootstrap values are given; only values >50% are shown. \*16S only, no H3.

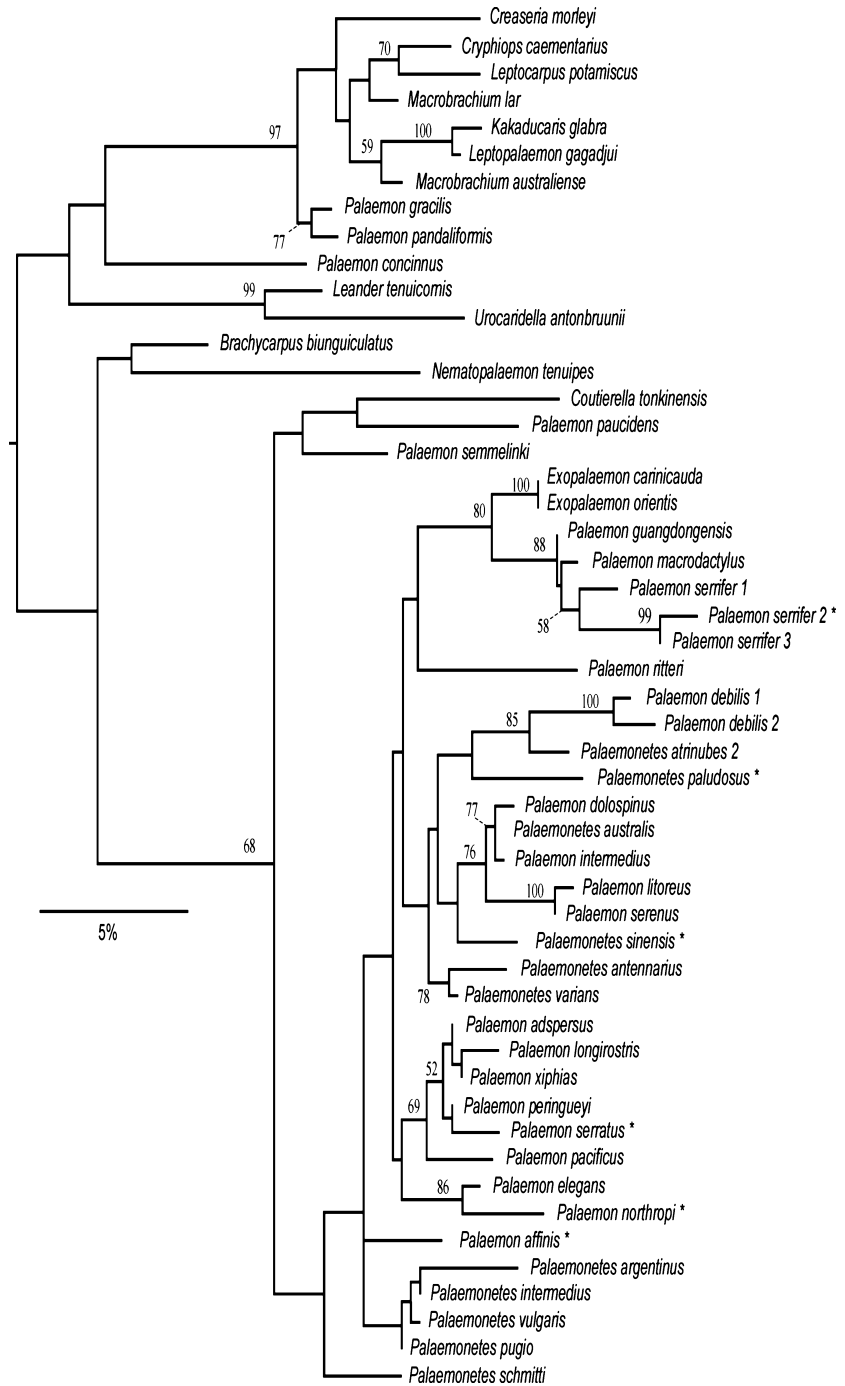
was of uncertain placement in our analysis but does not seem to have a strong affinity to the Australian species (Fig. 2).

The Atlanto-Pacific Clade comprises three smaller clades. The European *Palaemon adspersus* (the type species of *Palaemon*), *P. longirostris*, *P. serratus* and *P. xiphias* form a well-supported clade together with the South African *P. peringueyi* and the Indo-Pacific *P. pacificus*. *Palaemon elegans* is excluded from the clade containing the other European species and instead forms a clade with the western Atlantic species *P. floridanus* (Fig. 1) and *P. northropi*

(Fig. 2). A strong relationship between the two European species of *Palaemonetes* included in our analysis, *Pt. varians* (the type species of *Palaemonetes*) and *Pt. antennarius*, is demonstrated but their relationship to the other species of the Atlanto-Pacific clade is less well supported (Fig. 3).

*Palaemon debilis* and *Palaemonetes atrinubes* form a well-supported clade, the Indo-Pacific Clade, in all analyses (Figs 1–3). Although this clade contains two named species, *Palaemon debilis* and *Pt. atrinubes*, sequences for both of these species showed high levels of divergence and the existence of cryptic species in both taxa is postulated.

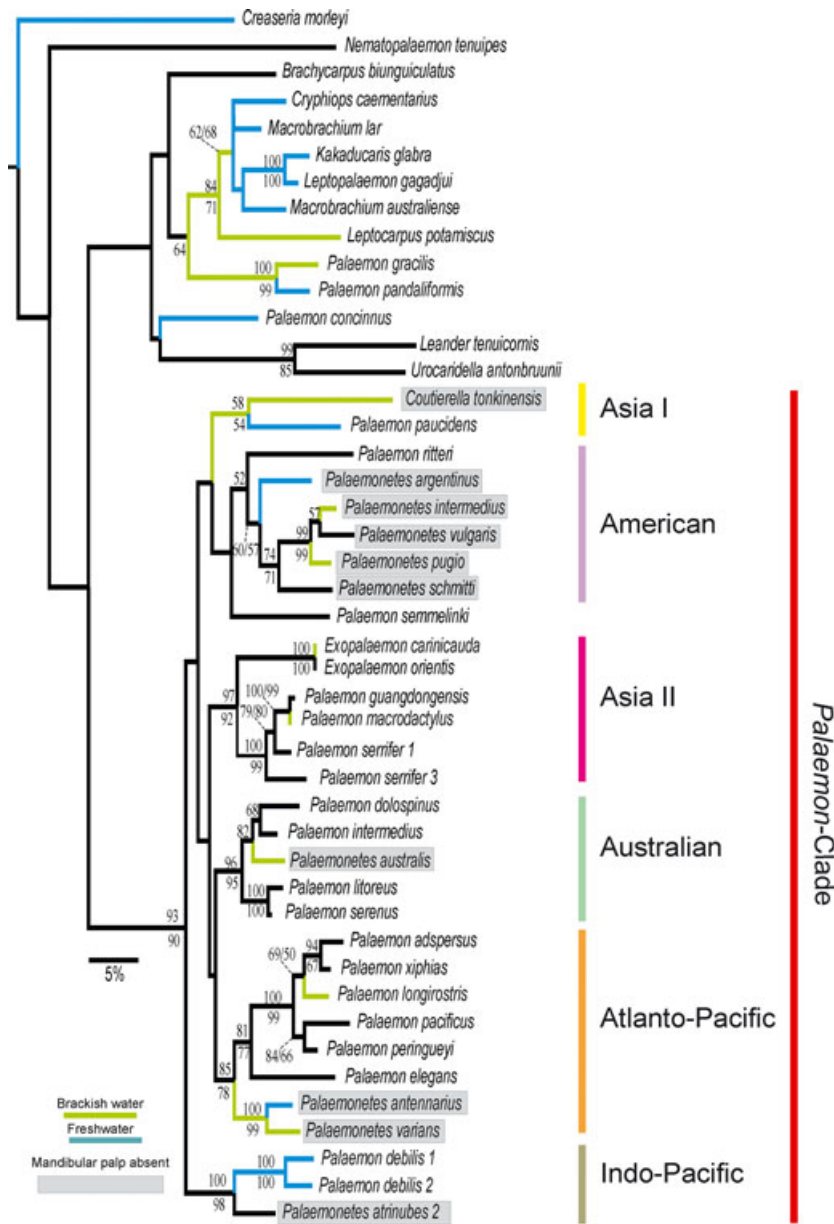




**Fig. 2** Maximum likelihood phylogram of H3 analysis. Maximum likelihood bootstrap values are given; only values >50% are shown. \*H3 only, no 16S.

*Kakaducaris glabra* and *Leptopalaemon gagadjui* (family Kakaducarididae) form a strongly supported clade nested within *Macrobrachium*. Amongst the other included Palaemoninae, *Cryphiops* (*Cryphiops*) *caementarius* forms a well-supported clade with *Macrobrachium*, *Leptocarpus potamiscus* and the kakaducarid genera; *Urocaridella antonbruunii* forms

a well-supported clade with *Leander tenuicornis*; however, the long branch length indicates that the relationship between these species is not particularly close; whilst *Nematopalaemon tenuipes*, *Creaseria morleyi* and *Brachycarpus biunguiculatus* have no obvious affinities with any other outgroup taxa in the combined tree (Fig. 3).



**Fig. 3** Maximum likelihood phylogram of combined analysis. Blue lineages are species occurring in freshwater, green are brackish water lineages and black are marine lineages. Shaded species lack a mandibular palp. Maximum likelihood (above line) and parsimony bootstrap (below line) values are given; only values >50% are shown. For interpretation of color references in figure legend, please refer to the Web version of this article.

**Discussion**

**Systematic implications**

The present analyses constitute the most comprehensive molecular phylogeny to date of Palaemoninae at the generic level, as well for *Palaemon* and *Palaemonetes* at species level, with a reasonably robust taxon sampling and utilising both mitochondrial and nuclear markers giving good resolution at both species and generic level. This approach has revealed several interesting patterns with potential implications for the current generic classification of the Palaemoninae: (1) most *Palaemon*, *Palaemonetes*, *Coutierella* and *Exopalaemon* form a well-supported monophyletic

clade in all analyses (the ‘*Palaemon*’ Clade); (2) *Palaemon concinnus*, *P. gracilis* and *P. pandaliformis* are excluded from the ‘*Palaemon*’ Clade; (3) within this large ‘*Palaemon*’ Clade, six smaller clades can be identified, largely reflecting current distribution of constituent taxa, with two, or possibly more, separate clades identified from Asia; (4) *Exopalaemon* constitute a monophyletic clade in all trees nested within the Asia 2 Clade; (5) *Urocaridella* appears as a sister taxon to *Leander*; (6) *Cryphiops* (*Cryphiops*) *caementarius* forms a clade with *Macrobrachium* with strong support, in agreement with the findings of Pereira (1997) and Pileggi & Mantelatto (2010); and (7) the kakaducarid



genera *Leptopalaemon* and *Kakaducaris* appear allied to *Macrobrachium* and therefore nested within the Palaemoninae supporting the conclusions of Page *et al.* (2008) and Bracken *et al.* (2009).

The main implication of our analyses is that *Palaemon*, *Palaemonetes*, *Exopalaemon* and *Coutierella* do not form reciprocal monophyletic clades supporting their current separate generic level status. Instead, they form a clade of mixed genera that, to an extent, reflects the geographic distributions of the species rather than their generic affiliation. The occurrence of the type species of both *Palaemon* and *Palaemonetes* (*P. adspersus* and *Pt. varians*, respectively) in the Atlanto-Pacific Clade supports the likely synonymy of these genera, which has previously been suggested by other workers (Kemp 1925; Chace 1972; Bray 1976; Pereira 1997; Knowlton & Vargo 2004). This is in contrast to the morphological phylogeny conducted by Pereira (1997) which differs in that the two genera are not as intermixed, but nevertheless largely integrate with each other.

*Palaemon concinnus*, *P. gracilis* and *P. pandaliformis* are excluded from the 'Palaemon' Clade in our analysis. *Palaemon gracilis* and *P. pandaliformis* are part of a morphologically homogeneous group in reduced salinity and freshwater in Central America. The current analysis confirms the strong relationship between these species but places them outside of *Palaemon* and closer to *Macrobrachium*. Morphologically, these species conform to the most recent definition of *Palaemon* (Walker & Poore 2003) which implies possible convergent evolution in the expression of certain characters, especially the placement of the carapace spine in a branchiostegal position. The morphological cladistic analysis of Pereira (1997) also placed these species in a monophyletic clade but within *Palaemon*, as a sister group to *Exopalaemon orientis*, *Nematopalaemon schmitti* and *Leptocarpus potamiscus*. *Palaemon concinnus* is also placed outside of *Palaemon* in our analysis but in Pereira's (1997) study it was included in a curious assemblage of *Palaemon* species comprising two Asian species, one widespread Indo-Pacific species, one eastern Pacific species and two European species. As currently classified, *P. concinnus* is unique within the genus in possessing a rudimentary appendix on the endopod of the first pleopod of males. This character is also found in several other genera within the subfamily (*Leander*, *Brachycarpus*, *Leandrites* and *Urocaridella*). A morphological reappraisal of *P. concinnus*, as well as *P. pandaliformis* and *P. gracilis* to ascertain their generic affinities thus appears desirable.

In our data, species of *Exopalaemon* form a strongly supported monophyletic clade within the Asia 2 Clade. *Exopalaemon* was originally described as a subgenus of *Palaemon* by Holthuis (1950) but later afforded generic level status by Chace & Bruce (1993), characterised by having a strong

basal crest to its rostrum. Within the subfamily, *Nematopalaemon* is the only other genus to possess a strongly crested rostrum, although several species of *Macrobrachium* are described as having a 'somewhat-crested' rostrum. Our data, therefore, suggest that *Exopalaemon* represents a derived clade of *Palaemon* in Asian waters. Recently, Baeza *et al.* (2009) and Baeza (2010) demonstrated the paraphyly of *Lysmata* Risso, 1816 (Hippolytidae) caused by the inclusion of *Exhippolysmata* Stebbing, 1915 (but see Fiedler *et al.* 2010). This result has striking parallels with the current analysis as *Exhippolysmata* is likewise currently separated from *Lysmata* by possessing a crested rostrum.

For many years, *Coutierella tonkinensis* was included in *Palaemonetes* until Bruce (1989) re-established *Coutierella* as a monotypic genus with the diagnostic features being a lack of a well-developed pleurobranch on the fourth thoracic segment, the presence of feebly developed, distinctly subspatulate chelae on the second periopods and the presence of three or more pairs of spines on the posterior margin of the telson as well as several specialised features of the mouthparts. Here, *C. tonkinensis* with *P. paucidens* form the weakly supported Asia 1 Clade, casting doubt on the validity of the generic status of *Coutierella*.

Possible species complexes were indicated from our analyses centred around *Palaemon debilis*, *P. serrifer* and *Palaemonetes atrinubes*. In each case, genetic distance between exemplars was greater than that observed for some other well-delineated species in the analyses. *Palaemon debilis* has previously been suspected of being a species complex on morphological grounds (De Grave & Al-Maslmani 2006) and the current data lend weight to this suggestion, with a ~9.5% difference in the two 16S rDNA sequences. The specimens of *Pt. atrinubes* from western and eastern Australia differed by ~18.5% in the 16S rDNA data. Three species can be identified in the material identified as *P. serrifer* from Korea, Taiwan and Singapore, respectively. Previously, morphological variability has been noted in *P. serrifer* (e.g. Kubo 1942; Holthuis 1950; Nguyễn 1992) and further analysis may reveal consistent morphological differences between these populations. The status of all these lineages should await a reappraisal of material of each species from across their respective geographic ranges. Reuschel *et al.* (2010) have also recently proposed cryptic species in the European *P. elegans* because of its high genetic diversity (2.5% in 16S and 8.7% in cytochrome oxidase subunit I), but this species was only represented by a single exemplar in our analysis.

A combined morphological and molecular approach with greater taxon sampling and using more genes and novel morphological characters would be desirable to help resolve whether it is justifiable to synonymise *Palaemon*, *Palaemonetes*, *Exopalaemon* and *Coutierella* or create new

genera to accommodate *P. concinnus*, *P. gracilis* and *P. pandaliformis*.

#### ***The mandibular palp and its absence in Palaemonetes***

Within the Palaemoninae, the mandibular palp is present in 13 of the 21 genera, absent in seven genera and variable in the remaining genus. The presence of a mandibular palp is generally considered the most primitive state in carideans. All genera derived from the basal node in our analyses possess a mandibular palp (variable in *Urocaridella*) suggesting that this may be the plesiomorphic condition in Palaemoninae.

The presence or absence of a mandibular palp is the sole character currently used to differentiate the genera *Palaemon* and *Palaemonetes*. This, however, only holds true for the adult form. A review of descriptions of larval Palaemoninae reveals that the mandibular palp is absent in all larval stages and Knowlton & Vargo (2004) concluded that the larval development of *Palaemon* and *Palaemonetes* are so similar that the two genera are extremely difficult to separate as larvae. Although Gore (1985) described the appearance of a mandibular palp as part of the normal pattern of larval development in decapods, in Palaemoninae the palp develops over a series of moults in juveniles rather than in the larval stages (Lewis & Ward 1965; Bray 1976; Pillai 1979; Walker & Poore 2003) coinciding with the development of sexual characters (appendix masculina in males and ovaries in females; Bray 1976; C. W. Ashelby, pers. obs.). The lack of reciprocal monophyly of *Palaemon* and *Palaemonetes* demonstrated here strongly indicates that the absence of, or rather failure to gain, a mandibular palp in *Palaemonetes* cannot be regarded as a synapomorphy, but rather as a homoplastic character state. Species that lack a mandibular palp are found in five of the clades identified in these analyses plus two further species in the H3 only analysis (Figs 2–3). Why it is absent in some species poses the question of its function and whether its absence can be attributed to differences in life style, habitat or ecology of the animals. Although the mandibular palp is found in many caridean genera, it has received little attention in terms of its functionality. A logical conclusion may be that it is used during feeding but Borradaile (1917) stated that it was not used in food manipulation in *Palaemon serratus* and probably had a sensory function, although limited as it is absent in so many species (i.e. those species currently assigned to *Palaemonetes*). In contrast, Bauer (2004) suggested that the palp may be used in cleaning of the mandibles. There seems to be little difference in feeding mechanism and diet between species that have a palp or lack one, based on the limited available studies (Forster 1951a,b; Sitts & Knight 1979; Siegfried 1982; Guerao 1995; Janas & Barańska 2008). A feeding-related function

would potentially suggest a dietary shift between small juveniles and adults, evidence for which is lacking. Garm (2004) observed that the palp of *Palaemon adspersus* was not seen to move independently of the mandible, nor did it have prey contact, but when the mandibles close it sweeps the lateral side of the labrum. This sweeping across the labrum is probably a grooming procedure (but not of the mandibles). More importantly, all mandibular palp setae are either mechano- or chemoreceptors (A. Garm, pers. comm.) implying a sensory function, possibly to sense the need for grooming.

Following this line of thought, the question of why the mandibular palp is absent mostly in freshwater species within the ‘*Palaemon*’ Clade is raised. Whether the failure to develop a palp is also related to the adaptation to freshwater, as most *Palaemonetes* are freshwater animals, is not known. However, this would seem unlikely, as the palp is present in *Macrobrachium*, which is predominately a freshwater genus and has been retained in several freshwater species of *Palaemon*. However, both the segmentation and presence of the mandibular palp have been demonstrated to be variable characters in a number of previous studies (e.g. Fujino & Miyake 1968; Chace 1972), notably amongst species that tolerate reduced salinities.

#### ***The invasion of freshwater***

Six species of *Palaemon* and 20 species of *Palaemonetes* are found in freshwater with five and six brackish water species, respectively, whilst some species of *Palaemon* have been reported from all three water types. This apparent ecological split may, in part, have contributed to them being regarded as distinct genera.

The transition between marine and freshwater environments represents a huge evolutionary change for animals. Of those that have made the transition to freshwater, crustaceans are amongst the most frequently reported, possibly because the morphological and physiological traits of this group, such as the ability to reduce membrane permeability, promote the adaptation to freshwater environments (Lee & Bell 1999). Decapods are well represented in freshwater with approximately 23% of known species inhabiting freshwater (Bond-Buckup *et al.* 2008; Crandall & Buhay 2008; De Grave *et al.* 2008; Yeo *et al.* 2008).

The term ‘freshwater species’ is frequently used in the literature without definition but, unless defined, the term may be very inclusive and not very informative (Bogan 2008). The definition of a freshwater animal remains controversial (Balian *et al.* 2008) and often problematic as in nature there is no neat dividing line between marine, brackish and freshwater. Rather, there is a continuous gradation (Boxshall & Defaye 2008) with many motile animals being diadromous. Whilst some workers have sought

to employ salinity tolerance ranges in the definitions (e.g. Lee & Bell 1999) and others have put life history traits of animals, such as abbreviation of larval development as a supplementary defining criterion (e.g. Cumberlidge & Ng 2009), such definitions are often unworkable because of a paucity of information. As there is a gradient from species that spend all their lives in freshwater through diadromous species to those that spend all their lives in marine waters, we here use the term freshwater species to include all species that occur for all or part of their lives in freshwater (Lévêque *et al.* 2005) in all or part of their range.

Representatives of eight caridean shrimp families are found in freshwater, dominated by the Atyidae and Palaemonidae (De Grave *et al.* 2008). The subfamily Palaemoninae is widely believed to have radiated into freshwater from ancestral marine stock (e.g. Sollaud 1923; Rabalais & Gore 1985; Freire *et al.* 2003; Murphy & Austin 2004) with the large genus *Macrobrachium* being essentially confined to freshwater and euryhaline water. Freire *et al.* (2003) and Augusto *et al.* (2009) hypothesise that freshwater palaemonid shrimp have taken a direct route via brackish waters, followed by penetration into freshwater habitats, with speciation driven by either separation of river basins by vicariant events or indicative of separate invasions of freshwater, as has been shown in atyid shrimps (Cook *et al.* 2006). Which of these processes has led to the present, disparate distribution of the freshwater species of Palaemoninae, particularly *Palaemonetes*, has remained unanswered. Laboratory studies on the salinity tolerance of the larvae of the freshwater species *Pt. kadiakensis* conducted by Strenth (1976) indicated that it has an inability to disperse over large oceanic stretches, supporting previous theories regarding the dispersal capabilities of freshwater animals. With the exception of *Pt. argentinus*, the nine American freshwater species of *Palaemonetes* (including *Calathbaemon bolthuisi*) recognised by Strenth (1976), as well as *Pt. antennarius* and *Pt. sinensis* from Europe and China respectively, were assumed to represent a monophyletic group based on antennal scale characteristics of the larvae. From this, it was theorised that freshwater *Palaemonetes* arose from one major colonisation event with dispersal between disparate regions occurring via land bridges or when land masses were closer together and with subsequent radiation in freshwater but with possible limited polyphyly (i.e. that of *Pt. argentinus*). The paucity of species in certain regions was believed to be due to competitive exclusion by *Macrobrachium* or extinction of intermediate species (Strenth 1976). The high morphological similarity of adults (Pereira 1997) partially supports Strenth's (1976) view that they represent a single invasion of freshwater with subsequent radiation. In contrast, we demonstrate a conflicting scenario and document the

colonisation of freshwater in five of the major geographically defined clades, in *P. pandaliformis* and *P. concinnus* and a further three potential colonisation events being suggested by the separate 16S and H3 trees (*Pt. paludosus*, *Pt. sinensis*, *E. annandalei* and *E. modestus*). This indicates that a minimum of ten independent invasions of freshwater has occurred in species currently assigned to *Palaemon* or included in the 'Palaemon' Clade and that species have independently evolved the ability to survive in freshwater in all major biogeographic regions and we can confidently discount Strenth's (1976) single colonisation followed by radiation hypothesis. The numerous species of the 'Palaemon' Clade occurring in marginal marine and brackish habitats indicates that the colonisation of freshwaters by this group may still be progressing.

The number of invasions demonstrated suggests that 'Palaemon' species have significant potential over evolutionary time for such habitat transitions. This is supportive of Lee & Bell's (1999) observation that once the evolutionary innovation to invade freshwater arises in a clade, freshwater invasion appears to occur repeatedly. A review of the literature suggests that ten demonstrable independent invasions of freshwater within a single worldwide genus may be unprecedented. Although multiple invasions of freshwater have been demonstrated at higher taxonomic levels (e.g. freshwater genera within families) or smaller geographic scales (e.g. Bartsch 1996; Boxshall & Juame 2000; Bogan 2008; Strong *et al.* 2008; Väinölä *et al.* 2008; Wilson 2008; Yeo *et al.* 2008), no other invertebrate genus seems to have achieved this number of independent invasions of freshwater.

The molecular evidence presented here casts serious doubt on the current generic classification of the Palaemoninae. In view of this, an in-depth appraisal of the currently employed morphological characters is required, including the search for new characters. We also demonstrate multiple invasions of freshwater occurring on an unprecedented scale.

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