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Probing marine *Gammarus* (Amphipoda) taxonomy with DNA barcodes

Abstract The genus *Gammarus* (Amphipoda) is one of the most speciose genera of Crustacea, yet much uncertainty remains concerning taxonomy and systematic relationships, particularly for brackish and marine forms. We used DNA barcode sequences from the mitochondrial cytochrome c oxidase I (COI) gene to probe the taxonomy of prominent members of marine and brackish water *Gammarus* of the North Atlantic, Baltic, Mediterranean and Black Seas. We investigated 16 putative *Gammarus* spp. at an average number of 9 specimens per species. This constitutes the most taxonomically and geographically comprehensive molecular study of marine *Gammarus* to date. Average between-species sequence divergence (26.8%) was much higher than intraspecific distances (0.8%), enabling clear molecular species identification and highlighting several possible misidentifications from previously published studies. Specimens of *Gammarus aequicauda* and *G. insensibilis* from the Black Sea were at least 14% distant from their putative conspecifics elsewhere. Placing these findings in a geographic context provides strong indication of cryptic speciation. Further, we detected phylogeographic splits in *G. oceanicus* and *G. duebeni*. Our analyses also suggest phylogenetic positioning of *G. marinus* with members of the genus *Echinogammarus*, thus confirming its classification as *Echinogammarus marinus*. We have demonstrated that comprehensive analyses of taxonomically complex groups using DNA barcodes can result in a diversity of complementary data on taxonomy, phylogeography and phylogenetics. The combination of these results, with further morphological and ecological data, will enable significant progress in our understanding of this ecologically important group of crustaceans.

Key words *Gammarus*, DNA barcoding, cytochrome c oxidase I, *cox1*, cryptic species, phylogeography, phylogenetics, *Echinogammarus*, marine, brackish, Amphipoda

Introduction

A vast amount of scientific literature recognises the importance of the amphipods of the genus *Gammarus* in aquatic ecosystems worldwide. *Gammarus* occur in a wide variety of freshwater, brackish and marine habitats, where they are often dominant members, playing a key role in the structure and function of aquatic communities. *Gammarus* often occur in large swarms and have a significant impact on the transfer of carbon in the food chain as detritivores, shredders, grazers or predators of smaller animals, eggs and larvae (e.g. Kelly *et al.*, 2002; Christie & Kraufvelin, 2003), and constitute an important food source for a variety of animals (e.g. Costa & Costa, 2000). There have been a growing number of accounts describing invasive gammarid species and the dramatic changes they produce in benthic fauna and community structures (Kelly & Dick, 2005; Kelly *et al.*, 2006a; Grabowski *et al.*, 2007; Piscart *et al.*, 2007). *Gammarus* are also widely used in ecotoxicological research (e.g. Clason & Zauke, 2000; Costa *et al.*, 2005; Fialkowski & Rainbow, 2006; Prato *et al.*, 2006) and are important model organisms for the study of host–parasite interactions (e.g. Kostadinova & Mavrodieva, 2005; Rolbiecki & Normant, 2005), co-evolution and ecology, including parasitic sex-ratio distortion (Ironside *et al.*, 2003) and parasite-mediated competition (MacNeil *et al.*, 2004).

Despite their ecological importance and the research interest they have long received, there are still important taxonomic uncertainties and problematic species identifications within *Gammarus*. Although many attempts have been made to employ morphological characters to resolve phylogenetic

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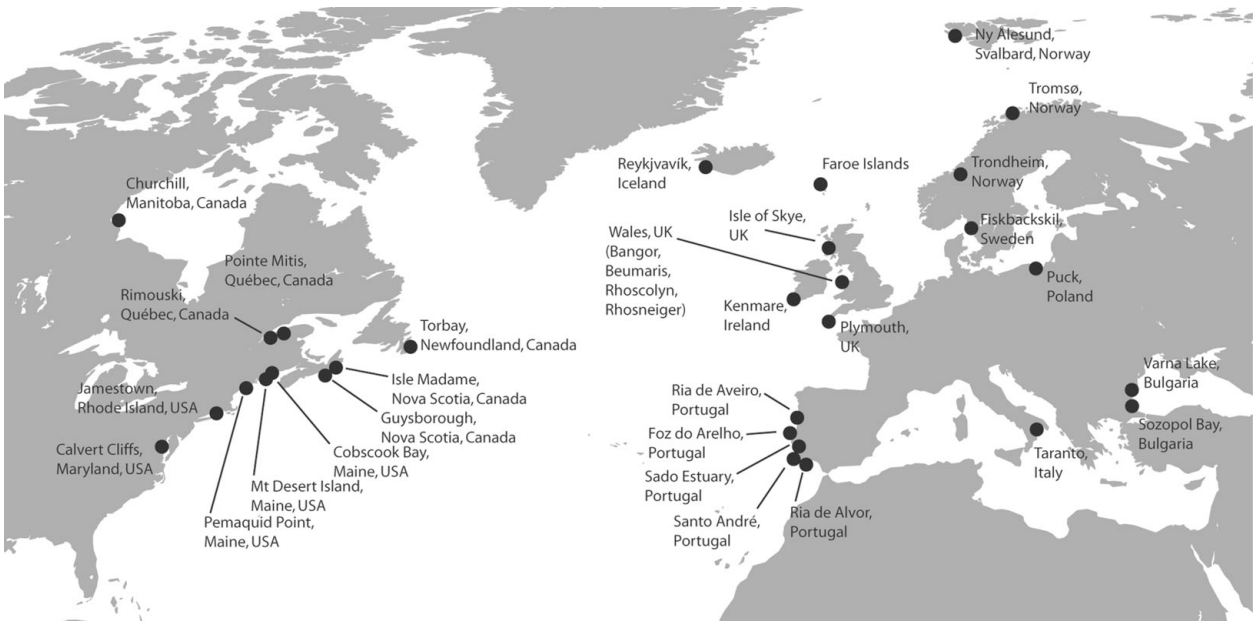

Figure 1 Main collection locations across the North Atlantic and adjacent seas of the *Gammarus* analysed in this study.

relationships within the Amphipoda, the results are tentative and based on only a few characters for most groups, with terminal taxa like Gammaridae largely abandoned (reviewed in Englisch *et al.*, 2003). *Gammarus* is a particularly speciose genus, with 204 species currently described (Väinölä *et al.*, 2008), the vast majority of which are freshwater species. However, even among marine species the taxonomy is complex and morphological identifications difficult, requiring very detailed inspection of specimens and considerable expertise. Species diagnoses demand inspection of diverse morphological characters, some of which often show considerable variation. Sexual dimorphism and ontogenetic variation further complicate identifications. Due to these difficulties it is not uncommon for specimens from this genus to be pooled and reported as *Gammarus* spp. in ecological studies. In the NE Atlantic and Baltic Sea the main taxonomic clarifications were not made until the mid 20th century, when several variants of the so-called *G. zaddachi* group were given species rank (Segerstråle, 1947; Spooner, 1947; Kinne, 1954). The *Gammarus* fauna of the Mediterranean Sea was only clarified in 1967 with the decisive work of Stock (1967), which provides a taxonomic key for seven species within the *G. locusta* complex. Despite such progress, the difficulties with taxonomic identification persist, with morphological identification of this fauna, particularly in the Black Sea, very poorly understood (Sezgin & Katagan, 2007).

Molecular markers have seldom been employed in *Gammarus* taxonomy. The few studies produced so far have used rather different approaches and hence have reduced comparative value (Meyran *et al.*, 1997; Costa *et al.*, 2004a; Rock *et al.*, 2007). Phylogenetic studies (Skadsheim & Siegismund, 1986; Englisch *et al.*, 2003; Hou *et al.*, 2007) have either included only a few *Gammarus* species or have been highly biased towards a certain faunal type and geographic region. The recent study by Hou *et al.* (2007) was expansive in their employment of four nuclear and mitochondrial genes to create a *Gammarus* phylogeny. While this study is an extremely valuable contribution to understanding the phylogenetic relationships and taxonomy of this large and complex genus, its contribution for understanding the North Atlantic *Gammarus* fauna lies mostly in the well-supported phylogenetic backbone it supplies, showing the relationships among eight marine and brackish-water North Atlantic species, and other species in the genus.

In this study, we used DNA barcodes to probe the taxonomy of prominent members of marine and brackish water *Gammarus* of the North Atlantic, Baltic, Mediterranean and Black Seas. DNA barcodes are established, standardised molecular tags for species identification, which are based on the premise that a single short region of the genome can provide enough nucleotide sequence information for species discrimination in large taxonomic assemblages (Hebert *et al.*, 2003). The established DNA barcode region for most groups of animals consists of sequence data of the mitochondrial gene cytochrome c oxidase subunit I (COI or *cox1*). Former studies in different vertebrate and invertebrate taxa demonstrated the ability of a 650 base pair region of the COI gene to deliver species-diagnostic barcodes (e.g. Ward *et al.*, 2005; Costa *et al.*, 2007). Our aim was to produce an assessment of the taxonomy of 16 putative species of marine *Gammarus* by analysing the DNA barcodes from multiple populations and individuals across each species range (mean = 9; range: 2–33 individuals).

Materials and methods

Samples and locations

Specimens were collected from the intertidal zone in a range of locations extending east–west from the western Black Sea to Hudson Bay, and north–south from Svalbard to southern Portugal (Fig. 1). A list of the specimens with collection details is given in Table 1.

Species	Location (number of specimens)	MOTU[a]	GenBank Accession	Source
Gammarus aequicauda	Bulgaria (5)	1	GQ341702-GQ341706	This study
Gammarus aequicauda	Italy (2)	2	GQ341707-GQ341708	This study
Gammarus chevreuxi	Portugal (5)	3	GQ341713-GQ341716	This study
Gammarus crinicornis	Belgium (2), Portugal (1)	4	GQ341717-GQ341719	This study
Gammarus duebeni	Canada (2) Denmark (2) England (2) Scotland (2) Iceland (5) Norway (4) Sweden (1) USA (4) Wales (4)	5	GQ341720-GQ341744	This study, Rock *et al.* (2007)
Gammarus finmarchicus	Maine, USA (1), Norway (1), Scotland (1)	6	GQ341745-GQ341747	This study
Gammarus insensibilis	Bulgaria (4)	7	GQ341709-GQ341712	This study
Gammarus insensibilis	Portugal (4)	8	GQ341748-GQ341751	This study
Gammarus lawrencianus	Maine, USA (5), Quebec, Canada (5)	9	GQ341752-GQ341761	This study
Gammarus locusta	Belgium (4), Portugal (5), Scotland (1), Wales (3)	10	GQ341762-GQ341774	This study
Echinogammarus (Gammarus) marinus	Iceland (4), Ireland (3), Scotland (1), Sweden (2), Wales (1)	11	GQ341691-GQ341701	This study
Gammarus mucronatus	Maine, USA (5) Nova Scotia, Canada (5)	12	GQ341775-GQ341784	This study
Gammarus obtusatus	Iceland (5), Maine, USA (7), Norway (4), Nova Scotia, Canada (5)	13	GQ341785-GQ341805	This study
Gammarus oceanicus	Churchill, Canada (4), Iceland (8), Maine, USA (3), Tromso, Norway (3), Poland (4), Quebec, Canada (3), Svalbard, Norway (7), Scotland (1)	14	GQ341806-GQ341838	This study
Gammarus salinus	Poland (8), Sweden (2)	15	GQ341839-GQ341848	This study
Gammarus setosus	Churchill, Canada (2), Quebec Canada (2) Svalbard, Norway (5)	16	GQ341849-GQ341857	This study
Gammarus tigrinus	Poland (4)	17	GQ341858-GQ341861	This study
Gammarus wilkitzkii	Svalbard, Norway (2)	18	- - - - -	F. Dufresne (unpublished data)
Gammarus zaddachi	Poland (2), Norway (1) Wales (5)	19	GQ341862-GQ341869	This study

Table 1 Species, collection locations, MOTU[a] and database accessions for sequences obtained in this study, and assembled from other studies.

[a] MOTU : molecular operational taxonomic unit.

Species	Location (number of specimens)	MOTU	GenBank Accession	Source
Gammarus aequicauda	Black Sea	20	AY926667	Macdonald *et al.* (2005)
Gammarus annulatus	Massachusetts, USA	9	AY926668	Macdonald *et al.* (2005)
Gammarus bousfieldi	Illinois, USA	21	EF570299	Hou *et al.* (2007)
Gammarus duebeni	Maine, USA	5	AY926669	Macdonald *et al.* (2005)
Gammarus lacustris	Olkhon Island, Lake Baikal	22	AY926671	Macdonald *et al.* (2005)
Gammarus lacustris	unknown	22	DQ889100	Costa *et al.* (2007)
Gammarus locusta	Nunavut, Canada	10	EF570324	Hou *et al.* (2007)
Gammarus minus	Illinois, USA	23	EF570326	Hou *et al.* (2007)
Gammarus oceanicus	Massachusetts, USA	14	AY926674	Macdonald *et al.* (2005)
Gammarus pseudolimnaeus	Illinois, USA	24	EF570333	Hou *et al.* (2007)
Gammarus pseudolimnaeus	unknown	25	- - - - -	This study
Gammarus pulex	Netherlands	26	EF570334	Hou *et al.* (2007)
Gammarus roeseli	Austria	27	EF570337	Hou *et al.* (2007)
Gammarus tigrinus	Not available	17	DQ300215 DQ300217 DQ300222 DQ300223 DQ300232 DQ300239 DQ300244	Kelly *et al.* (2006b)
Gammarus tigrinus	Netherlands	17	EF570348	Hou *et al.* (2007)
Chaetogammarus marinus	Norway	11	AY926655	Macdonald *et al.* (2005)
Chaetogammarus obtusatus	Nova Scotia, Canada	11	AY926656	Macdonald *et al.* (2005)
Echinogammarus ischnus	Black Sea and Caspian Sea and drainage systems	28	AY326115 to AY326126	Cristescu *et al.* (2004)
Echinogammarus trichiatus	Romaine and Ukraine	29	AY529050 and AY529051	Cristescu and Hebert (2005)
Eulimnogammarus cyaneus	Lake Baikal, Bolshie Koty	30	AY061801	Väinölä *et al.* (2001)
Eulimnogammarus viridis	Lake Baikal	31	AY926664	Macdonald *et al.* (2005)
Jesogammarus debilis	Beijing, China	32	EF570351	Hou *et al.* (2007)
Jesogammarus hebeiensis	Beijing, China	33	EF570352	Hou *et al.* (2007)

Table 1 Continued.

Based on morphological identifications, our specimens were assigned to 16 *Gammarus* and one *Echinogammarus* species. Morphological comparison was conducted after Stock (1967), Bousfield (1973) and Lincoln (1979). In some instances the identification of reference specimens was confirmed by the Natural History Museum London (M. Lowe, pers. comm.).

We retrieved available sequences of *Gammarus* matching the COI barcode region, including those with known synonymous species names, from major public DNA data repositories (International Nucleotide Sequence Database Collaboration; INSDC). For comparative purposes, we also included sequences from the genera *Chaetogammarus*, *Echinogammarus* and *Eulimnogammarus*, as well as two species of *Jesogammarus* which were used as the outgroup. Species names and accession numbers are provided in Table 1. A total of 33 putative species (see below in this section for detailed explanation) and 223 specimens were assembled for further analysis.

DNA extraction, amplification and sequencing

Since our dataset was produced from the merged datasets of F.O. Costa, J. Rock and C.M. Henzler, three protocols were used (as described in Henzle, 2006; Costa *et al.* 2007 and Rock *et al.*, 2007). All protocols were similar and the latter is given here. DNA was extracted from ethanol-preserved specimens using a slightly modified version of a standard

salt extraction method (e.g. Miller *et al.*, 1988). Approximately 640 bp of the COI gene was amplified from all individuals using Folmer *et al.*'s (1994) universal primers (LCO1490: 5′–GGTCAACAAATCATAAAGATATTGG-3′, HCO02198: 5′–TAAACTTCAGGGTGACCAAAAAATCA-3′) or versions of these primers that were degenerate across a variety of crustaceans (UCOIF: 5′–TAWACTTCDGGRTGRCCRAAAAAYCA-3′; UCOIR: 5′–ACWAAYCAYAAAGAYATYGG-3′; these primers were used for *Gammarus obtusatus* and *Gammarus mucronatus*). Polymerase chain reaction (PCR) amplifications were carried out in 15 μL reactions: 100 mM Titanium *Taq* PCR Buffer (contains $MgCl_2$; Clontech, Palo Alto, CA), 200 mM each dNTP, 200 mM each primer, 10–50 ng of genomic DNA, and 5 mM Titanium *Taq* DNA Polymerase (a mixture of DNA polymerase and TaqStart Antibody; Clontech, Palo Alto, CA). Amplification was carried out with an annealing temperature of 50 °C for both primer sets. For all PCR amplifications, products were purified using the PerfectPrepPCR Cleanup kit (Eppendorf AG, Hamburg, Germany). Sequencing was carried out in both directions using BigDye terminators v. 3.1 (Applied Biosystems, Foster City, CA) and sequenced on an automated AB 3730 sequence analyser. Sequences were edited and aligned manually and in Sequencher v. 4.2.2 (Gene Codes Corp., Ann Arbor, MI, 2000).

Data analyses

The program ModelGenerator (Keane *et al.*, 2006) was used to select the maximum likelihood model for both nucleotide and amino acid alignments. Estimation of the maximum likelihood tree was performed using the program RAxML-7.0.4 (Stamatakis 2006, Stamatakis *et al.*, 2008). Codon positions were specified as independent data partitions allowing the optimisation of α-shape parameters, GTR-rates, and base frequencies for each. Bootstrap pseudoreplicates (N = 1000) were used to estimate confidence in tree topology. Maximum likelihood trees were also estimated using PhyML (Guindon & Gascuel 2003) using the GTR+I+G model and approximate likelihood ratio test (aLRT) scores were obtained as additional estimates of confidence in tree topology (Anisimova & Gascuel 2006).

In order to illustrate the branching pattern of more basal nodes, nucleotides were translated to amino acid sequences and a neighbour-joining (NJ) tree was built in MEGA4 (Tamura *et al.*, 2007) based on the Jones–Taylor–Thornton (JTT) matrix (Jones *et al.*, 1992) and determining branch support with 1000 bootstrap replicates.

We applied the Distance Summary (DS) tool available in the Barcode of Life Data System (BOLD; Ratnasingham & Hebert, 2007) to our data. Briefly, the DS analysis performs an automated computation of pairwise divergences at different taxonomic levels. A preliminary analysis revealed very large divergences within *G. aequicauda* and *G. insensibilis*, suggesting the presence of multiple species under the same species assignments. To accommodate these observations in the DS calculation we defined separate molecular operational taxonomic units (MOTU; Floyd *et al.*, 2002). By applying MOTU to our analyses, taxa could be identified through sequence identity. Identity in sequence need not correspond to identity of OTU as measured by other models (biological or morphological). This approach allowed the assignment of putative species to clusters that emerge from the molecular divergence data, and hence enabled testing species groupings under various scenarios. In this case, we attributed separate MOTUs to reciprocally monophyletic groups of specimens with more than 3% divergence and according to geographic origin (e.g. Black Sea specimens). The DS summary was complemented by the calculation of the quotient between congeneric and conspecific divergences, hereafter referred to as 'taxonomic resolution ratio' (TRR).

Results

Intra- and inter-specific divergences

The analysis of pairwise COI nucleotide divergences for all *Gammarus* species in our dataset (not including INSDC data), consistently showed a much higher between-species versus within-species divergence (Table 2). The within-species divergence averaged 0.86% (range of 0–4.3), while between-species divergence was close to 27% (range of 5.2–34.2), leading to a TRR value of 32.6. The maximum within-species distance corresponded to distances between the two putative subspecies of *G. duebeni celticus* and *G. duebeni duebeni* (4.3%). The minimum distance among species was detected between *G. insensibilis* from Black Sea and *G. aequicauda* from Italy (5.2%).

All pre-defined MOTUs clustered in generally well-supported monophyletic groups, independently of the evolutionary model and tree-building method used (Fig. 2; a detailed tree with non-collapsed branches can be found in Appendix A, which is available as 'Supplementary data' on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1477200009990120). The topology of the two nucleotide trees was virtually identical for the shallow and highly supported nodes of the tree, allowing clear species discrimination by observation of the clustering patterns. Deeper nodes of the trees showed an overall decrease in node support and more differences among topologies. For instance, in contrast to RAxML, PhyML analysis positioned *G. finmarchicus*, *G.chevreuxi* and *E. marinus* with the Mediterranean phylogroup (as defined in section 3.4). However, such rearrangements were not associated with high bootstrap support and where nodes were well-supported there was high congruence between tree-building methods.

Taxonomic assignments

Seven taxonomic mismatches were observed (labelled a–g, Fig. 2), representing several different forms of incongruence between taxonomic assignments. In the case of *G. aequicauda* (a), our samples from Italy formed a separate MOTU (27% K2P, TRR of 42) from a single Black Sea INSDC sequence (Macdonald *et al.*, 2005), which was also divergent from the second clade of *G. aequicauda* consisting of our Black Sea specimens (Fig. 3). For *G. insensibilis* (b) our samples from the Black Sea and Portugal formed separate MOTUs with a

Taxon[a]	Pairwise divergences	n Comparisons	Min distance	Mean[b] distance	Max distance	TRR[c]
All *Gammarus*[d] (17 putative species, 169 seqs.)	Within a Species	1364	0	0.82 ± 0.03	4.30	32.6
	Within a Genus	12832	5.19	26.76 ± 0.03	34.23	
G. aequicauda Italy/ ***G. aequicauda*** Black Sea (7 sequences)	Within a Species	11	0	0.64 ± 0.09	1.00	42.1
	Within a Genus	10	26.31	26.94 ± 0.13	27.49	
G. insensibilis Portugal/ ***G. insensibilis*** Black Sea (8 sequences)	Within a Species	12	0	0.63 ± 0.12	1.23	23.14
	Within a Genus	16	14.04	14.58 ± 0.08	15.19	
G. oceanicus Global/ ***G. oceanicus*** Rimouski/Maine (33 sequences)	Within a clade	366	0	0.24 ± 0.01	0.91	8.9
	Between clades	162	1.66	2.13 ± 0.03	3.1	
G. duebeni duebeni Global/ ***G. duebeni celticus*** Wales (25 sequences)	Within a clade	254	0	0.31 ± 0.01	0.82	12.8
	Between clades	46	3.44	3.98 ± 0.03	4.30	

Table 2 Pairwise COI barcode nucleotide divergences for 17 putative *Gammarus* species collected for this study, using K2P distances (%).

[a] Number of species with more than 1 sequence, and number of sequences analysed, reported within parentheses.
[b] Data reported as K2P distance ± SE.
[c] TRR = taxonomic resolution ratio (see Data analysis in Materials and methods).
[d] Distance summary determined using all identified species and sequences obtained from this study.

divergence of *c*. 15% (Fig. 3, Table 2). In the case of *G. annulatus* (c), this single INSDC sample (Macdonald *et al.*, 2005) was embedded within our well-supported *G. lawrencianus* clade (Fig. 4). There was also discordance with a single *G. locusta* sample from Hou *et al.* (2007) (d), which clustered with our *G. setosus* clade. Similarly, a single INSDC *Chaetogammarus obtusatus* (Macdonald *et al.*, 2005) (e) was embedded in our *E. marinus* clade. Three of our specimens from Svalbard morphologically identified as *G. zaddachi* (f) grouped instead with *G. oceanicus* (Fig. 5). Finally, specimens of *G. pseudolimnaeus* (g) from Costa *et al.* (2007) and from Hou *et al.* (2007) also did not match.

Phylogeographic structure

Deep phylogeographic structure was observed within the geographic range of two well-sampled species, *G. oceanicus* and *G. duebeni*. Two clades diverging ∼2% from each other (Table 2) were observed for *G. oceanicus*: one large clade was comprised of specimens predominantly from the Baltic Sea and NE Atlantic, although individuals from Churchill, Canada were also associated (Fig. 5, MOTU 14a). A second clade contained populations distributed south of the St. Lawrence River along the NW Atlantic coast (MOTU 14b). Two distinct clades differing by 4% are also seen in *G. duebeni* (Table 2; see Rock *et al*. 2007).

Phylogenetic analyses

Two putative phylogroups were detected in our data, and summarised further using phylogenetic reconstruction with deduced amino-acid data (Fig. 6; a detailed tree with non-collapsed branches can be found in Appendix B, which is available as 'Supplementary data' on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1477200009990120). The most cohesive phylogroup was formed by the complex of species of the *G. locusta* group (*sensu* Stock, 1967), namely *G. aequicauda* (3 MOTUs), *G. crinicornis*, *G. insensibilis* (2 MOTUs) and *G. locusta*, to the point that these species are generally not distinguishable any more as independent clades. The other consistent phylogroup was formed by the northern European species *G. salinus*, *G. setosus*, *G. wilkitzkii* and *G. zaddachi*. More hierarchy of relatedness is retained in the amino-acid analysis of this group, in accord with the nucleotide analyses (Fig. 2), with *G. salinus* and *G. zaddachi* most closely related. These two large phylogroups, in turn, display a common ancestral node in both amino acid and nucleotide trees (although with low node support). In the amino acid tree they are also joined by *G. finmarchicus*, *G. chevreuxi*, *G. duebeni*, *G. lacustris* and *G. oceanicus*, forming a clade of 13 marine and brackish European species.

Another group emerging from the amino-acid data is the one formed by *Echinogammarus ischnus*, *E. trichiatus* and

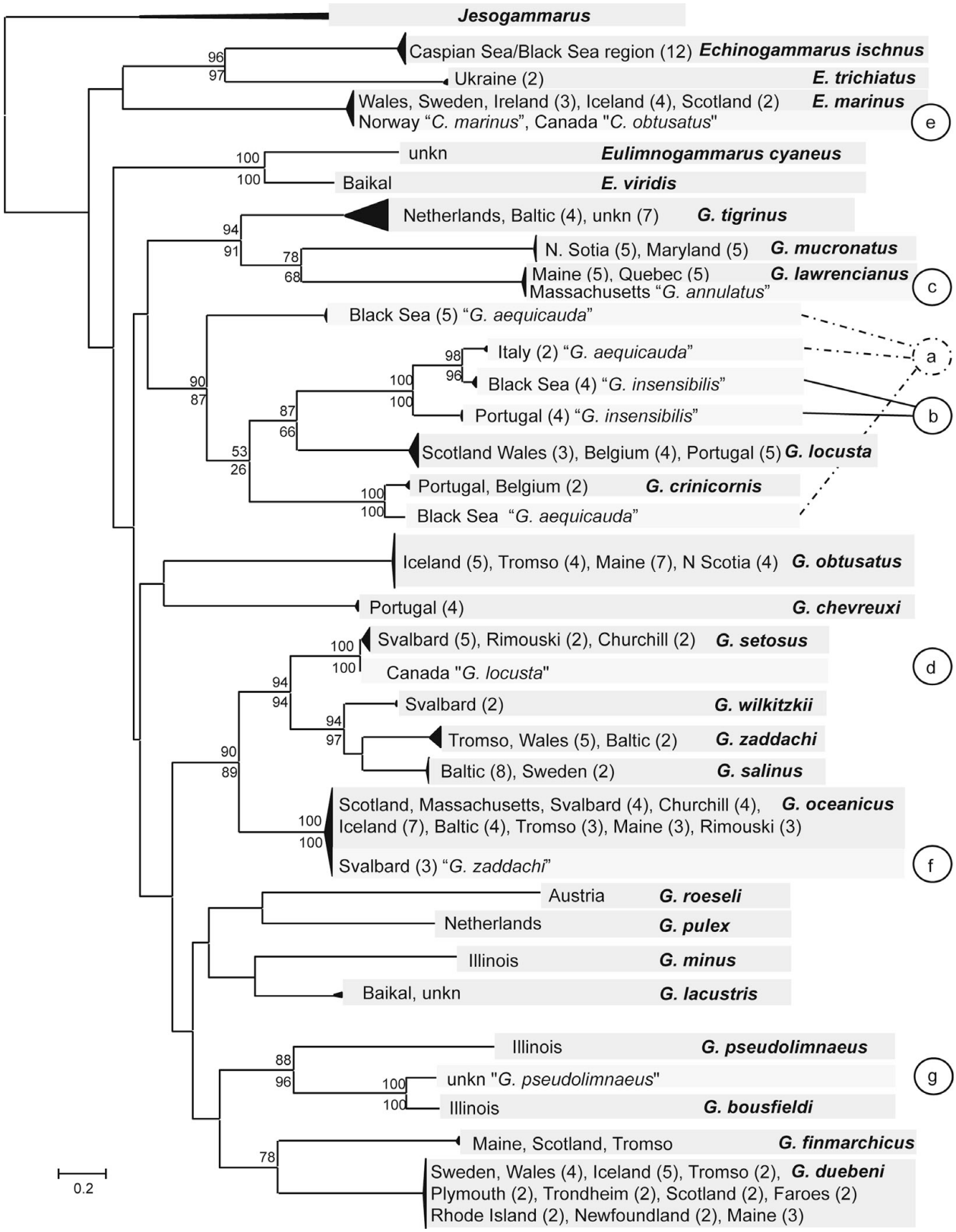

Figure 2 Maximum likelihood tree of *Gammarus* based on nucleotide sequences of the cytochrome oxidase I gene: schematic overview of the RAxML tree with species clusters collapsed and highlighted (a detailed tree with non-collapsed branches can be found in Appendix A, which is available as 'Supplementary data' on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1477200009990120). Support values where > 50 are given with bootstrap values above and aLRT values (where in accord with PhyML topology) below the line. Taxonomic mismatches are labelled *a* to *g*. Sample sizes where greater than 1 are given in parentheses. Unkn = unknown geographic origin for INSDC samples.

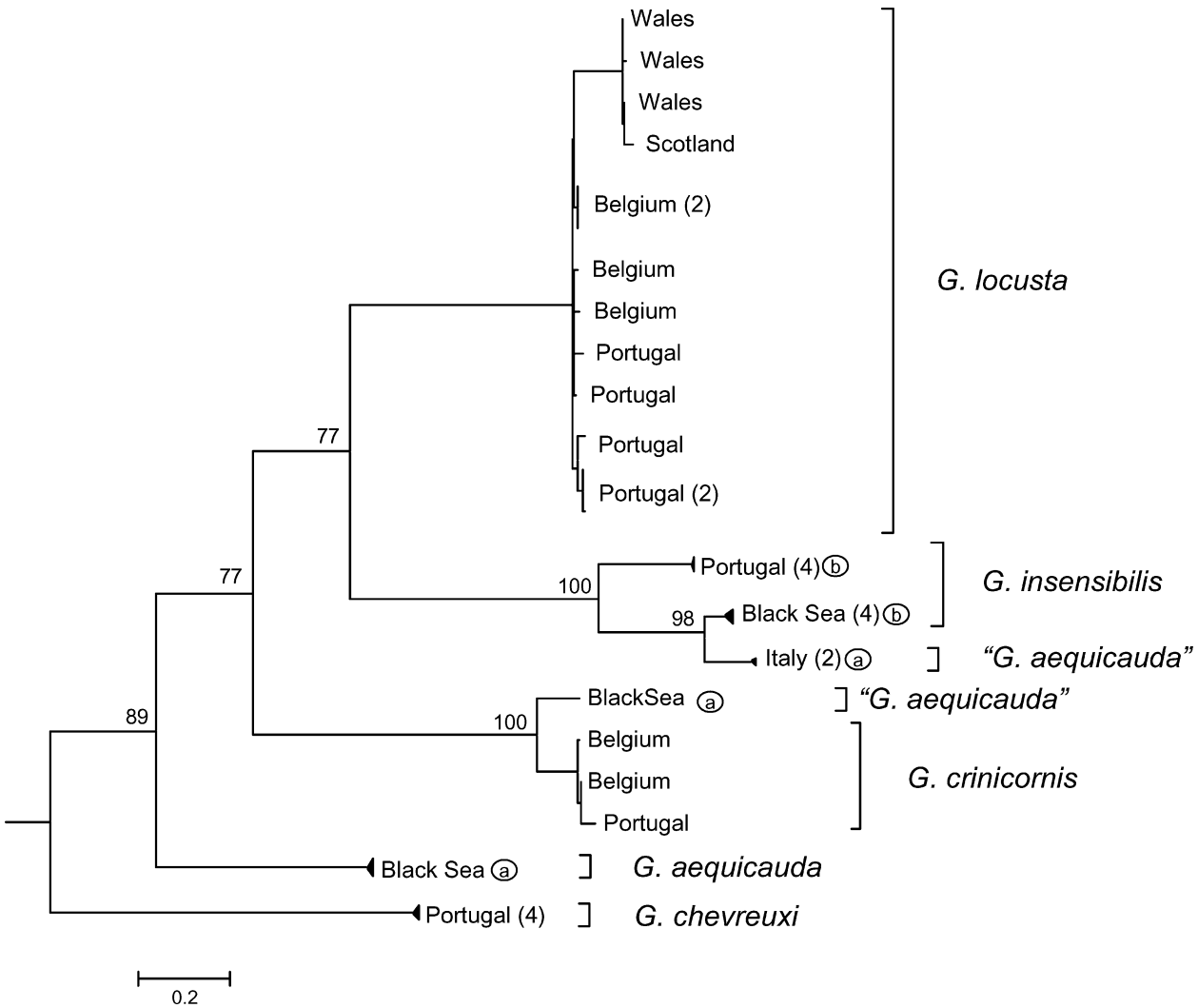

Figure 3 Sub-tree detail (from Fig. 2) of sections corresponding to *G. locusta* and related species. Bootstrap values are given where >50.

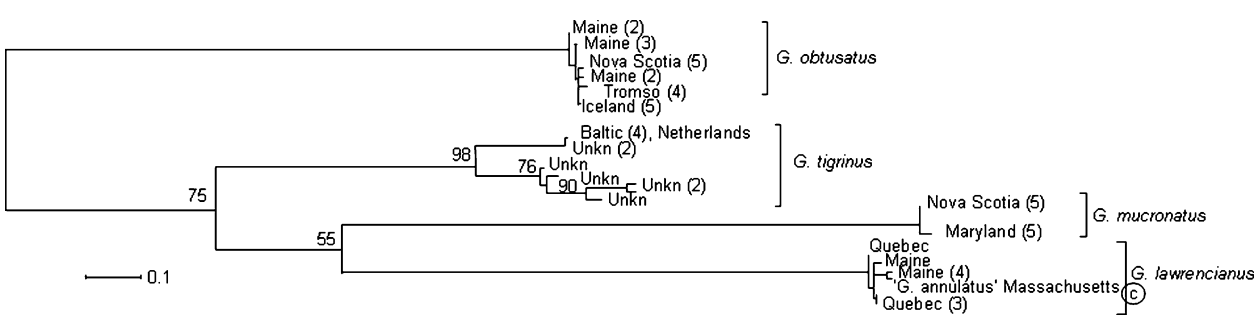

Figure 4 Sub-tree detail (from Fig. 2) of sections corresponding to *G. obtusatus* and the N. American species. Bootstrap values are given where >50.

E. marinus. These species remain reasonably divergent from each other and the node support is moderate, however this group was recovered in both nucleotide (Fig. 2) and amino acid (Fig. 6) analyses. The N. American species *G. mucronatus*, *G. tigrinus* and *G. lawrencianus* also grouped together consistently, with moderate support in both amino acid and nucleotide trees.

Discussion

COI barcodes were very effective in discriminating *Gammarus* species with near-cryptic morphology. The level of species resolution (TRR *c.* 30×) in *Gammarus* is within the range observed for other animal groups (e.g. Hebert *et al.*, 2003; Costa *et al.*, 2007; Ward, 2009). However, both the conspecific

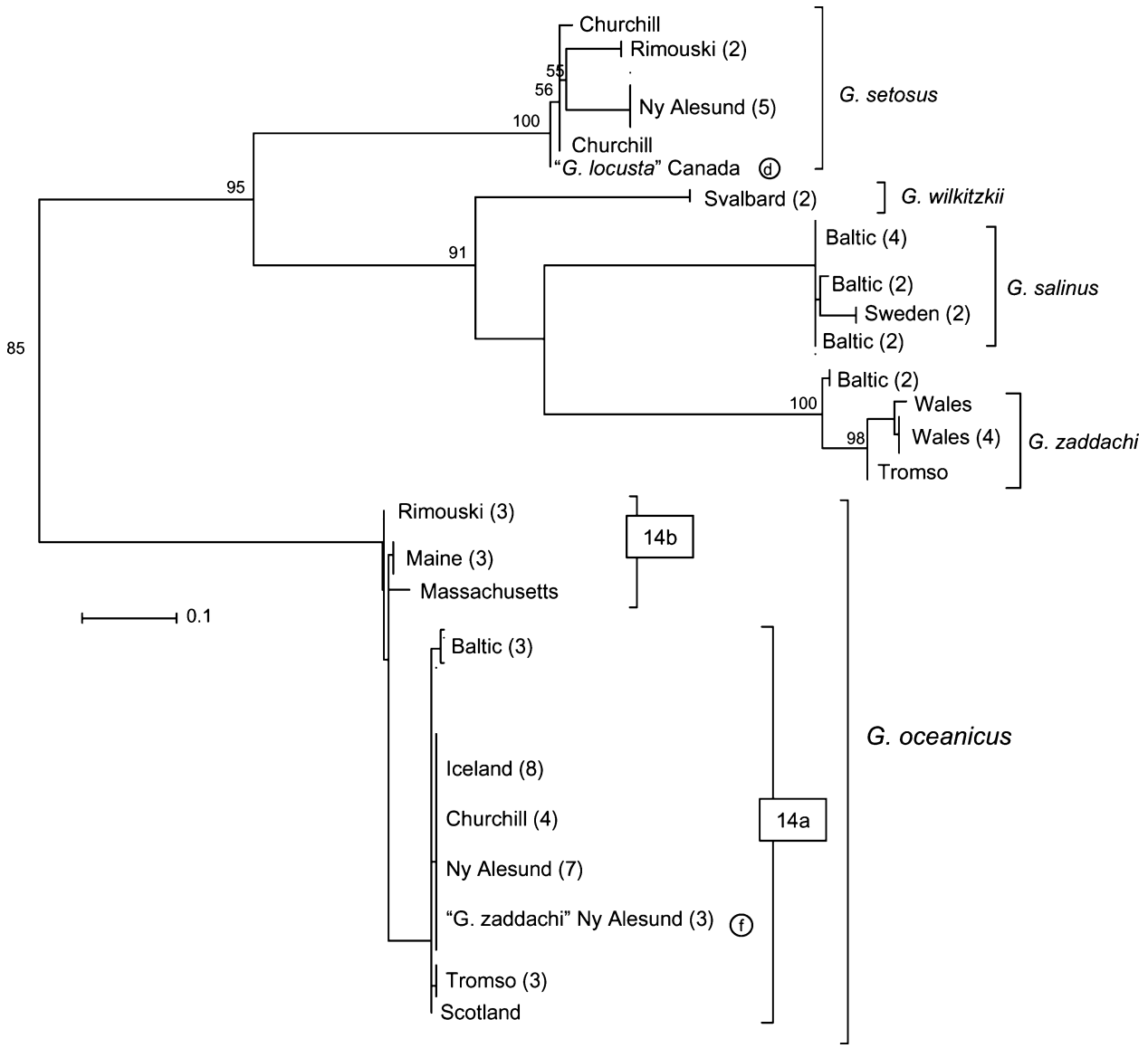

Figure 5 Sub-tree detail (from Fig. 2) of sections corresponding to *G. oceanicus*, *G. zaddachi* and related species. Bootstrap values are given where > 50.

and congeneric divergences appear to be in the upper limits of the reported variation range, the former probably due to the comprehensive geographic coverage of some species in our study.

Taxonomic assignments

Gammarus of the Mediterranean

A number of taxonomically relevant issues emerged despite the moderate number of species analysed, both within our dataset and by comparison with INSDC sequence data. The most striking examples come from the Black Sea specimens of *G. aequicauda* and *G. insensibilis*, which were genetically distinct from individuals of the same species outside the Black Sea. In his review of these and other *G. locusta* complex species, Stock (1967) stated that further taxonomic work was required for both *G. aequicauda* and *G. insensibilis*, suggesting that the former required further splitting. He opted to describe two 'forms' of *G. aequicauda*: one form from a brackish pool in Turkey, closely resembling the type specimens from brackish waters in Crimea, and one from nearly fresh water in southern France. Since the type specimens of *G. aequicauda* are from Crimea, in the Black Sea, it is more logical to assume that our specimens from the Black Sea represent the 'true' *aequicauda*, with specimens from Italy representing Stock's second 'form'. There is also the possibility of misidentification with *G. plumicornis*, another *Gammarus* of the Mediterranean *G. locusta*-complex, which is 'remarkably similar' to *G. aequicauda* (Stock, 1967). Nevertheless, even among Black Sea specimens, our *G. aequicauda* does not match the *G. aequicauda* specimen from the Black Sea of Macdonald *et al.* (2005). The latter appears to be closely related to *G. crinicornis*, its nearest neighbour with a K2P distance of 6.8%, and a species known to occur in the Black Sea. However, Macdonald

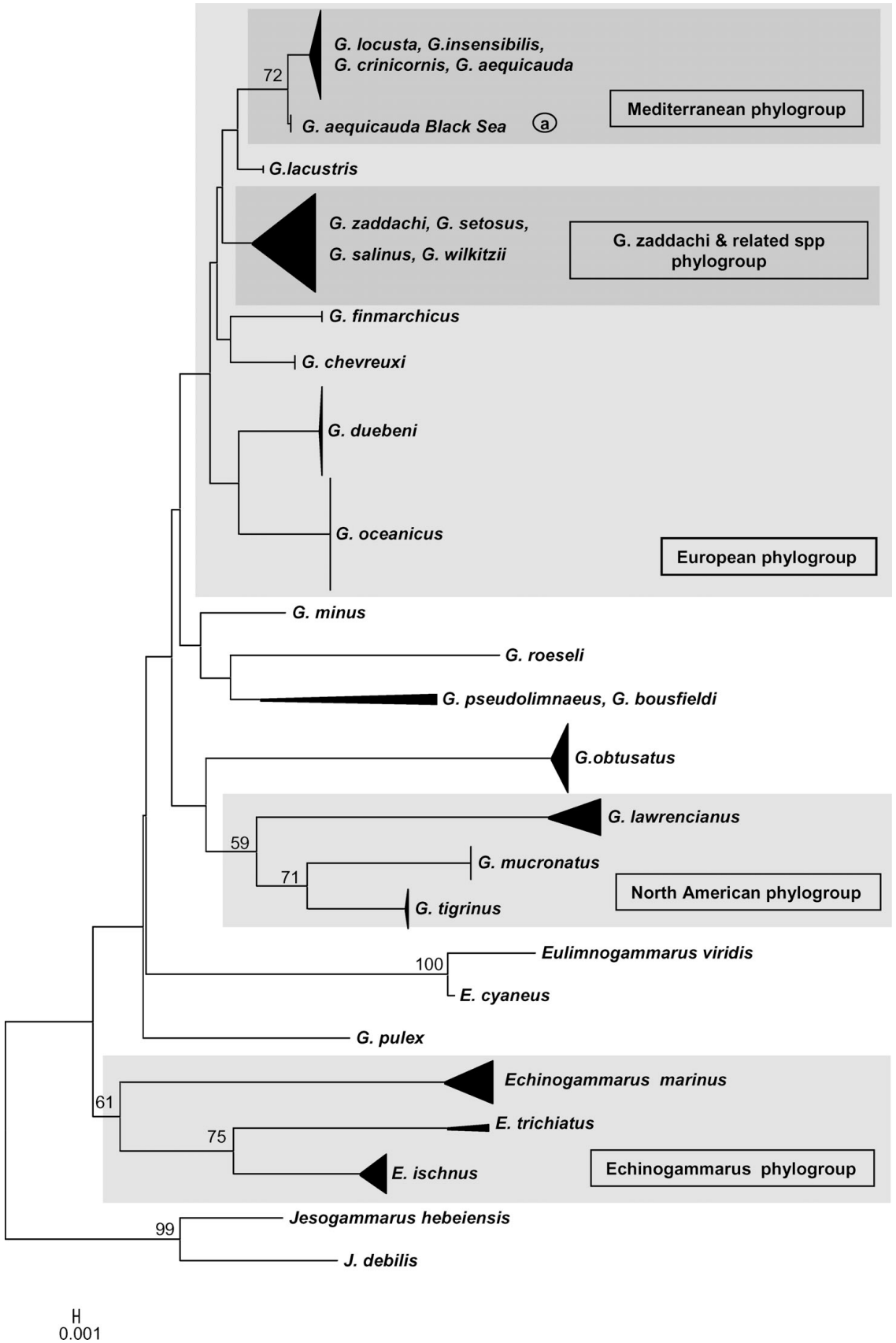

Figure 6 Neighbour-joining tree of *Gammarus* based on amino-acid sequences of the cytochrome oxidase I gene: schematic overview of the tree with main species clusters collapsed and highlighted (a detailed tree with non-collapsed branches can be found in Appendix B, which is available as 'Supplementary data' on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1477200009990120). Bootstrap values are given where > 50.

et al.'s (2005) sequence is also divergent from *G. crinicornis* sampled in our study, suggesting that it might in fact represent *G. subtypicus* Stock, 1966. This is the only other *Gammarus* known from the Black Sea that is not represented in our study, and it is morphologically similar to *G. crinicornis* (Sezgin & Katagan, 2007).

Taxonomic discordances involving *G. insensibilis* involved similarities with *G. inaequicauda.* The specimens of *G. insensibilis* from the European Atlantic coast appeared to constitute the southern form of a single species cline of which the northern form is *G. inaequicauda* as described by Stock (1967), who suggested that further taxonomic work would be required to determine if these are in fact a single polytypic species. *Gammarus inaequicauda* was later recognised to occur in the Baltic Sea (Segerstråle, 1971; Jażdżewski, 1973), but to date it is perhaps one of the lesser known species of European marine and brackish *Gammarus*. The species identity of our specimens of *G. insensibilis* from Sado estuary, on the west coast of Portugal, was confirmed by Krzysztof Jażdżewski. University of Lodz, Poland (pers. comm.). Still, Stock (1967) confirmed the occurrence of this species in the Black Sea, Mediterranean and the Atlantic European coast north to Rade de Brest, France, and Lincoln (1979) confirmed its occurrence in the British Isles. Along this range there are at least two well-known potential phylogeographic breaks – the Bosphorus (Papadopoulos *et al.*, 2005) and the Strait of Gibraltar (Patarnello *et al.*, 2007), which might be expected to cause significant genetic differentiation in this species.

Before Stock's (1967) comprehensive and authoritative review, the taxonomy of Mediterranean *Gammarus* was chaotic. Despite the separation of the *G. locusta* complex into seven species, Stock (1967) argued that there was still some hidden diversity that needed to be clarified and our data appear to confirm such suspicions. As can be confirmed from Stock's (1967) review, the morphological taxonomy of this group is so intricate that they could be considered cryptic or near-cryptic species. Therefore, DNA barcodes are particularly useful to assist taxonomic clarification and routine monitoring in this group. The completion of the DNA barcode library of the *G. locusta*-complex, with data for *G. plumicornis*, *G. subtypicus* and *G. inaequicauda* is therefore crucial. Further, the narrow connection of the Black Sea with the Mediterranean through the Bosphorus Strait suggests a fruitful ground for vicariant speciation, as demonstrated in recent work on chaetognaths (Peijnenburg *et al.*, 2004).

Gammarus of the western North Atlantic

Our data demonstrate that the *Gammarus* specimen collected in Creswell Bay in Canada, and assigned by Hou *et al.* (2007) to the species *G. locusta*, is in fact *G. setosus*. Indeed, it is well established that the *G. locusta* L. 1758 range is restricted to the NE Atlantic and Baltic Sea, and *G. locusta* is not known to occur in Arctic waters (reviewed in Costa & Costa, 2000; Stock, 1967; Lincoln, 1979; Gaston and Spicer, 2001; Costa *et al.*, 2004b), so this was most likely a misidentification. Furthermore, both *G. locusta* and *G. setosus* are well represented in our dataset, with multiple specimens from different locations (including *G. setosus* from Churchill, Manitoba) and identification confirmed independently by different experts.

The only representative of *G. annulatus* in our dataset (Macdonald *et al.*, 2005) matched unambiguously with *G. lawrencianus* in a well-supported clade forming a single MOTU. We sampled *G. lawrencianus* throughout its range, including northern Newfoundland, which is far beyond the recorded range of *G. annulatus* (which extends from southern New England to Sable Island, off Nova Scotia; Bousfield, 1973). Because the *G. annulatus* sequence matched sequences from specimens outside its range, and as the species are morphologically very similar, we are inclined to assume this specimen is indeed *G. lawrencianus*. However, until further data are available for both species, we cannot discount the possibility that the two species share barcode haplotypes, or indeed are a single species.

Additional taxonomic discordances were detected between a *Chaetogammarus obtusatus* specimen (Macdonald *et al.*, 2005), which matched our *E. marinus*, and a mismatch between our specimen of *G. pseudolimnaeus* and that of Hou *et al.* (2007). In the former instance, the species *C. obtusatus* is not listed under this genus in either ERMS or the Integrated Taxonomic Information System (ITIS), and is instead in the genus *Echinogammarus* (E. Dahl, 1938, source: ITIS); our data indicate that this sequence of *C. obtusatus* is in fact *Echinogammarus marinus* (Leach, 1815, see discussion concerning systematics below). Strangely, however, the specimen is listed by Macdonald *et al.* (2005) as being from Canada, where *E. marinus* does not occur (suggesting perhaps that mislabelling of the specimen occurred in the lab). In the case of *G. pseudolimnaeus*, two specimens were found to diverge by 26%, presenting strong evidence that they are in fact two separate species.

Gammarus of the Svalbard intertidal zone

In addition to the two gammarid species known to be present in the intertidal zone of Svalbard (*G. oceanicus* and *G. setosus*; Klekowski & Węslawski 1990), a third putative species was recently collected. Whereas the former species inhabit rocky low-subtidal zones, these specimens were collected from the silty, nutrient-rich convergence of glacial run-off and tidal zone. They were identified as *G. zaddachi* from morphological characteristics (after Lincoln, 1979), with independent confirmation by M. Lowe (pers. comm.), which was surprising as *G. zaddachi* has no previous record on Svalbard. Barcoding of four specimens, however, yielded sequences with 100% identity to *G. oceanicus*. As all individuals observed at this collection site were markedly smaller than nearby *G. oceanicus* (~11.5 mm shorter in length from first pereon to last epimeral plate; J. Rock unpub data), we considered that they might represent ontogenetic variation in morphology with ecological partitioning of different life history stages (as has been observed in other amphipods; Stevens *et al.*, 2006). However, the presence of well developed though non-setose oostegites suggested they were late-instar juveniles or non-breeding adult females (J. Ironside, pers. comm.). The decoupling of genetic and adult phenotypic variation has been well documented in other amphipods, e.g. where disproportionately higher genetic

to morphological diversity has resulted in cryptic speciation (Müller *et al*., 2000; Witt *et al.*, 2006; Lefébure *et al*., 2007). Here, however, two genetically similar, but morphologically divergent forms occur in close proximity but inhabit different microhabitats. It is possible that this is a case of phenotypic plasticity in body size/maturation rate, as the glacial outflow environment (vs. rocky intertidal) offers limited retreat sites for larger animals and is extremely temporal in existence. Certainly introgression of mtDNA between species, a molecular phenomenon occasionally reported in other fauna (Fredsted *et al*., 2006) is unlikely to have occurred here, as the closest known populations of *G. zaddachi* are in northern Norway and are of much larger body size (J. Rock, pers. obs.). As COI is known to have low resolving power for defining some very closely related and/or recently diverged species (Mallet & Willmott, 2003; Hickerson *et al.*, 2006) nuclear markers are required to further resolve the taxonomy and evolutionary mechanisms at work in Svalbard gammarids.

Phylogeographic insights

In addition to detecting taxonomic discordances, COI sequence analysis revealed geographic structure for some species in our dataset. Again, *G. oceanicus* offers an interesting example. The two clades observed for this species matched a geographic arrangement demarcating individuals from the Baltic Sea to Hudson Bay in one clade (MOTU 14a), and individuals south of the St. Lawrence River in the other (MOTU 14b), which is similar to phylogeographic patterns observed for a number of coastal marine invertebrates in the North Atlantic (Wares & Cunningham, 2001). During the last glacial maximum (*c*. 20 000 years BP), many species with amphi-Atlantic ranges were forced to withdraw to southern refugia, isolating western and eastern Atlantic populations. After the retreat of the glaciers, NE Atlantic populations in some species expanded westwards and recolonised western Atlantic shores. In *G. oceanicus*, western Atlantic populations appear to have survived glaciation, with an isolation period apparently long enough to prevent subsequent gene flow among west and east Atlantic populations. A comprehensive phylogeographic study (Henzler, 2006) presents the pattern we have observed here for *G. oceanicus*. Northern and southern North American clades also appeared in our more limited sampling of *G. mucronatus* and *G. lawrencianus*, supporting a major phylogeographic break between New England and Chesapeake Bay revealed previously (Henzler, 2006). Geographic structuring in *G. tigrinus* (ITS1) also matched divergence of southern (Carolinian province) and northern phylogroups previously established with nuclear markers (Kelly *et al.*, 2006a). Although native to the northwest Atlantic, *G. tigrinus* has been recently introduced to the British Isles, from which it has expanded into north European coasts and the Baltic Sea (Kelly *et al.*, 2006b). Our *G. tigrinus* sequences from Poland clustered with the northern phylogroup, confirming Kelly *et al.*'s (2006b) suggestion that COI haplotypes could be used to identify the original introductory populations from the northwest Atlantic.

Phylogeographic divergence between the eastern and western Atlantic was not observed in *G. duebeni*, with extensive geographic sampling from both regions comprising a single clade (MOTU 5a). However, a second well-supported clade of specimens from a location in Wales (MOTU 5b) diverged by ~4%, representing the subspecies *G. duebeni celticus* (see Rock *et al.*, 2007). Of note is a similar level of divergence between *E. marinus* populations in the NE Atlantic, and between populations of *G. locusta* from Wales/Scotland versus continental Europe. Potential structuring in these species deserves further investigation as they suggest patterns potentially associated with glacial refugia for *Gammarus* also described by Rock *et al.* (2007). Geographic structuring was also suggested for several other eastern Atlantic species including *G. setosus, G. zaddachi and G. salinus,* with some level of divergence for the latter two associated with populations in the Baltic.

Phylogenetics and systematics

Nucleotide sequences of COI barcodes have been shown to perform very well in discriminating genetic groups at – or even below – the species level, although they often do not perform as well recovering deeper phylogenetic groupings (Hajibabaei *et al*., 2006). By examining clustering patterns using amino acid data, we aimed to detect potentially informative multispecific phylogeographic groups within our dataset. For example, species from southern Europe that were clearly separated by nucleotide sequences (*G. aequicauda*, *G. crinicornis*, *G. insensibilis*, *G. locusta*) were virtually identical at the amino-acid level, supporting the '*G. locusta*-group' (Stock, 1967) as a distinct systematic and evolutionary lineage. Similarly, a clade emerges from the amino-acid data including *G. zaddachi*, *G. salinus*, *G. wilkitzkii* and *G. setosus*. The so-called '*G. zaddachi*-group' is usually perceived as composed of *G. zaddachi*, *G. salinus*, *G. oceanicus* and *G. duebeni.* Our analyses extended close affinity with cold-water species *G. wilkitzkii* and *G. setosus*, but excluded *G. oceanicus* and *G. duebeni*. Earlier phylogenetic studies on *Gammarus* using allozymes (Skadsheim & Siegismund, 1986) were only partially effective in unravelling relatedness within the group, but also found close affinity between *G. salinus* and *G. zaddachi*. Our amino-acid data also reinforced the phylogenetic affinity of *E. marinus* with other *Echinogammarus*, revealing a consistent cluster with *E. ischnus* and *E. trichiatus*, which are well demarcated from other *Gammarus* species. Although a number of authors have considered '*marinus*' to belong to the genus *Echinogammarus* (e.g. Dick *et al*., 2005; D. Platvoet: Amsterdam Zoological Museum; http://nlbif.eti.uva.nl/bis/amphipoda.php; Fauna Europea: http://www.faunaeur.org/index.php), rather than *Gammarus* or *Chaetogammarus*, this is not followed by all authors (ERMS: Bellan Santini and Costello, 2001; ITIS: http://www.itis.gov; Lincoln, 1979). Here we add new sequence data that support the assignment of *Echinogammarus* as the appropriate genus name and encourage its widespread use.

Concluding remarks

COI sequence data provided generally unambiguous species discrimination of *Gammarus*, and enabled cross-checking of identifications among studies by different authors. The several mismatches found among such studies only confirm the

need for a standardised, unambiguous and routine molecular identification tool for such taxonomically complex groups. The current study provides a valuable reference library against which DNA barcodes of marine *Gammarus* obtained in different regions can be checked in the future. Such a universal taxonomic screening tool, when integrated with ecological and geographic data, could impart a significant improvement on our knowledge of the taxonomy, crypticism, distribution ranges and phylogeography of marine *Gammarus*. For example, our data highlighted hotspots of speciation and evolution in marine and brackish water *Gammarus*. Range-wide sampling provided strong indication of cryptic speciation, in particular for *G. aequicauda*, *G. insensibilis* and *G. duebeni*. Good sample sizes and geographic coverage also yielded indication of population structure in multiple *Gammarus* species. Finally, our results supplied relevant information concerning the phylogeny of *Gammarus*, confirming the cohesiveness of southern European species, suggesting a phylogenetic separation of North American and European species, and corroborating the likely systematic position and genus for an as yet taxonomically unsettled species (*E. marinus*).

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