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Cryptic diversity of the 'cosmopolitan' harpacticoid copepod *Nannopus palustris*: genetic and morphological evidence

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Abstract

Nannopus palustris **Brady, 1880 is a free-living widely distributed harpacticoid copepod, which has been formerly assumed to be a single, cosmopolitan but highly variable species. We compared several geographically distant *N. palustris* populations in terms of their morphology and genetics. Populations from the White Sea (WS), the North Sea (NS), the Black Sea (BS) and two sympatric morphs from South Carolina, USA (SC notched and SC straight morphs), were considered. The NS, BS and to a lesser extent SC notched specimens were morphologically similar and partly coincided to the 'canonical' description of the species. By contrast, WS population showed remarkable anatomical and morphometric peculiarities that correspond to some earlier descriptions. Genetic analyses of mitochondrial (cytochrome *b*) and nuclear (28S rDNA) genes demonstrated the significant distinctness among WS, both SC and (NS +BS) populations, the latter two being genetically indistinguishable. Concordance between mitochondrial and nuclear gene trees and morphological data supports that *N. palustris* is in fact composed of several pseudo-sibling species, which are genetically and morphologically divergent. Neither correlation between genetic divergence and geographical distance nor significant intrapopulation diversity was found for these species. Taxonomic status, distribution and phylogenetic relationships of the species within the *Nannopus* genus need to be reconsidered. A further subdivision of species complexes might have important implications for the analysis of biodiversity of benthic copepods and consequently for the interpretation of their (species-specific) ecological function.**

Keywords: cryptic species, genetic diversity, Harpacticoida, meiofauna, morphology, *Nannopus palustris*

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Introduction

Harpacticoid copepods (Crustacea, Copepoda), aside from nematodes, are ubiquitous and often the most abundant component of marine meiofauna (Giere 2009). Unlike many macrobenthic groups, harpacticoids recruit

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directly to the benthic environment, that is, have no long pelagic dispersal phase. Nevertheless, many harpacticoids, as well as many other meiobenthic species, have been reported to be widely distributed or even cosmopolitan (Wells 1986; Chertoprud *et al.* 2010), despite the limited reproductive and dispersal potential (the so-called meiofauna paradox, Giere 2009). Recent studies reveal that many former cosmopolitan meiobenthic 'species' constitute complexes of genetically, and often morphologically, diverging sibling species (Gourbault & De Craemer 1996; Todaro *et al.* 1996; Schizas *et al.* 1999; Rocha-Olivares *et al.* 2001; Staton *et al.* 2005). Moreover, morphological stasis and convergent evolution of character states are known to occur in harpacticoid copepod evolution (see e.g. Rocha-Olivares *et al.* 2001; Easton *et al.* 2010), so species may be overlooked when 'traditional' morphology alone is used for species delimitation. Especially for widely distributed, highly abundant and ecologically important organisms, a correct delineation of the species is essential in biodiversity research. Therefore, the synergistic application of detailed morphological characterization and molecular analysis can lead to new insights and provide pivotal information to ecologists.

A striking example of this issue is *Nannopus palustris* Brady, 1880 (Nannopodidae: Harpacticoida)—a free-living widely distributed copepod, which has been reported from shallow marine and brackish waters from the Arctic to the tropics (Fig. 1). *N. palustris* was formerly assumed to be a single, cosmopolitan, but highly variable species, with a wide range of halo-and thermotolerance (Wells 1971).

Numerous morphological variations have been repeatedly reported for this harpacticoid, which are not consistent with the early descriptions of Canu (1892) and Scott (1902) (e.g. see Montschenko & Polishchuk 1969; Wells 1971; and references therein). These differences were usually attributed to ecological or geographical variability. In particular, populations inhabiting the intertidal zone of South Carolina, USA, have 80–90% of the females with straight caudal rami terminal setae and up to 10% with an unusual notch on the basal part of the principle caudal rami terminal seta (Coull & Fleeger 1977). Genetic sequencing of the South Carolina population showed that 'notched' and 'straight' (non-notched) morphs were genetically distinct in both 28S rDNA and mitochondrial Cyt*b* haplotypes and presumably represent different cryptic species. Ironically despite extensive sampling, no males have been found with the notched form morphologically or genetically (Staton *et al.* 2005).

Thus, further analysis of more populations from wider geographical range is necessary to untangle this curious pattern. The main purpose of this study was to collect and compare *N. palustris* from different geographic locations using a combination of gene-sequencing and morphology to determine levels of divergence among the remote populations. Better knowledge of this divergence and a further delineation of species can largely contribute to our understanding of the distribution of species worldwide, their (species-specific) ecological role in marine sediments and the overall interpretation of marine biodiversity. Ecological function is intimately linked to biodiversity, and loss of genetic diversity

Fig. 1 Geographic distribution of *N. palustris* (based on Lang 1948; C.B. Wilson taxonomic cards on WoRMS (Appeltans *et al.* 2012); the Ocean Biogeographic Information System and the Encyclopedia of Life databases). The open circles represent the sampling locations included in the current study.

because of local disturbance could destabilize or disrupt of ecological function (Hooper *et al.* 2012).

Materials and methods

Sampling location and processing

Specimens of *Nannopus palustris* were collected from three distant sites.

1 The White Sea population (WS) was sampled in October 2011, at the intertidal zone near the White Sea Biological Station of Moscow State University (66°34′N, 33°08′E). Sediments were medium-silted poorly sorted sand (modal particle size 0.7–0.9 mm); water salinity was 20–22, water temperature 6.6–7.2 °C. Earlier, *N. palustris* abundance of 61–118 ind/10 cm^2 was reported from this site (Rybnikov *et al.* 2003). Totally, 81 copepods were collected at low tide and preserved in 96% ethanol.

2 The North Sea population (NS) was sampled at the Paulina intertidal flat in the Westerschelde estuary, SW Netherlands (51°21′N, 3°43′E), in the silty sediments of the high intertidal zone. Sea water temperature at the time of collection was 16 °C, salinity typically ranged between 25 and 28. In October 2011, 69 copepods were collected at low tide and preserved in 96% ethanol.

3 The Black Sea population (BS) was sampled at the Grygorivsky Liman, a small bay in the NW part of the sea (46°63′N, 31°02′E). Seventeen alcohol (96% ethanol)-preserved copepods for the genetic study were collected in August 2003 and additional six in July 2011 from the top centimetre of the silty sediment at 15 m depth. Also, 61 formalin-preserved copepods for the morphological study were collected by a Petersen bottom grab at the Odessa Bay (46°30′ N, 30°45′E) from the silty sediment (10 m depth) at July 2011. Near-bottom water temperature in these sites was 9–12 °C, salinity ranged 15–18.

In the laboratory, sediments were decantated over a 250-μm sieve. Specimens were picked out with a glass Pasteur pipette under a light microscope and finally stored in 96% ethanol.

4 The data on *N. palustris* population from the South Carolina, USA (Staton *et al.* 2005), were also used for comparison. Samples were collected monthly for 1 year from October 2001 to September 2002 from the sloping (20% grade) mud flat at Oyster Landing in the North Inlet-Winyah Bay, National Estuarine Research Reserve (33°18′N, 79°13′W). The mean annual sediment/water interface temperature ranged from 10 ± 0.6 °C (January) to 29 ± 0.5 °C (August); the typical salinity was 30–33. Details of this site and sampling procedures were as described by Staton *et al.* (2005).

Morphological analysis

Prior to dissection, measurements of body dimensions (full body length without caudal rami and maximal prosome width) were conducted on all collected copepods using an ocular micrometre. Up to ten specimens from the every studied location were dissected in glycerol and the parts individually mounted in lactophenol under coverslips, which were subsequently sealed with transparent nail varnish.

To compare our material with the literature data (Canu 1892; Scott 1902; Sars 1909; Coull & Fleeger 1977; Staton *et al.* 2005), copepods were examined for 10 characters that were previously listed and discussed by Wells (1971) as the key markers of morphological variability. These key characteristics are as follows: number of segments of the antennule (A1), number of setae on the exopod of the antenna (A2), number of setae on the distal segment of leg (pereiopod, P) first exopod, type of inner seta on the distal segment of P3 exopod, number of setae on the inner edge of the distal segment of P3 endopod, number of setae on the inner edge of the distal segment of P4 exopod, number of setae on the P5 exopod of the female, number of egg sacs, type of P3 endopod of males and the form of the caudal ramus. In addition, we also checked the number of setae on the basis of the A2.

To analyse the variation of length and width (separately) of the populations, a Newman–Keuls *post hoc* test for multiple comparisons of the means (Glantz 2005) was performed. For this test, three SC morphs (notched, fat and thin, data from Staton *et al.* 2005) were analysed separately. Data on two populations from the Spitzbergen (Wojtasik & Kur 2007) were also included.

DNA amplification and sequencing

Specimens processed in 2003. Black Sea samples were analysed in the Coull laboratory at the University of South Carolina. DNA from seven Black Sea individuals was extracted according to Schizas *et al.* (1997), with the exception of a 10-fold increase in proteinase K per reaction, as outlined by Staton *et al.* (2005). From this extraction, 2–3 μL was used as a template for DNA amplifications (Saiki *et al.* 1988), which were performed according to Schizas *et al.* (1999) and Staton *et al.* (2005). Briefly, the D3 region of 28S rDNA was amplified using the primers D3A (GAC CCG TCT TGA AAC ACG GA) and D3B (TCG GAA GGA ACC AGC TAC TA) (Litvaitis *et al.* 1994). Mitochondrial cytochrome *b* apoenzyme

(Cyt*b*) was amplified with primers cytb424 (GGW TAY GTW YTW CCW TGR GGW CAR AT) and cytb876 (GCR TAW GCR AAW ARR AAR TAYCAY TCW G) (Staton *et al.* 2005). Amplification products were purified with Exonuclease I (NEB Catalogue #M0293L) and Antarctic phosphatase (NEB Catalogue #M0289L) (ExoAP). ExoAP purification solution consisted of 5% 10× AP buffer, 10% ExoAP and 85% dH$_2$0. Four microlitre of amplification product was incubated with 4 μL of ExoAP at 37 °C for 15 min, 80 °C for 15 min then held at 15 °C until removed from the thermal cycler. Purified products were sequenced using 1/16th strength reactions because of the high sensitivity of the capillary sequencer. Briefly, sequencing reactions were carried out as follows: 3.5 μL of 2.5× sequencing dilution buffer, 0.5 μL BigDye Terminator v3.1 mix, 1 μL primer, 2.0 μL DNA template and 3.0 μL dH$_2$0. Sequencing reactions were carried out with 39 thermal cycles of the following steps: 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min and held at 15 °C after the final cycle. Sequencing reactions were cleaned and precipitated by adding 1 μL of 1.5 M NaOAc + 250 mM EDTA (pH 8) and 40 μL of 95% EtOH. Samples were agitated on a Microplate Genie for 1–2 min and then allowed to sit for 15 min at −20 °C. Samples were then centrifuged for 45 min at 1500 *g*. After centrifugation, reaction fluids were decanted, and microplates were sealed, labelled and stored at −20 °C. Precipitated sequences were sent to the Savannah River Ecology Lab and sequenced on an Applied Biosystems 3130 capillary sequencer. Sequences were aligned and edited in Sequencher 4.1TM (GeneCodes, Ann Arbor, MI, USA). Concatenated sequences were exported from SequencerTM for phylogenetic analysis.

Specimens processed in 2011. Samples were analysed in the Molecular Genetics Lab of Pertsov White Sea Biological Station, Lomonosov Moscow State University. Promega Wizard SV Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) was used for tissue lysis and DNA purification following the manufacturer's protocol. The same primers as above were used for polymerase chain reaction (PCR) amplification. Loci were amplified using an Encyclo PCR kit (Evrogen Joint Stock Company, Russia). Amplification was performed in a total volume of 25-μL reaction mixture containing 1× PCR buffer, 1 μL of 10 μM of primer pair mix, 1 μL of template, 0.2 mM of each dNTP and 0.5 units Taq polymerase. Reaction mixtures were heated to 94 °C for 120 s, followed by 35 cycles of 15 s at 94 °C, 30 s at a specific annealing temperature and 45 s at 72 °C, and then a final extension of 7 min at 72 °C on Veriti® Thermal Cycler. Annealing temperature was set to 47 °C for the Cyt*b* primer pairs and 50 °C for the 28S primer pair. The Promega PCR Purification Kit protocol (Promega) was employed to purify amplification products. Amplification products were sequenced in both directions. Each sequencing reaction mixture, including 1 μL BigDye (Applied Biosystems, Perkin–Elmer Corporation, Foster City, CA, USA), 1 μL of 1 μM primer and 1 μL of DNA template, ran for 40 cycles of 96 °C (15 s), 50 °C (30 s) and 60 °C (4 min). Sequences were purified by ethanol precipitation to remove unincorporated primers and dyes. Products were re-suspended in 12 μL of dimethylformamide and electrophoresed in an ABI Prism_3500 sequencer (Applied Biosystems).

Overall, 62 unique sequences were analysed for Cyt*b* and 57 for 28S genes (see Appendix S1). Sequences for both genes were obtained from the same individuals with a few exceptions.

Phylogenetic analysis

Obtained sequences were processed by the MUSCLE (Edgar 2004) algorithm in MEGA5 software kit (Tamura *et al.* 2011) providing 296- and 266-bp alignments for 28S and Cyt*b*, respectively (see Appendices S2 and S3). Data sets were tested for best suiting nucleotide substitution models by MEGA5 using Bayesian information criterion (BIC), resulting in Jukes-Cantor model for 28S, and Tamura 3-parameter model with gamma distribution of rates among sites for Cyt*b*. Phylogenetic reconstructions were made for both data sets by maximum likelihood method in MEGA5, and statistical support for internodes was tested by bootstrapping in 1000 replications. Additionally, we performed Bayesian inference of phylogeny in MRBAYES 3.2 (Ronquist & Huelsenbeck 2003). High-resolution trees were generated in MEGA5. GenBank sequences of *Tigriopus californicus* (accession numbers GQ141001.1 and AF363324.1) were used as out groups for rooting the mitochondrial Cyt*b* and 28S rDNA gene trees, respectively.

To test the concordance between two matrices of between-population genetic divergence (measured as number of base substitutions per site from averaging over all sequence pairs between groups), we performed the Mantel test of matrix association with 5000 random permutations. This test was also used to examine the relationship between genetic divergence and geographic distance among populations. Geographic distances were measured as the shortest, continuous water-surface distance between sampling locations.

Results

Morphological variations

We did not attempt a complete morphological analysis of *Nannopus palustris*, but rather an in-depth comparison between the studied populations based on 11 key characteristics that serve as the main source for variation (see before, Wells 1971). In our analysis, the descriptions given by Coull & Fleeger (1977) and Staton *et al.* (2005) were used for the South Carolina populations. Relying on the genetic results (Staton *et al.* 2005), we have considered fat and thin morphs as a single 'species' (SC straight) because both morphs were similar. Based on Sars' (1909) and Wells' (1971) opinions that the original description by Brady is imperfect and fragmentary, and the fact that Canu's (1892) and Scott's (1902) identifications of the species are correct and generally accepted, we refer to their descriptions as 'normal' or 'canonical'. We also include the description of Sars (1909) as important and relevant to our comparative analysis (Table 1). See Materials and methods for the set of characters considered.

All examined populations differed in some way from the 'canonical' description of *N. palustris* (Table 1). They had in common only five of eleven characters. On the one hand, except for the type of apical spine of P3 endopod of the male, there was no marked distinction in setal formula between the SC straight population and both the NS and the BS populations. Specimens from these three populations looked similar morphologically, and all of them differed from the 'canonical' description just in number of setae on the inner edge of the distal segment of P4 exopod. According to Wells (1971) and Coull & Fleeger (1977), the form of the P3 endopod of the male is similar to the 'canonical' *N. palustris* and SC specimens.

The WS copepods, on the contrary, were separate and significantly differed from the other populations (see Table 1). They had five characteristics that distinguish them from NS and BS specimens (and six—from the 'canonical' description). Meanwhile, WS specimens were almost identical to the Norwegian *N. palustris* described and drawn by Sars (1909) except for the number of seta on the basis of the A2 (but it could be a mistake in his drawing) and the number of ovisac. A sharp spine on the P3 endopod of males was the only common character discriminating all three European

Table 1 Variation in the key morphological characteristics of *Nannopus palustris* Brady

Character	'Canonical'*	North Sea, Norway †	Atlantic coast, North Inlet, South Carolina, USA‡		North Sea, The Netherlands (NS)	Black Sea (BS)	White Sea (WS)
			Straight	Notched			
A1 ♀	5 segments	5 segments	n/a		5 segments	5 segments	5 segments
Exopod A2	4 setae	4 setae	n/a		4 setae	4 setae	4 setae
Basis A2	2 setae	1 seta	n/a		2 setae	2 setae	2 setae
P1 Exopod distal segment	2 setae	1 seta	2 setae		2 setae	2 setae	1 seta
P3 Exopod inner seta of distal segment	Long	Short	Long		Long	Long	Short
P3 Enp. inner edge of distal segment	3 long setae§	2 setae—1 short and 1 long	3 setae—1 short and 2 long		3 setae—1 short and 2 long	3 setae—1 short and 2 long	2 setae—1 short and 1 long
P4 Exopod inner edge of distal segment	3 setae	1 seta	2 setae		2 setae	2 setae	1 seta
P5 ♀ Exopod	5 setae	5 setae	5 setae		5 setae	5 setae	5 setae
Ovisac	2 sacs	1 sac	2 sacs		2 sacs	2 sacs	2 sacs
P3 ♂ Endopod	Blunt bifid apical spine	n/a	Blunt bifid apical spine	Unknown	Sharp spine	Sharp spine	Sharp spine
Caudal furca	2 apical setae, innermost thick and tapering	♀ outer seta very short, inner thick with basal tooth	2 apical setae, innermost thick and tapering	♀ outer seta very short, inner thick with basal tooth	2 apical setae, innermost thick and tapering	2 apical setae, innermost thick and tapering	♀ outer seta very short, inner thick with basal tooth

n/a, data not available.

*Wells' reference (1971: 515) to Canu (1892) and Scott (1902).

†Sars (1909).

‡Coull & Fleeger (1977), Staton *et al.* (2005), and our own observations.

§Wells (1971) indicated three long setae on the inner edge of distal segment of the P3 exopod, but Canu's drawing (1892) clearly showed 1 short and 2 long setae.

populations from the American ones and from the 'canonical' description.

Sex ratio and morphometry

In all three populations collected for this study, males were found, and the male/female ratio was similar: 13:87% for WS and NS populations (38 and 69 individuals, respectively) and 21:79% for BS population (61 individuals). Our North and White Sea samples were taken in October, so the per cent of males might be even higher in the summer.

Morphometric data (Fig. 2) also showed that SC notched and WS populations were most clearly distinct in their size and shape. The SC notched females were appreciably shorter and wider, while the WS individuals had more slender proportions, and their females were larger than others. The Newman–Keuls *a posteriori* test of female mean lengths and widths confirmed that both SC notched and WS populations differed significantly ($P < 0.01$) from all other populations (Table 2). Specimens from the North Sea (NS), SC straight morph and specimens from the Spitzbergen (two populations collected from the tidal flats with water temperature 6.6–11.9 °C and salinity 0–9.3, data from Wojtasik & Kur 2007) were rather close together in terms of their size. BS population occupied an intermediate position.

Body size is presumably very responsive to environmental conditions that affect individual growth; therefore, the morphometric data could reflect environmental differences among the samples (e.g. in temperature, salinity, etc.) rather than interpopulation/interspecific variations. However, the two most contrasting populations (SC notched and WS) differ significantly from all others, irrespective of their habitat conditions, including the ecologically similar ones (WS vs. NS and Spitzbergen; or SC notched vs. SC straight).

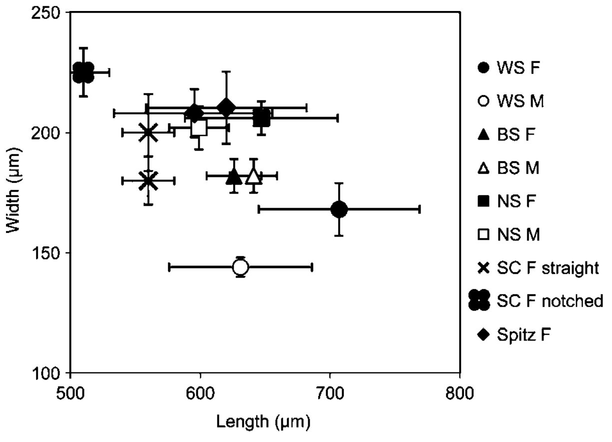

Fig. 2 Ranges of body dimensions (mean and std. deviation) for different *N. palustris* populations. Data on SC notched and two straight (fat & thin) morphs are from Staton *et al.* (2005), data on two Spitzbergen populations ("Spitz") are from Wojtasik & Kur (2007). F – females (filled symbols), M – males (open symbols).

Genotypic variability

Nannopus palustris specimens from three populations (WS, NS and BS) were sequenced for 28S rDNA and for mitochondrial Cyt*b* genes. Previously published sequences for the same genes from South Carolina morphs (SC straight and SC notched, Staton *et al.* 2005) were also included in the overall analysis.

The 28S rDNA phylogenetic tree (Fig. 3a) grouped all sequences into a monophyletic cluster, which included four divergent clades with strong bootstrap support (BP = 53–100%), indicating that all four lineages were genetically distinct from each other. The BS and NS populations were genetically indistinguishable and very close to the SC notched clade (0.3–0.4% difference) but far from the SC straight clade (2.1–2.2%). The WS clade (supported by BP = 100%) appeared to be basal and most distinct (average difference 3.9%). High pairwise sequence identities were observed within each clade indicating high levels of intrapopulation homogeneity.

The Cyt*b* tree (Fig. 3b) had a topology that was consistent with the 28S rDNA tree (Mantel correlation between the two matrices was 0.877, $P = 0.002$) but showed rather high divergence level between the clades. NS and BS haplotypes were most similar (20.5%) and grouped closer to SC notched ones (average difference 47.1%). The WS haplotypes, most separate, were relatively closer to the SC straight form (with difference of 50.2%). The average difference between these two groups was as high as 66.2%. Again, haplotypes within each clade were genetically indistinguishable from one another.

The genetic divergence between populations showed no relationship with geographic distances, neither in nuclear nor in mitochondrial genes; the respective Mantel correlations were 0.233 ($P = 0.305$) and 0.361 ($P = 0.137$).

Thus, according to both nuclear and mitochondrial genes, the analysed haplotypes assorted into four divergent clades, of those two (SC straight and SC notched) were sympatric, one set of haplotypes was shared between to geographically remote populations (NS and BS), whereas WS haplotypes were most genetically separated from all populations analysed.

Discussion

Morphological variations

It is known that cryptic species of small metazoan invertebrates are particularly difficult to identify using

Table 2 Results of the Newman–Keuls post hoc test of differences for mean length (under diagonal) and width (below diagonal). Diagonally are the numbers of individuals measured (underlined). Significant values: typed in bold ($P < 0.01$), typed in *italic* ($P < 0.05$). Only data on females are presented

Populations	SC notched	SC fat	SC thin	Spitz 1	Spitz 2	BS	NS	WS
SC notched	30	**8.55**	**9.51**	**8.04**	**9.25**	**24.31**	**11.48**	**15.33**
SC fat	**7.26**	30	0.38	3.24	**4.92**	**13.83**	**7.15**	**11.24**
SC thin	**17.13**	**6.89**	28	*3.24*	**4.96**	**13.54**	**7.12**	**11.19**
Spitz 1	**7.15**	2.52	**11.53**	43	1.69	3.23	*3.87*	**7.87**
Spitz 2	**4.46**	2.58	**9.05**	0.76	30	0.55	1.78	**5.44**
BS	**21.41**	**7.00**	0.97	**14.34**	**10.39**	48	2.03	**7.20**
NS	**9.87**	2.18	**13.20**	1.16	1.65	**17.77**	61	**4.26**
WS	**22.31**	**12.08**	**4.11**	**16.80**	**13.19**	**6.73**	**17.45**	53

morphological criteria alone (Rocha-Olivares *et al.* 2001). In harpacticoid copepod systematics, setal formulae are one of the main criteria for species diagnosis (Huys & Boxshall 1991; Wells 2007). In our case, there are clear differences in setation between several populations formally attributed as *Nannopus palustris*.

The WS population demonstrates the most remarkable differences from 'canonical' *N. palustris* given in Canu (1892) and Scott (1902) and summarized by Wells (1971). These differences include six of the eleven morphological traits examined here, so this discrepancy could not be treated as 'minor intraspecific variations'. On the other hand, WS specimens have more in common with the description in Sars (1909), differing only in number of setae on the basis of the antenna; this similarity had earlier been pointed out by Chislenko (1967). He discovered a single female *N. palustris* in silty sand at the Karelian coast of the White Sea, not far from our sample area. His drawings of antennule +rostrum, P1, P4 exopod and P5 exopod are identical to Sars (1909) and our WS specimens. Later, Arlt (1983) drew P4 and P5 of a female found in coarse sand in the western part of the Baltic Sea (between the Bomholm and Rügen islands), and his specimen was also similar to Sars'.

Our WS population significantly stands out against others in terms of average body size and proportions. Its length (female: 0.71 ± 0.07 mm) exceeds the typical values of 0.55–0.65 mm stated for adult *N. palustris* females (Lang 1948; Wojtasik & Kur 2007; and our data) but is close to the 0.75 mm value reported earlier for the White Sea individuals (Chislenko 1967; Kornev & Chertoprud 2008) and to that given for Norwegian specimens by Sars (1909) (0.7 mm).

By contrast, the NS and BS specimens examined in this study are morphologically similar and both are closer to the original descriptions of Canu (1892) and Scott (1902). However, they noticeably differ from this 'canonical' form in setation of P4 exopod and type of P3 endopod of male (Table 1). Earlier, Montschenko & Polishchuk (1969) discovered that Black Sea females of *N. palustris* differed from these earlier described by Jakubisiak (1938), Chislenko (1967) and Sars (1909), but fully complied with Gurney's (1932) description. However, both Gurney's (1932) and even Sars' (1909) pictures, which presented in the Lang's monograph (1948), are not identical to the classical description of Canu (1892) and Scott (1902). Another interesting point about Black Sea *N. palustris* is that Montschenko & Polishchuk (1969) drew the inner furcal seta with a small bulb, which also has been observed occasionally in our Black Sea females.

Curiously enough, of all populations considered here, the one that most corresponds to the 'normal' set of characters is the SC straight form from South Carolina. In particular, only these males have the typical blunt bifid apical spine on leg 3 endopod. These specimens, however, differ from 'canonical' *N. palustris* in number of setae on inner edge of distal segment of leg 4 exopod (Table 1) and also in the presence of P2–P4 additional minute inner seta on the inner edge of the terminal segments of exopods and endopods of P2–P3 (Coull & Fleeger 1977). Another South Carolina form (SC notched) has the same leg setation but is clearly distinct in the specific form of the terminal seta of the caudal ramus. This character makes the SC notched population closer to the WS population; though, they differ in many other morphological features, as well as in morphometry. Moreover, the SC notched form remains the only 'no-males' population—a striking exception that still needs an explanation (see Staton *et al.* 2005 for discussion).

Many of these discrepancies can be resolved assuming that previous authors dealt with several closely related but different species, one as the 'classic' *N. palustris* described by Canu and Scott and another one found by Sars (as he at first considered it himself, see Sars 1909: 308). If that is the case, only the SC

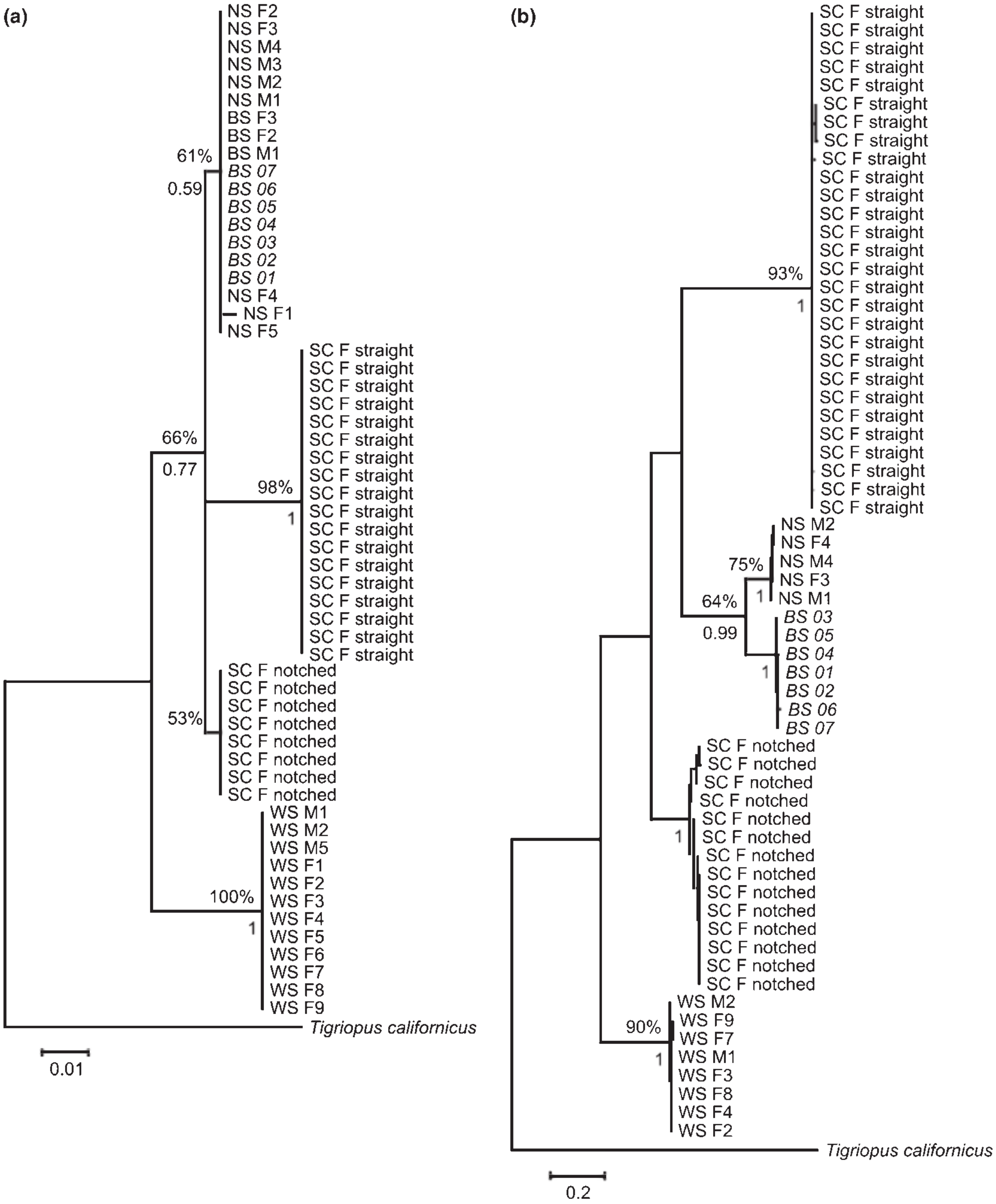

Fig. 3 Maximum-likelihood tree topology inferred from 28S rDNA (a) and Cyt*b* (b) sequences. The sequences of the BS copepods collected in 2003 typed in *italic*. Branch lengths represent estimated number of substitutions per site. Numbers above and under branches indicate clade credibility values from MEGA5 (bootstrap support percentage) and MrBayes (Bayesian posterior probability), respectively. Only values >50 are included.

straight form could be attributed, under some reservations, to the first species, while the WS specimens are close to the second one. Thus, there are at least three lines (WS, NS+BS and SC notched) obviously divergent from the 'normal' set of morphological traits previously described. The morphological differences among these major lineages are congruent with the patterns of genetic differentiation.

Genetic analysis

Our data reaffirm considerable genetic heterogeneity among different *Nannopus* populations. Phylogenethic trees based both on mitochondrial Cyt*b* and on 28S rDNA clearly indicate that specimens from WS, (NS+BS), as well as straight and notched SC morphs belong to separate clades.

The BS and NS populations, despite their geographical isolation, seem to be almost identical genetically and morphologically and thus could be considered as single species. They do not show any pronounced divergence of nuclear genes, and the divergence of their Cyt*b* sequences (20%) is in the range of typical intraspecific level. That is not surprising in view of noticeable atlanto-boreal features and possibly Atlantic origin of the modern Black Sea harpacticoid fauna (Chertoprud *et al.* 2010).

This BS+NS clade most closely resembles the SC notched population, especially by nuclear genes; though, the difference between them in mitochondrial Cyt*b* is rather significant. Mitochondrial genes have higher mutation rates and smaller effective size and evolve faster than the nuclear genes (Avise 2000). According to our data, morphologically these clades differ only in the form of the caudal ramus. Therefore, the BS+NS and SC notched clades could be considered as initial stage of divergence from a common ancestry.

The SC straight and WS clades are genetically distant and morphologically dissimilar to others. The genetic divergences among these clades are rather large (in the range of 44–78% for mitochondrial genes and up to 4.29% for nuclear ones). These values significantly exceed the levels of intraspecific divergences reported for harpacticoids and some other invertebrate taxa (17–27% for mitochondrial and 2–4% for nuclear genes; e.g. Burton & Lee 1994; Schizas *et al.* 1999; Rocha-Olivares *et al.* 2001; Derycke *et al.* 2007; and references therein), providing evidence that each clade might justifiably be assigned to a different species.

Divergence values obtained for Cyt*b* nucleotide sequences are unexpectedly high. One possible explanation may be that some nonorthologous gene copies have been PCR amplified in mtDNA. However, we believe they are of the same highly variable gene. Their reading frame was not broken, most substitutions were in third codon position, and predicted proteins had same conserved sites of Cyt*b* (N-terminus)/b6/petB superfamily. Moreover, the divergences of sequences for usually more conservative 28S were also rather high, at least for the WS clade. Two sets of the BS sequences, obtained independently in two different laboratories from different samples, supplement each other (for Cyt*b*) or match perfectly (for rDNA sequences). So far, molecular studies of several harpacticoid genera also have discovered a surprising level of genetic diversity. High levels of intra- and interspecific divergence seem to be widespread phenomena among members of the order Harpacticoida (Schizas *et al.* 2002; Easton *et al.* 2010).

Most individual haplotypes belonging to the same clade yielded low levels of within-clade haplotype divergence (<4% for Cyt*b* and much less for 28S rDNA). As noted above, 28S rDNA sequences obtained independently from BS samples collected in the years 2003 and 2011, coincided to each other. Moreover, little genetic variation in Cyt*b* and 28S genes has been found between 'straight-morph' populations from Massachusetts, South Carolina and Louisiana (J.G. Baguley, unpublished). These results contrast with high intraspecific diversity of haplotypes within a location, which has also been reported in other meiobenthic species (e.g. Burton & Lee 1994; Schizas *et al.* 1999, 2002; Staton *et al.* 2001; Derycke *et al.* 2007, 2010). For example, Rocha-Olivares *et al.* (2001) observed high degree of divergence in the harpacticoid copepod *Cletocamptus deitersi* among North American regions (Alabama, Louisiana, California and Mexico). Such low intrapopulation diversity of *N. palustris* may be an artefact of limited or inadequate sampling within our three collecting sites (BS, NS and WS populations), but this is obviously not the case for the extensively sampled North American populations. It may suggest either demographic effects or some selective forces acting on the *N. palustris* genome at the local or regional scale.

Genetic distance was not correlated with geographic distance between localities. Moreover, different species of the '*N. palustris*' complex demonstrate very different patterns of distribution. The two sympatric populations, that is, straight and notched SC ones, are highly dissimilar. Taking into account the above-mentioned early literature, coexistence of several *Nannopus* species in the North Sea is also rather possible. In sympatry, distinct clades imply that a reproductive barrier has allowed the accumulation of genetic differences. At the same time, specimens from the North and Black seas are morphologically indistinguishable and share a single 28S rDNA sequence, despite these populations occupy not only geographically remote but ecologically different habitats: full-saline tidal flat (NS) and low-saline sublittoral sediments (BS). This BS+NS clade may have a truly wide geographic distribution and ecological range, or its distribution may be a result of founder effects with one of the two locations serving as the source population.

Therefore, the distribution of haplotype clades among the sampling locations does not conform to the 'isolation-by-distance' model, which would predict that genetic distance between populations within a species flock increases as geographic distance and degree of geographical isolation increases (Slatkin 1993). This pattern seems to be unusual for the species with limited dispersal capacity that is restricted to linear habitats of coastal, mainly estuarine environments. In these situations, clinal biogeographic patterns with speciation at the ends of species ranges are expected to be most likely because of oceanographic discontinuity and prevailing current directions (Edmands 2001; Chen & Hare 2011). Indeed, some other studies on harpacticoids showed a strong spatial structure in genetic data [e.g. Schizas *et al.* (1999) for *Microarthridion*; Rocha-Olivares *et al.* (2001) for *Cletocamptus*; Edmands (2001); Handschumacher *et al.* (2010) for *Tigriopus*], whereas others showed a weak or no spatial structure [e.g. Schizas *et al.* (2002) for *Microarthridion* or Denis *et al.* (2009) for *Tigriopus*]. Perhaps such deep and spatially uncorrelated genetic divergence occurred long ago, and these species are on independent evolutionary tracks. The phylogenies based on both nuclear and mitochondrial genes are generally concordant. Such concordant phylogeographic patterns among unlinked genes most probably reflect the important role of historical factors limiting gene flow among populations (Avise 2000). Cryptic species are apparently widespread phenomena among many meiobenthic taxa. However, this fact *per se* cannot solve the 'paradox of meiofauna', because at least some of these cryptic species turn out to be widely distributed themselves. Further investigation is necessary to elucidate species dispersal mechanisms and ecological relationships in more detail. The current data are, however, too limited for any reliable interpretation of the worldwide *Nannopus* distribution pattern or an explanation thereof.

Conclusions

It is obvious that the situation with this 'well-known cosmopolitan species' is entangled and uncertain. This study confirms the earlier results of Staton *et al.* (2005) and provides strong genetic and morphological evidence that *Nannopus palustris* is composed of several pseudo-sibling species that possess significant, yet previously unrecognized, anatomical differences. These species (formerly all recognized as *N. palustris*) are awaiting a revision and proper description to prevent further confusion. Our work has also demonstrated that the form of caudal rami is not strictly linked to other morphological or genetic differences and should be considered together with other morphological characters.

More individuals from the known geographic distribution of *N. palustris* must be examined morphologically and genetically before any reliable phylogenetic analysis can be proposed. Such information is crucial to any assessment of marine harpacticoids diversity, biogeography and evolution.

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L.G. and M.D.T. are interested in ecology and biogeography of harpacticoids. They carried out the sampling and material pretreatment, and L.G. performed preparation and morphological analysis. T.N., D.S. and N.M. are molecular geneticists and evolutionary biologists. They performed DNA extraction, sequencing and phylogenetic analysis. J.G.B. is a meiobenthic ecologist with particular interests in harpacticoid biodiversity, biogeography and cryptic speciation. J.G.B. performed DNA extraction, amplification and sequencing of Black Sea specimens (2003). A.A. has broad interests in the invertebrate ecology, biodiversity and biogeography. He initiated and designed the research. All authors contributed to the design of the study, data interpretation and manuscript editing.

Data accessibility

DNA sequences: Genbank accessions JQ733048–JQ733088; JX287510–JX287523. Spreadsheet with detailed information about sampling locations and the Genbank accession number for each sequence in each individual is uploaded in Appendix S1 (Supporting information). Final DNA alignment files used for phylogenetic trees generating (in NEXUS format) are also uploaded in Appendices S2 and S3 (Supporting information).

Supporting information

Additional Supporting Information may be found in the online version of this article.

Appendix S1 List of individual sequence data used for the analysis.

Appendix S2 Final 28S DNA alignment file (in NEXUS format).

Appendix S3 Final Cyt*b* DNA alignment file (in NEXUS format).