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Species Diversity of *Ramphogordius sanguineus/Lineus ruber*-like Nemerteans (Nemertea: Heteronemertea) and Geographic Distribution of *R. sanguineus*

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Heteronemerteans, such as *Lineus ruber*, *L. viridis*, *Ramphogordius sanguineus, R. lacteus, Riseriellus occultus*, and *Micrura varicolor*, share many similar external characters. Although several internal characters useful for distinguishing these nemertean species have been documented, their identification is based mostly on coloration, the shape of the head, and how they contract, which may not be always reliable. We sequenced the mitochondrial COI gene for 160 specimens recently collected from 27 locations around the world (provisionally identified as the above species, according to external characters and contraction patterns, with most of them as *R. sanguineus*). Based on these specimens, together with sequences of 16 specimens from GenBank, we conducted a DNA-based species delimitation/identification by means of statistical parsimony and phylogenetic analyses. Our results show that the analyzed specimens may contain nine species, which can be separated by large genetic gaps; heteronemerteans with an external appearance similar to *R. sanguineus/Lineus ruber/L. viridis* have high species diversity in European waters from where eight species can be discriminated. Our 42 individuals from Vancouver Island (Canada) are revealed to be *R. sanguineus*, which supports an earlier argument that nemerteans reported as *L. ruber* or *L. viridis* from the Pacific Northwest may refer to this species. We report *R. sanguineus* from Chile, southern China, and the species is also distributed on the Atlantic coast of South America (Argentina). In addition, present analyses reveal the occurrence of *L. viridis* in Qingdao, which is the first record of the species from Chinese waters.

Key words: "*Lineus ruber*" complex, *Ramphogordius sanguineus*, cytochrome *c* oxidase subunit I (COI), statistical parsimony network, phylogeny, geographic distribution, Nemertea

INTRODUCTION

The phylum Nemertea is a poorly characterized metazoan taxa. In addition to the ~1300 described species (Gibson, 1995; Kajihara et al., 2008), numerous species that are new to science have been collected, but not described or nominated, and our knowledge of nemertean biodiversity remains uneven across different parts of the world. For example, about 300 species have been recorded from the Pacific coasts of Asia, but it has been conjectured that the number of species in these areas is probably much higher (Chernyshev, 2014). In a much smaller sea area, the waters off Hong Kong, 39 species have been recorded (including inadequately described species), but many more species of nemerteans as yet unidentified have been collected from the region (Gibson and Sundberg, 2003). The slow pace of

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identifying and describing nemertean taxa may be caused by the low number of biologists studying this taxon, and the difficulties in species identification. The lack of obvious external characters make these worms difficult to identify at the species level in many cases. The use of internal characters requires histological sectioning, and homoplasy in characters is another obstacle (Schwartz and Norenburg, 2001; Strand and Sundberg, 2005; Roe et al., 2007; Sundberg and Strand, 2010; Strand et al., 2014). Additionally, many nominated taxa have neither been soundly defined nor adequately described, and Gibson (1985) reckons that at least 30% of nemertean species are dubious.

The "*Lineus ruber*" complex exemplifies the difficulty and the tangle status of nemertean taxonomy. The complex was considered to consist of *Lineus viridis* (Müller, 1774), *Lineus ruber* (Müller, 1774), *Lineus sanguineus* (Rathke, 1799) (now *Ramphogordius sanguineus* (Rathke, 1799)) and *Lineus pseudolacteus* Gontcharoff, 1951 (Gontcharoff, 1951), with the last of these now considered a synonym of *R. sanguineus* (see Runnels, 2013). The first three nominated species have numerous synonyms and have been used as a name for the others (Gibson, 1994, 1995). Among these species, *L. viridis* was regarded for many years as a green variant of *L. ruber*, before Gontcharoff (1951) found differences in their larval development. Chernyshev (2004) restored the genus *Poseidon* Girard, 1852 for *L. ruber* and *L. viridis*, but this taxonomic proposal has not been widely used in recent publications. These two species have been known to have a parallel circumpolar distribution in the north hemisphere (Gibson, 1994, 1995), but *L. ruber* has also been reported from few sites of the south hemisphere (Wheeler, 1934; Strand et al., 2014). *Ramphogordius sanguineus* is a species reported from the temperate coasts of Europe, Asia, North and South America, and New Zealand (Bierne, 1983; Gibson, 1995). Although it can be distinguished from *L. ruber* and *L. viridis* by several anatomical characters and the pattern of regeneration (Coe, 1943; Moretto et al., 1976; Riser, 1993, 1994; Chernyshev, 2004), both the "standard" morphological method (i.e. by means of checking serial sections) and regeneration test are not applicable in the routine identification of nemerteans, as commented on by Sundberg and Strand (2010). We believe that in most cases specimens reported under these names may have been identified based solely on the examination of living individuals. Differences in coloration and behavior are useful and widely accepted characters for distinguishing these three species. *Ramphogordius sanguineus* has a tendency to contract into a spiral coil when disturbed, while *L. ruber* and *L. viridis* contract without coiling (Coe, 1943; Gibson, 1994; Riser, 1994; Roe et al., 2007). However, these approaches may not be consistently reliable, as there are strong similarities and intraspecific variations in body color (e.g., *R. sanguineus* is known to exhibit olive, red, brown, and green coloration, which may overlap with the colors of the other two species) (e.g., Coe, 1943; Riser, 1994; Chernyshev, 2004; Roe et al., 2007) and the both of coloration and contracting behavior may depend on the age and the physiological condition of specimens. In addition, several other species, e.g. *Ramphogordius lacteus* Rathke, 1843, *Micrura varicolor* Punnett, 1903, *Riseriellus occultus* Rogers et al., 1993, *Lineus pseudoruber* (Friedrich, 1935), and the unnamed "aberrant type" of Rogers et al. (1995), are also similar/identical to the above species in external appearance. *Ramphogordius lacteus* has the same contracting behavior as *R. sanguineus*, and the both species are similar in the external appearance of the head (Riser, 1994). *Micrura varicolor* has green and red varieties (Punnett, 1903). Though it may be distinguished from the other species by its caudal cirrus, this structure is likely to be broken off in most specimens, and in such cases the species shows considerable resemblance to *L. ruber*/*L. viridis* (Punnett, 1903; Malin Strand, personal observation). *Riseriellus occultus* is greenish, brownish, or blackish in color; it tends to coil up into a tight spiral when touched (a typical character of *R. sanguineus*) (Rogers, 1993). Though *L. pseudoruber* can be distinguished from *L. ruber* by anatomical characters and the "aberrant type" can be separated from *L. ruber* and *L. viridis* by allele frequency data of 13 enzyme loci, they are identical to *L. ruber*/*L. viridis* in external characters (Friedrich, 1935; Rogers et al., 1995). Moreover, all these forms (*R. lacteus*, *M. varicolor*, *R. occultus*, *L. pseudoruber*, and the "aberrant type") are distributed in European waters, which overlaps with the recorded distribution of "*Lineus ruber*" complex. Given that there is high similarity in external characters of these species and the examination of internal characters is not a foolproof method for nemertean identification (Strand and Sundberg, 2011), it would not be surprising if many of the previous records are proved to belong to another species by future studies on more powerful data such as DNA sequences.

DNA barcoding (Hebert et al., 2003) is increasingly widely used for species identification. For metazoans, the mitochondrial cytochrome *c* oxidase subunit I (COI) gene is usually selected as the target DNA sequence for barcoding analyses (Hebert et al., 2003, 2004a, b; Mark, 2003; Schindel and Miller, 2005). Such a DNA-based approach has been successfully used also in delimiting/identifying nemertean species (e.g., Chen et al., 2010; Fernández-Álvarez and Machordom, 2013; Strand et al., 2014; Leasi and Norenburg, 2014; Alfaya et al., 2015) and DNA information in general has been suggested to replace internal characters as the base for species descriptions (Sundberg and Strand, 2010; Strand and Sundberg, 2011; Strand et al., 2014).

With regard to the aforementioned species, some DNA sequences have been submitted to GenBank (those of COI gene, see Table 1). Previous molecular studies involving these taxa have focused mostly on phylogenetic analyses (e.g., Sundberg and Saur, 1998; Thollesson and Norenburg, 2003; Andrade et al., 2012; Strand et al., 2014). Although biochemical methods have been used in delimitating some of these species (Bierne et al., 1993; Rogers et al., 1993, 1998), few results for species delimitation and identification employing DNA markers have been reported. Runnels (2013) analyzed the phylogeography and species status of *R. sanguineus* using the mitochondrial *nad6* sequences of specimens from 29 geographic locations. Results of her analyses were congruent with previous morphological and histocompatibility observations (Bierne et al., 1993; Riser, 1993), and suggested that the five species that were previously diagnosed based on geographic location (i.e., *Lineus socialis* Leidy, 1855; *Lineus nigricans* Bürger, 1892; *Lineus vegetus* Coe, 1931; *L. sanguineus*; *L. pseudolacteus*)

should all be synonymized to the single species *R. sanguineus*. Here, we present the results of statistical parsimony network analysis, based on the COI marker, for specimens provisionally identified as the aforementioned species from external morphology. The dataset include sequences of 160 specimens recently collected from 27 localities around the world, and 16 sequences downloaded from GenBank, with most of the present sampling locations not overlapping with those of Runnels (2013). Main aims are to uncover the species diversity of heteronemerteans with an external appearance of *Ramphogordius sanguineus*/*Lineus ruber*/*L. viridis*, to clarify questionable records, and to gain a better understanding of the geographic distribution of *R. sanguineus*.

MATERIALS AND METHODS

Specimens and DNA extraction

A total of 160 specimens, which were formerly identified as *Ramphogordius sanguineus*(?), *Lineus ruber*(?), *L. ruber*/*viridis*, or *Micrura varicolor* according to external characters, were collected from 27 localities along the coasts of Europe, Asia, and North and South America. Their sampling information and GenBank accession numbers of partial COI sequences are summarized in Table 1.

The total genomic DNA of each specimen was isolated with genomic DNA isolation kit (Omega, USA) following the manufacturer's protocol and stored at –20°C.

Sixteen COI sequences of *R. lacteus*, *R. sanguineus*, *L. viridis*, *L. ruber*, and *R. occultus* downloaded from GenBank are also shown in Table 1. Several other sequences that had been registered as these species in GenBank were not included in the present analyses, either because they did not match well with the COI region used by the present study or were unlikely sequenced from nemerteans.

PCR amplification and DNA sequencing

Partial COI gene was amplified by using the primer pair LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) (Folmer et al., 1994) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Thaewnonngiw et al., 2004; Rawlings et al., 2007). PCR reaction volume amounted to 50 μl containing 25-μl Easy*Taq* Super Mix (Transgen, China), 1-μl each primer, 1-μl DNA template, and 22-μl distilled H_2O. The PCR reactions were performed with a Mastercycler Gradient 5341 thermal cycler (Eppendorf, Germany). Thermal cycling was set up with 5 min of denaturation at 94°C, followed by 32 cycles comprising denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and elongation at 72°C for 1 min, and finally completed with an elongation at 72°C for 5 min. All PCR products were purified with the PCR purification kit (Omega, USA) and sequenced with an ABI 3730 sequencer (Applied Biosystems).

Data analysis

All sequences were aligned and edited using the program BioEdit ver. 7.0.1 (Hall, 1999). The possibility of sequence saturation was evaluated in DAMBE ver. 5.2.34 (Xia and Xie, 2001). Phylogenetic trees were generated from the aligned COI sequence data in PHYML ver. 3.0 (Guindon et al., 2010). The best-fit DNA substitution model was determined under the Akaike Information Criterion (AIC) by using the software Modeltest ver. 3.7 (Posada and Crandall, 1998). Parameters of maximum likelihood (ML) algorithms were set as: 1000 times bootstrap replications, 4 for nucleotide substitution category, BioNJ for the starting tree, and SPR for branch swapping option. Bayesian inference (BI) was carried out with MrBayes ver. 3.2.2 on XSEDE (Miller et al., 2010) based on the best-fit model selected by MrModelTest ver. 2.2 (Nylander, 2004). Two sets of four independent Markov chains were run for 1×10^6 generations sampling every 1000 generations. The first 25% of the generations were discarded as burn-in. The consensus tree and the Bayesian posterior probability were produced and computed using the remaining samples. Stationarity was considered to be credible when the average standard deviation of split frequencies was below 0.01 (Huelsenbeck et al., 2001).

We used the software TCS ver. 1.18 (Clement et al., 2000) to construct the haplotype networks with a maximum connectivity limit of 95%. Moreover, the uncorrected *p*-distances within network or

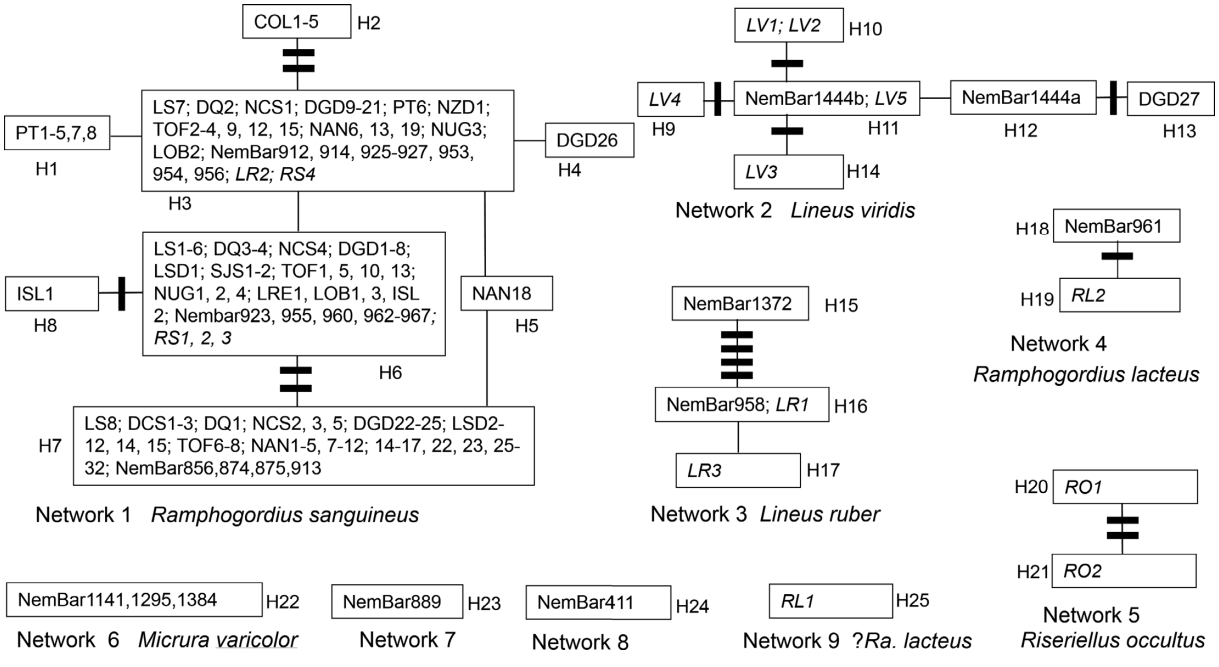

Fig. 1. Haplotype networks for the COI gene of the studied specimens under the 95% parsimony criterion. Each bar represents a missing haplotype. Codes of specimens see Table 1. Codes of specimens/sequences from GenBank are shown in italics.

Table 1. Information on specimens used in the present study, including that of the sequences downloaded from GenBank.

Species formerly identified	Locality	Labcode	Number of specimens	GenBank accession number	Reference
Specimens sequenced for the present study					
Ramphogordius sanguineus	Lüshun, Liaoning, China	LS1-8	8	KP213923–KP213930	This work
R. sanguineus	Dachangshan Dao (Is.), Liaoning, China	DCS1-3	3	KP213884–KP213886	This work
R. sanguineus	Daqin Dao (Is.), Changdao, Shandong, China	DQ1-4	4	KP213913–KP213916	This work
R. sanguineus	Nanchangshan Dao (Is.), Changdao, Shandong, China	NCS1-5	5	KP213974–KP213978	This work
R. sanguineus	Dagong Dao (Is.), Qingdao, Shandong, China	DGD1-26, DGD27	27	KP213887–KP213912, KP213883	This work
R. sanguineus	Lingshan Dao (Is.), Qingdao, Shangdong, China	LSD1-5	14	KP213931–KP213944	This work
R. sanguineus	Sijiao Shan (Is.), Shengsi, Zhejiang, China	SJS1, 2	2	KP213992, KP213993	This work
R. sanguineus	Pingtan, Fujian, China	PT1-8	8	KP213984–KP213991	This work
R. sanguineus	Naozhou Dao (Is), Zhangjiang, Guangdong, China	NZD1	1	KP213983	This work
R. sanguineus	Tofino, BC, Canada	TOF1-15	13	KP213994–KP214006	This work
R. sanguineus	Nanaimo, BC, Canada	NAN1-32	29	KP213945–KP213973	This work
R. sanguineus	Nuevo Gulf, Puerto Madryn, Argentina	NUG1-4	4	KP213979–KP213982	This work
R. sanguineus	Las Represas beach, Tapia de Casariego, Asturias, Spain	LRE1	1	KP213922	This work
R. sanguineus	Lobadiz beach, Ferrol, Galicia, Spain	LOB1-3	3	KP213919–KP213921	This work
R. sanguineus	Islares beach, Castro-Urdiales, Cantabria, Spain	ISL1, 2	2	KP213917, KP213918	This work
R. sanguineus	Colera, Cap de Creus, Catalonia, Spain	COL1-5	5	KP214007–KP214011	This work
R. sanguineus	Crosby, England, UK	NemBar923, 925–927, 953–956	8	KR606035, KR606037–KR606039, KR606040–KR606043	This work
R. sanguineus	Rhos-on-Sea, Wales, UK	NemBar960, 962–967	7	KR606036, KP606044–KR606049	This work
R. sanguineus?	Rhos-on-Sea, Wales, UK	NemBar961	1	KR606057	This work
R. sanguineus	Coquimbo, intertidal, Chile	NemBar856	1	KR606050	This work
R. sanguineus	Totoralillo, intertidal, Chile	NemBar874–875	2	KR606051–KR606052	This work
R. sanguineus	Punta Tumbes, Caleta Canteras, Chile	NemBar912–914	3	KR606053–KR606055	This work
Lineus ruber	Crosby, England, UK	NemBar958	1	KR606056	This work
L. ruber	Tromsö, Norway	NemBar1372	1	KP697741	This work
L. ruber?	Coquimbo, Chile	NemBar889	1	KR606058	This work
L. ruber/*viridis*	Saltö, Sweden	NemBar411	1	KR606059	This work
Micrura varicolor	Gullesfjorden, Norway	NemBar 1295	1	KP697754	This work
M. varicolor	Sandnes sunde, Norway	NemBar1384	1	KP697757	This work
M. varicolor	Sildegapet, Norge, Håkon, Norway	NemBar1141	1	NORGE019-14.COI-5P [1]	This work
M. varicolor	Sandspollen, Norway	NemBar1444a, 1444b	2	KP697759, KP697760	This work
Sequences from GenBank					
R. sanguineus	Penmon, Wales, UK	RS1 (=NemBar924)	1	KC812599	Strand et al. (2014)
R. sanguineus	Rhos-on-Sea, Wales, UK	RS2 (=NemBar968)	1	KC812598	Strand et al. (2014)
R. sanguineus	Anglesey, Wales, UK	RS3	1	AJ436938	Thollesson and Norenburg (2003)

Continued.

Table 1. Continued.

Species formerly identified	Locality	Labcode	Number of specimens	GenBank accession number	Reference
R. sanguineus	Crosby, England, UK	RS4 (=NemBar798)	1	GU392025	Strand and Sundberg (2011)
R. lacteus	Playa de los Genoveses, Cabo de Gata, Almeria, Spain.	RL1	1	KF935519	Kvist et al. (2014)
R. lacteus	Le Cabellou, Concarneaeu, Finistère department, Brittany, France	RL2	1	HQ848583	Andrade et al. (2012)
L. ruber	Crosby, England, UK	LR1	1	KC812602	Strand et al. (2014)
L. ruber	Punta Tumbes, Caleta Canteras, Chile	LR2 (=NemBar915)	1	KC812595	Strand et al. (2014)
L. ruber	Anglesey, Wales, UK	LR3	1	GU733828	Chen et al. (2010) [2]
L. viridis	Penmon, Wales, UK	LV1	1	KC812597	Strand et al. (2014)
L. viridis	Penmon, Wales, UK	LV2	1	KC812596	Strand et al. (2014)
L. viridis	Sylt Island, Nordfriesland, Schleswig-Holstein, Germany	LV3	1	HQ848579	Andrade et al. (2012)
L. viridis	Sylt Island, Nordfriesland, Schleswig-Holstein, Germany	LV5	1	NC012889	Podsiadlowski et al. (2009)
L. viridis	Manset, ME, USA	LV4	1	AJ436936	Thollesson and Norenburg (2003)
R. occultus	Crosby, Wales, UK	RO1	1	KC812600	Strand et al. (2014)
R. occultus	Rhos-on-Sea, Wales, UK	RO2	1	HQ848582	Andrade et al. (2012)

[1] Register number of BOLD. A GenBank number will not be available until it is mirrored by GenBank.
[2] This sequence was submitted to GenBank by Chen et al. (2010) but was not mentioned in the reference. Here we announce the locality of this specimen.

between networks were determined using MEGA ver. 5 (Tamura et al., 2011).

RESULTS

The aligned dataset contained 606 base pairs (bp) of COI sequences from 176 individuals. They presented no base insertions and deletions and comprised 25 unique haplotypes. Statistical parsimony analysis revealed nine distinct networks (Fig. 1). Network 1 (with eight haplotypes) was composed of 154 individuals, 42 from two localities in Canada, 71 from nine localities in China, 11 from four localities in Spain, four from Nuevo Gulf (Patagonia, Argentina), 19 from four localities in UK, and seven individuals (including the LR2, previously reported as *L. ruber* in Strand et al. (2014)) from three localities in Chile. Network 2 contained eight individuals (six haplotypes), among which six were from European waters, one was from the Atlantic coast of USA, and one from China. Network 3 (three haplotypes) was constituted by four *L. ruber* specimens with two from England, one from Wales, and one from Norway. Network 4 (two haplotypes) contained a specimen from France and a specimen from Wales, with the former having been reported as *R. lacteus* and the latter having been questionably identified as *R. sanguineus* (Table 1). Network 5 contained two *R. occultus* individuals (two haplotypes) from Wales. Network 6 contained three individuals (one haplotype) of *M. varicolor* from Norway. Network 7 as well as Network 8 was composed of a single specimen with questionable identification, from Chile and Sweden, respectively. The other specimen (RL1) that was collected from the Mediterranean coast of Spain and reported as *R. lacteus* was assigned to Network 9.

The uncorrected *p*-distances within and between networks are shown in Table 2 and Table 3, respectively. The within network distances were low, with the mean values of each of the nine networks varying from 0.00% to 0.66%, and the maximum value found between individuals of Network 1 being 1.16%. The averaged values of the uncorrected *p*-distances between specimens from different networks varied from 10.89% (between Networks 2 and 8) to 20.46% (between Networks 4 and 7), which were much higher than the within-network distances.

Table 2. Within-network uncorrected *p*-distances (%) of COI sequences.

Haplotype network	Number of individuals	Number of haplotypes	Range	Mean
Network 1	154	8	0.00–1.16	0.53
Network 2	8	6	0.00–0.66	0.42
Network 3	4	3	0.00–1.00	0.66
Network 4	2	2	0.33	0.33
Network 5	2	2	0.50	0.50
Network 6	3	1	0.00–0.00	0.00
Network 7	1	1	–	–
Network 8	1	1	–	–
Network 9	1	1	–	–
Overall average				0.49

The possibility of sequence saturation test shows that the COI sequences did not reach supersaturation, suggesting that these COI sequences could be used for reconstructing phylogenetic trees. As best-fit nucleotide substitution model, GTR + I + G was used for both ML and BI analyses. The topology of BI tree was in accordance with ML tree (Fig. 2). Individuals/haplotypes from different networks (Fig. 1)

Table 3. Averaged uncorrected *p*-distances (%) for COI sequences between networks.

	Network 1	Network 2	Network 3	Network 4	Network 5	Network 6	Network 7	Network 8	Network 9
Network 1									
Network 2	16.62								
Network 3	15.87	12.77							
Network 4	14.52	17.72	16.73						
Network 5	16.91	16.92	16.44	17.24					
Network 6	15.30	15.95	16.44	18.15	17.57				
Network7	18.81	18.61	18.05	20.46	19.72	18.32			
Network 8	15.72	10.89	12.10	17.16	15.59	15.18	17.99		
Network 9	13.43	17.83	17.60	12.21	16.42	16.34	20.13	15.68	

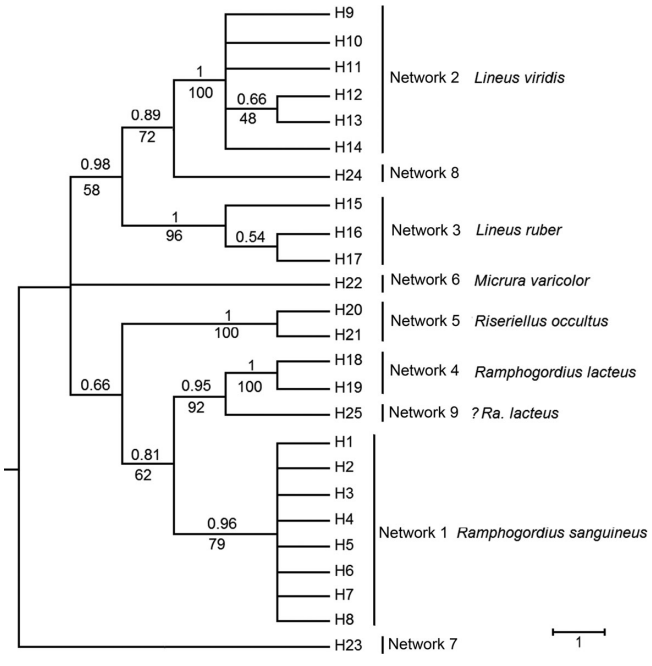

Fig. 2. The phylogenetic tree reconstructed for COI gene haplotypes by Bayesian inference (BI) and maximum likelihood (ML). Numbers above branches are Bayesian posterior probability values. Numbers below branches are bootstrap support values for ML analysis.

Table 4. Occurrence of COI haplotypes of *Ramphogordius sanguineus* (Rathke, 1799) (= Network 1) in different geographic regions. Data shown as number of individuals followed by percentage frequency (calculated only for regions of $n > 10$).

Haplotype	Europe	Argentina	China	Canada	Chile	Total
H1	0/0	0	7/9.9	0/0	0	7/4.5
H2	5/16.7	0	0/0	0/0	0	5/3.2
H3	8/26.7	1	18 /25.4	9/21.4	3	39/25.3
H4	0/0	0	1/1.4	0/0	0	1/0.6
H5	0/0	0	0/0	1/2.4	0	1/0.6
H6	16/53.3	3	20/28.2	4/9.5	0	43/27.9
H7	0/0	0	25/35.2	28/66.7	4	57/37.0
H8	1/3.3	0	0/0	0/0	0	1/0.6
Total number of specimens/ haplotypes	30/4	4/2	71/5	42/4	7/2	154/8

were clustered into separate clades with high bootstrap support (Fig. 2).

Because of low variation in COI sequences (11 variable sites and eight informative sites) within Network 1 (*R. sanguineus*, see Discussion), tree-based analyses showed no resolution for relationships among haplotypes/individuals of this clade (Fig. 2), whereas some interesting results can be seen in the distribution of haplotypes. As shown in Table 4, three major haplotypes (H3, 6, 7) cover more than 90% of the 154 individuals. Of these, H3 and H6 are distributed in all five geographically distant/isolated regions, but H7 only occurs in the Pacific. Additionally, H2 is shared only by the five individuals from Mediterranean waters (coast of Catalonia, Spain), and H1 is possessed only by eight (of the nine) individuals from Pingtan, China.

DISCUSSION

Empirical studies indicate that specimens assigned to a haplotype network by a statistical connection probability set to 95% in statistical parsimony analysis correspond to Linnaean names (Pons et al., 2006; Hart and Sunday, 2007; Bond and Stockman, 2008). Our analysis splits the 176 individuals into nine distinct networks (Fig. 1) that are supported by phylogenetic trees (Fig. 2). Except for Networks 7, 8, and 9 (for which mean intra-network genetic distances are unavailable because they contain a single specimen) and network 6 (for which no genetic distances were detected among the three specimens analyzed), the inter-network variations are 18.3–62.0 times higher than the intra-network variations (Tables 2, 3), which are higher than the 10 × threshold for species level differences as defined by Hebert et al. (2004a). This further supports the possibility that each of the nine networks represents a separate species. The results also suggest that the species identification of previous studies generally supported each other, e.g., Thollesson and Norenburg (2003) and Strand et al. (2014) for *R. sanguineus* (Network 1, RS1–3); Thollesson and Norenburg (2003), Podsiadlowski et al. (2009), Andrade et al. (2012), and Strand et al. (2014) for *L. viridis* (Network 2, LV1–5) and *R. occultus* (Network 5, RO1, 2); and Chen et al. (2010) and Strand et al. (2014) for *L. ruber* (Network 3, LR1, 3) (Fig. 1). The specimen LR2 from Chile assigned to Network 1 (Table 1; Fig. 1) was referred to as *L. ruber* in Strand et al. (2014), but has later been corrected to be *R. sanguineus* by Per Sundberg (pers. comm.). Thus we think that the species identification of these studies was reliable and their COI sequences (Table 1) (exceptions see below) could be used

as reference sequences for DNA taxonomy. However, two specimens of *R. lacteus* (RL1/KF935519 and RL2/HQ848583; Table 1) were classified into two different networks with high genetic distance (12.21%) and sister to each other in phylogenetic trees (Figs. 1, 2; Table 3). Since the location (northwestern France) of RL2 (as well as NemBar961, from Wales, UK, see further) (Table 1), is near the type locality of *R. lacteus* (Norwegian waters) (Rathke, 1843), it is likely that Network 4 refers to *R. lacteus* while Network 5/RL1 (from the Mediterranean coast of Spain) represents an unknown cryptic species. This is congruent with the result of an unpublished work that identified a cryptic lineage from *L. lacteus* specimens (Nicolas Bierne, pers. comm.).

Of the 151 specimens provisionally identified as *R. sanguineus*/*sanguineus*(?), 149 specimens, which were considerably variable in coloration (Fig. 3A–H), were confirmed to be *R. sanguineus* by statistical parsimony and phylogenetic analyses (Figs. 1, 2). However, the specimens DGD27 (from Qingdao, China) and NemBar961 (*R. sanguineus*?, from Wales, UK) were assigned to the clades of Network 2 (*L. viridis*) and Network 4 (*R. lacteus*?), respectively (Figs. 1, 2). Two (NemBar1444a, 1444b) of the five individuals formerly identified as *M. varicolor* are revealed to be *L. viridis*. These further indicate that it may not be always possible to correctly identify these nemerteans based solely on external morphology and/or behavior.

The specimen NemBar411, which was provisionally identified as *L. ruber*/*viridis*, forms a mono-individual network/clade in our analyses (Figs. 1, 2). This specimen was collected from Saltö, Sweden. Two obscure forms, *L. pseudoruber* (from Kiel Bay, Germany; Friedrich, 1935) and the cryptic "aberrant type" (from Britain and France waters; Rogers et al., 1995), were reported from adjacent seas. Whether they belong to the same species remains to be determined by future studies. Similarly, the Chile specimen NemBar889 (*L. ruber*?), which has the external appearance and contraction pattern of *L. ruber*, constitutes another mono-individual network/clade (Figs. 1, 2), indicating that there are at least two externally similar species around the coast of Coquimbo, Chile. The NemBar889 may be phylogenetically distant from the other forms analyzed (NCBI blast shows the most similar species (sequence identities 84–85%) are *Dendrorhynchus sinensis* Yin and Zeng, 1985, *Lineus longissimus* (Gunnerus, 1770) and *Cerebratulus leucopsis* (Coe, 1901)), but this must await further study.

The specimen DGD27 was collected among algae from the intertidal zone of Qingdao, China, in August 2010. It was a young individual about 8.0 mm long and 0.8 mm wide after being relaxed, and showed a greenish color (Fig. 3I). This is the first record of *L. viridis* from Chinese waters. Given that the collection site is very close to the port of Qingdao, one of the busiest international ports in the world, there is a possibility that *L. viridis* is a recently introduced species in this region. In other Asian waters, Takakura (1898) reported a form with a greenish or dark brown color from Koajiro Bay, Japan, under the name *Lineus gesserensis* (Müller, 1788), which has been applied to what are now known as *L. viridis* and *L. ruber* (Gibson, 1994, 1995). According to Takakura's (1898) brief description on external features, it was also possible that his specimens referred to *R. sanguineus* (see further).

Ramphogordius sanguineus and its various synonyms

Fig. 3. Photographs for some specimens of the present study. **(A–H)** *Ramphogordius sanguineus* (Rathke, 1799), each from: **(A)** Lingshan Dao, Shandong, China; **(B)** Nanchangshan Dao, Shandong, China; **(C)** Dagong Dao, Qingdao, Shandong, China; **(D)** Los Chalanos beach, Muros de Nalón, Asturias, Spain; **(E)** Dachangshan Dao, Liaoning, China; **(F)** Los Chalanos beach, Muros de Nalón, Asturias, Spain; **(G)** Nanaimo, British Columbia, Canada; **(H)** Cerro Avanzado beach, Nuevo Gulf, Patagonia, Argentina. **(I)** *Lineus viridis* (Müller, 1774), a young specimen from Dagong Dao, Qingdao, Shandong, China. Scale bars (for micrographs only) = 1.0 mm.

has been reported from many areas of the world, including the Arctic waters, coastal regions on both sides of the Atlantic and Pacific Oceans, and waters off New Zealand (Fig. 4). It was previously recorded from only single Chinese locality (Qingdao) (Yin et al., 1986; as *L. vegetus*). Our study reveals that *R. sanguineus* is widely distributed along Chinese coasts, including cold-temperate (from Dachangshan Dao southward to Lingshan Dao), warm-temperate (Sijiao Shan and Pingtan), and tropical (Naozhou Dao) waters (Table 1; for the division of marine biogeographic regions see Briggs and Bowen (2012)). *Lineus vegetus* was also reported from Japanese waters by Inaba (1988) and Iwata (1997). Although both records were thought to be questionable (Kajihara, 2007), the occurrence of *R. sanguineus* in Japanese seas has been confirmed in a recent unpublished M. Sc. thesis (Runnels, 2013). Since the brief description for *Lineus gesserensis* sensu Takakura (1898) could apply to all *L. ruber*, *L. viridis*, and *R. sanguineus*, and the latter two have been confirmed from Japanese and nearby Chinese waters (see above), Takakura's (1898) record was more likely to refer to *R. sanguineus* or *L. viridis* (or the both) than to *L. ruber*.

Along the Pacific coast of North America, *R. sanguineus* was reported from Washington (USA) to Ensenada (Mexico) (Coe, 1940; Corrêa, 1964; Roe et al., 2007). Our results show that all 42 individuals collected from two locations in Canada should be assigned to this species, which is very common under stones, among algae, oysters, mussels, and other fouling organisms in the intertidal zone in the Vancouver Island. Coe (1901, 1905, 1940) reported the distribution of *L. ruber* along the Pacific coast from Alaska to Monterey, California, while Roe et al. (2007) commented that "neither senior author has seen *Lineus ruber* or *Lineus viridis* (both well known to Norenburg) during many years of collecting along coasts of central California, Puget Sound, and Alaska" and "likely to be confused with reddish variety of *Ramphogordius sanguineus* (*Lineus vegetus*)". This argument is well supported by the present study. It seems that the distribution of *L. ruber* may not be as wide as having been considered previously (e.g., Gibson, 1995) and its occurrence in Pacific waters cannot be confirmed by the existing molecular data.

Isler (1902) reported *R. sanguineus* (as *L. nigricans*) from Punta Arenas, Chile. Riser (1994) re-checked Isler's (1902) slides and found that they should belong to an unknown species. However, the occurrence of *R. sanguineus* on the Pacific coast of South America (Chile) can be confirmed by DNA identification (i.e. specimens NemBar856, 874, 875, 912–914, and LR2 from three sites in Chile; see above). On the Atlantic coast of South America, Bierne (1983) collected *R. sanguineus* from Uruguay. Here we provide the first record of *R. sanguineus* from Patagonian waters (Nuevo Gulf).

In contrast to the regions discussed above, the distribution of *R. sanguineus* in European waters and the Atlantic coast of North America is less questioned. It has been recorded from coasts of Russia (White Sea), Italy, France, Belgium, Sweden, the British Isles, and Spain in Europe (e.g. Vernet and Anadón, 1991; Gibson, 1995; Maslakova, 2006), and from Newfoundland (Canada) to northern Florida, the Gulf coast westwards to Texas (USA), and Bermuda in the North America (Gibson, 1995; Brunel et al., 1998).

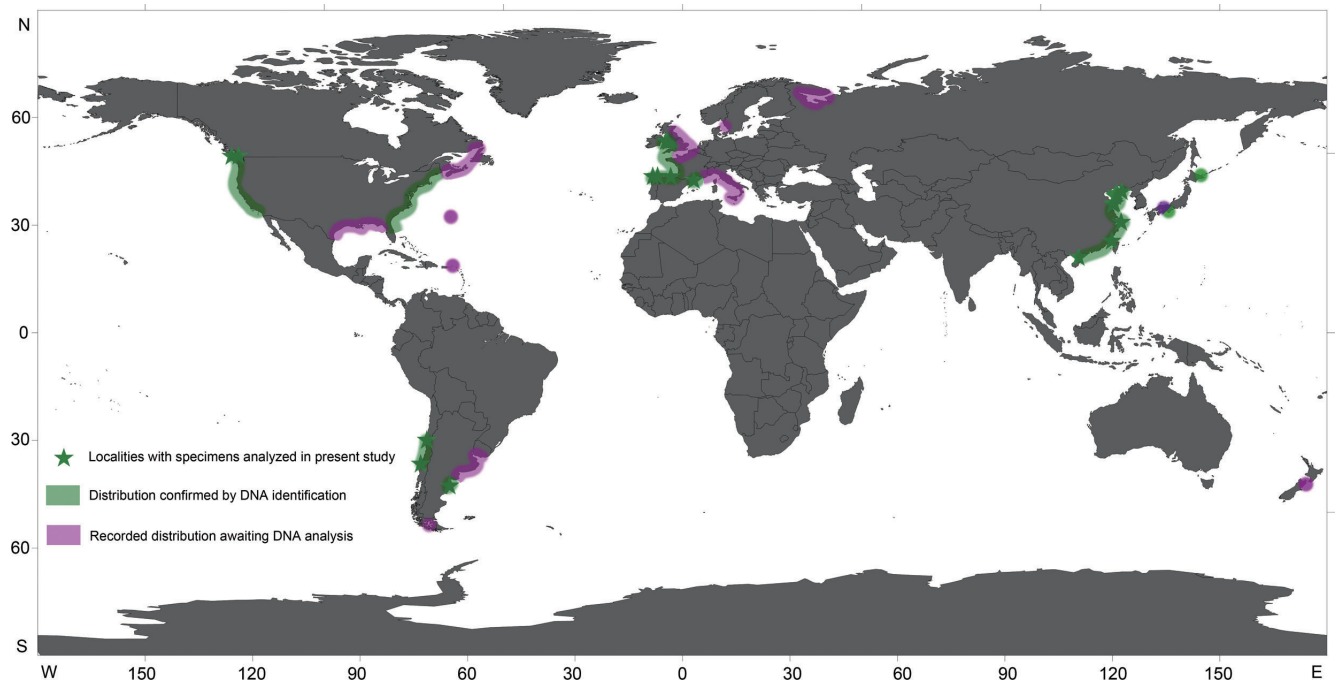

Fig. 4. Geographic distribution recorded for *Ramphogordius sanguineus* (Rathke, 1799). Green bands show coastal areas that the occurrence of *R. sanguineus* has been confirmed by DNA analyses (Runnels, 2013; present study); violet bands roughly indicate the coastlines where *R. sanguineus* has been reported in literature (Isler, 1902; Corrêa, 1961; Bierne, 1983; Inaba, 1988; Vernet and Anadón, 1991; Gibson, 1994, 1995; Riser, 1994; Brunel et al., 1998; Maslakova, 2006) but has not been reconfirmed with DNA analyses.

Although *R. sanguineus* (or its synonyms) reported in some of the numerous studies may in fact refer to other species, such as *L. ruber* and *L. viridis*, its occurrence in Spain, UK, France, and the Atlantic coasts of USA has been confirmed by molecular analyses (Runnels, 2013; present study).

The geographic distribution recorded to date for *R. sanguineus* is shown in Fig. 4. As indicated in previous studies (Bierne, 1983; Gibson, 1995), *R. sanguineus* has been recorded from worldwide temperate waters. However, it has never been found in Far East seas of Russia (Alexei Chernyshev, pers. comm.); two of the authors (MS and PS) doubt that they have encountered this species during many years fieldwork along the Swedish coasts; and the specimens reported from Punta Arenas (Chile) was thought to be another species (Riser, 1994; see above). Thus higher latitude coasts of cold-temperate regions (see Briggs and Bowen, 2012) are unlikely preferred habitats for *R. sanguineus*, its occurrence in the White Sea (Maslakova, 2006) needs to be confirmed by DNA analysis. The closest recorded location to the equator was the Virgin Islands (Corrêa, 1961), whereas this identification based on external appearance of preserved fragments is questionable (Riser, 1994). It seems that the distribution of *R. sanguineus* can extend only to the northern edge of tropical waters (in the northern hemisphere) such as Naozhou Dao, Guangdong, China (this study) and Link Port groin (near Fort Pierce), Florida, USA (Runnels, 2013). Therefore, similar to many other widely distributed marine organisms, *R. sanguineus*, has an "antitropical" pattern of distribution as defined by Hubbs (1952).

As indicated in previous studies (e.g., Riser, 1994; Runnels, 2013), several biological features (e.g., fragmentation, cyst formation, habitat preference of fouling community) could benefit the dispersal of *R. sanguineus* by rafting with debris in ocean currents or on boat hulls, which often are covered with fouling organisms. Runnels (2013) commented that, "the frequency and speed of ship transport likely overshadows the contribution of rafting to the dispersal of this species greatly enhancing the probability of gene flow". This author also documented by far the soundest evidence for the recent dispersal of this species (dispersed from Rhode Island to South Carolina, where the anthropogenic rocky substrate was recent). The wide distribution of three major COI haplotypes (H3, 6, 7) (Table 4) supports high-level exchange among geographically distant/isolated regions. However, if recent dispersal by ships is accepted as frequent, it is difficult to reconcile this with the fact that the most prevalent haplotype in the Pacific Ocean (H7, which is present in the 37.0% of the studied individuals and the 47.5% of the Pacific specimens) is not present in Atlantic individuals. Future phylogeographic studies with more intensive sampling and more powerful molecular markers may help to elucidate the distribution pattern of the species.

In conclusion, heteronemerteans of an external appearance similar to *Ramphogordius sanguineus*/*Lineus ruber* show high species diversity, particularly in European waters, where eight species were discriminated. *Ramphogordius sanguineus* is widely distributed in temperate waters, with the confirmed distribution including the European waters, the Atlantic and Pacific coasts of both North and South America, the northwestern Pacific coasts, and maybe also New Zealand coast. Though its distribution can extend to tropical waters, it has never been recorded from circumequatorial waters and the record from Arctic waters awaits validation by molecular analysis. The records of *L. ruber* from some geographic areas such as Chile and the Pacific Northwest likely refer to *R. sanguineus*. Existing data show that the distribution of *L. ruber* seems to be restricted to the Arctic and North Atlantic waters, while the occurrence of *L. viridis* in Pacific waters (Qingdao, China) is confirmed by DNA identification.

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