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ORIGINAL PAPER

Occasional sexual reproduction significantly affects the population structure of the widespread, predominantly asexually reproducing marine worm *Lineus sanguineus* (Nemertea: Pilidiophora)

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Abstract
Asexual reproduction by fissiparity has only been described from very few of the approximately 1300 nemertean species that otherwise mainly reproduce sexually. The best studied fissiparous species, *Lineus sanguineus* (Rathke 1799), is a cosmopolitan heteronemertean species inhabiting intertidal habitats of temperate coasts. Although sexual reproduction has never been described, molecular data suggest that sexual reproduction substantially shapes the genetic structure of the investigated populations. In an attempt to clarify the extent of sexual reproduction, three gene fragments (COI, 16S, ITS) were sequenced for 108 specimens sampled in 8 European localities. The results of a phylogenetic analysis and haplotype network showed no clear distinction between different populations, thus indicating the presence of sexual reproduction. Furthermore, we provide circumstantial evidence for the presence of a comparably long-lived planktonic larval stage as present in the closest relatives of *L. sanguineus*. To further understand the impact of abiotic factors on sexual reproduction and fissiparity, the effect of different temperature and illumination regimes on reproductive behavior and fragmentation was studied in specimens from a population from Bergen, Norway that share the same haplotype. Experimental setups represented summer (long light period and elevated temperature) and winter (short light and decreased temperature) conditions. Under winter conditions, a higher number of animals remained sexually mature and at least one specimen shed eggs on one occasion. Thus, although short light and/or low temperatures are most likely the influential factors on sexual maturity, the factors that influence fissiparity are less clear. The results of this study further solidify the cosmopolitan status of *L. sanguineus* and clarify the population structuring of this species. In addition, the study provides first data on the dynamics of sexual and asexual reproduction modes on which future investigations will have to expand, especially regarding genetic and physiological aspects.

Keywords Spiralia · Lophotrochozoa · Biogeography · Fissiparity · AMOVA · Heteronemertea

Introduction

In the animal kingdom, many species are capable of regenerating lost body parts, with regeneration of posterior body parts being fairly common (Bely and Nyberg 2010). On the other hand, fewer lineages are capable of regenerating a head. Within Nemertea, several species have independently gained this remarkable ability, one of the latest examples being *Baseodiscus delineatus* examined in Japan where male specimens reproduce by fissiparity in populations that appear to lack females (Ikenaga et al. 2019; Zattara et al. 2019). The best studied nemertean species in this respect is *Lineus* (=*Ramphogordius*) *sanguineus* that has already been studied for more than 200 years for its spontaneous fragmentation and regenerative capacities (e.g. McIntosh 1873–1874; Coe 1929, 1930b; Sivaradjam and Bierne 1981; Bierne 1990). Individuals of this species are known to naturally fragment into small pieces under certain conditions. These include warm temperatures, abundant food, and enough oxygen (Reutter 1967). From each fragment containing parts of the central nervous system, a complete individual can be regenerated, including the regeneration of a new head (Coe 1929, 1930b, 1934). These outstanding regenerative abilities are thought to provide the basis for asexual reproduction by fissiparity, which appears to be the

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dominant mode of reproduction in this species (Gontcharoff 1951; Bierne 1970; Gibson 1972).

Lineus sanguineus is a cosmopolitan heteronemertean species occurring in temperate waters (Riser 1994; Gibson 1995; Caplins and Turbeville 2011; Runnels 2013; Kang et al. 2015). Distribution records include European coasts, Atlantic and Pacific coasts of North and South America, and north Asian Pacific coasts (Gibson 1982, 1995; Kang et al. 2015). The species is found in the intertidal zone under rocks and stones, and in association with mussels and oysters on rocky shores (McIntosh 1873–1874; Coe 1943; Gibson 1982, 1995; Caplins and Turbeville 2011; Hiebert and Hunt 2015). *Lineus sanguineus* presents strong intraspecific color variation rendering species identification difficult (e.g. McIntosh 1873–1874; Gontcharoff 1951; Riser 1993, 1994; Caplins and Turbeville 2011). Due to its external appearance, *L. sanguineus* is often confused with the similar-looking species *Lineus ruber, Lineus viridis* or *Lineus clandestinus* (Kang et al. 2015; Krämer et al. 2017). In most phylogenetic analyses, *L. sanguineus* formed part of the "*L. ruber*" complex (Sundberg and Saur 1998; Thollesson and Norenburg 2003; Andrade et al. 2012), but more recent molecular analyses revealed a closer relationship to *Riseriellus occultus*, *Lineus lacteus* and *Lineus longissimus* (Ament-Velásquez et al. 2016). The closest related sister group is *Lineus* (=*Ramphogordius*) *pseudolacteus*, a hybrid species of *Lineus lacteus* and *Lineus sanguineus* that has so far only been recorded in Brittany (Gontcharoff 1951; Ament-Velásquez et al. 2016).

Due to the cosmopolitan distribution of *L. sanguineus*, the species was formerly seen as four separate species based on their respective distribution: *L. sanguineus* (European Atlantic coasts), *Lineus nigricans* (Mediterranean Sea), *Lineus socialis* (North American Atlantic coasts), and *Lineus vegetus* (North American Pacific coasts) (Gibson 1995). More recently, several morphological and molecular investigations concluded that there is only one species with a wide distributional range (Bierne et al. 1993; Riser 1993; Runnels 2013; Kang et al. 2015). This contradicts a generally observed phenomenon whereby assumed cosmopolitan species hide cryptic speciation, thus significantly reducing the distributional range of each of the species (e.g. Dawson and Jacobs 2001 in cnidarians, Gómez et al. 2002 in rotifers, and Leasi et al. 2016 in the hoplonemertean genus *Ototyphlonemertes*). This unusual cosmopolitan distribution can either be the result of long-distance dispersal via long-lived planktonic larvae or by dispersal of adult specimens or encysted fragments (Riser 1993, 1994; Caplins and Turbeville 2011; Kang et al. 2015). Adults could be transported by natural (rafting) or by anthropogenic means (in the fouling community of ship hulls) (Runnels 2013; Kang et al. 2015). Due to the so-far lacking evidence of a planktonic larvae, dispersal by ship was suggested as the most likely dispersal vector for a predominantly asexually reproducing species, such as *L. sanguineus* (Riser 1994; Runnels 2013; Kang et al. 2015).

This asexual reproduction by fissiparity sets *L. sanguineus* apart from other closely related species as neither species, except for *L. pseudolacteus*, is known to reproduce asexually. A reproductive system that is solely based on asexual reproduction also has a severe impact on population structuring as no larvae are present that could account for long-distance dispersal and genetic exchange ensuring little genetic structuring between remote populations (Jablonski 1986; Palumbi et al. 1997; Avise 2000; Cahill et al. 2017). One therefore would expect significant genetic structuring between distant populations, but only low levels of genetic diversity within the populations if the species reproduced solely asexually (Johnson and Threlfall 1987; Palumbi 1992; Bürger 1999). So far only few studies of population structure on *L. sanguineus* that could give evidence about the amount of asexual reproduction exist although it is a cosmopolitan species and has been studied extensively for more than 200 years (Kang et al. 2015; Ament-Velásquez et al. 2016; Zattara et al. 2019).

Based on current genetic evidence, sexual reproduction has to take place in *L. sanguineus* at least to some extent, although there is no definite proof of it (Riser 1994; Kang et al. 2015; Ament-Velásquez et al. 2016). In this study, we sequenced gene fragments of the mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rRNA (16S) as well as the nuclear internal transcribed spacer region (ITS) to examine population structure and haplotype diversity. Moreover, we performed phylogenetic analyses based on each gene fragment and a combined dataset. The results were analyzed with special emphasis on indication of sexual reproduction.

All of the more closely related heteronemertean species possess long-lived, planktonic pilidium larvae (e.g. Beckers et al. 2015 for *R. occultus*). This leads to the assumption that some kind of larvae has to be present in *L. sanguineus*, too. As no mature specimens were ever found between May and October, sexual reproduction was assumed to take place between mid-winter and early spring in the northern hemisphere (McIntosh 1873–1874; Coe 1899; Gontcharoff 1951; Bierne 1970; Gibson 1972; Riser 1994). Spermatogenesis seems to take place in a regular manner (Gontcharoff 1951; Döhren et al. 2010), whereas oogenesis is often reported as abortive, especially in specimens sampled in Brittany (Coe 1943; Gontcharoff 1951; Riser 1994). Thus, there are no observations of fertilization or egg deposition, let alone larval development of *L. sanguineus*. Observations on the closely related species *L. lacteus* and *L. ruber* revealed that photoperiod or temperature may have an effect on gonad maturation, with low temperatures and long periods of darkness triggering gonadogenesis (Vernet and Bierne 1988, 1993). Based on these findings we decided to further

investigate the effect of light and temperature on sexual maturation in specimens from a Northern-European population of *L. sanguineus* sharing the same haplotype expecting low temperatures and a short photoperiod to promote sexual maturity. Twenty specimens produced by asexual reproduction were used. Fragmentation, growth and the state of sexual maturity were recorded for several months under different temperature and light regimes.

Material and methods

Specimens and sampling sites

108 specimens of *Lineus sanguineus* (Rathke, 1799) were collected between 2010 and 2020 from eight European localities (Fig. 1). These included Germany (Helgoland, Sylt), Norway (Bergen), and France (Banyuls, Concarneau, Ile de Groix, Roscoff, Wimereux). Specimens were found either under rocks, in rock fissures or associated with mussels (*Mytilus edulis*). Photographs were taken with a digital camera (Canon EOS 600D) mounted on a dissection microscope (Zeiss Stemi 2000). A tissue sample of each individual was preserved in absolute ethanol (99.9%) for DNA extraction. Specimens sampled in 2014 in Bergen were collected alive and kept at the Institute of Evolutionary Biology in Bonn at 18 °C and under a light-darkness regime of 16 h:8 h for the experiments on reproduction.

Nucleic acid purification and PCR amplification

DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's extraction protocol. For this study, three loci were amplified: the partial

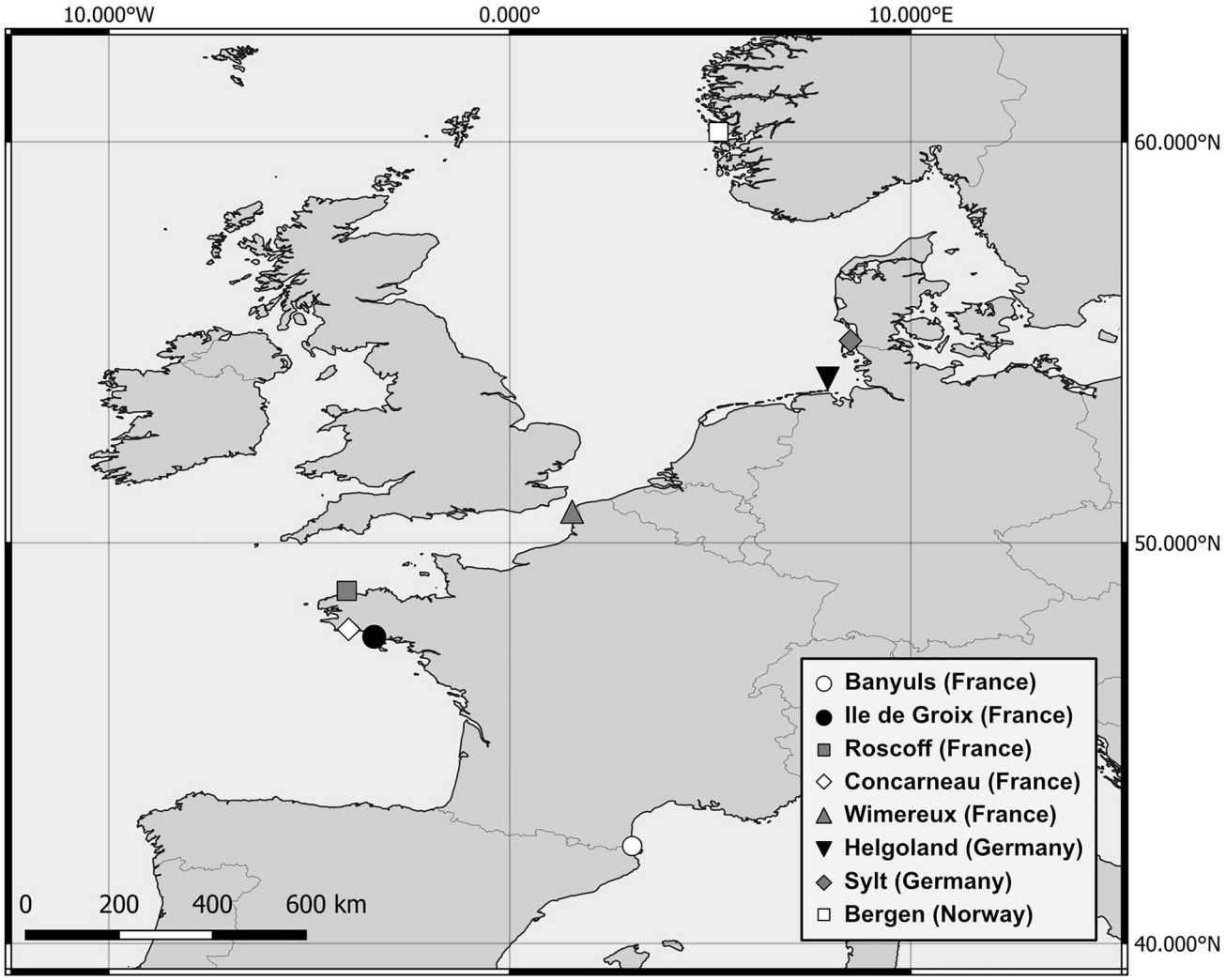


Fig. 1 Specimens of *Lineus sanguineus* were collected from eight European localities, including five localities in France, two localities in Germany, and one locality in Norway

mitochondrial COI and 16S rRNA and the nuclear ITS gene fragment (ITS1, 5.8S rRNA, and ITS2) using the following primers: for COI LCO1490 [GGTCAACAAATCATAAAGATATTGG] and HCO2198 [TAAACTTCAGGGTGACCAAAAAATCA] (Folmer et al. 1994), for 16S 16Sar-L [CGCCTGTTTATCAAAAACAT] and 16Sbr-H [CCGGTCTGAACTCAGATCACGT] (Palumbi et al. 1991), and for ITS ITS-28S [TTTTCAACTTTCCCTCACGG] and ITS-18S [CATTTGAGGAAGTAAAAGTCGTAAC] (Krämer et al. 2017). Polymerase chain reactions for COI and 16S were performed using Hot-Master Taq polymerase (Invitrogen™) whereas Dream Taq™ PCR Master Mix (Thermo Fisher) was used for ITS. PCR cycling was initiated with 2 min at 94 °C, followed by 40 cycles (30 s at 94 °C, 60 s at 48 °C and 60 s (COI, 16S)/120 s (ITS) at 72 °C), and terminated with a 2 min (COI, 16S)/ 1 min (ITS) final elongation at 72 °C. Amplified products were purified using illustra ExoProStar 1-Step (GE Healthcare) following the manufacturer's instructions. Sanger sequencing was conducted by LGC Genomics (Berlin, Germany) using only forward primers (COI, 16S) and forward and reverse primers (ITS) for sequencing (Sanger et al. 1977). All sequences have been deposited in the GenBank database (accession numbers provided in Table 1).

Sequence analysis

Sequences were edited with BioEdit version 7.2.5 (Hall 1999) and aligned using MAFFT version 7 (Katoh et al. 2019). For sequence alignment G-INS-I strategy with default parameters (scoring matrix for nucleotide sequences: 200PAM/K = 2, gap opening penalty: 1.53, offset value: 0.0) were chosen. All ambiguous positions were excluded with Gblocks version 0.91b (Castresana 2000) using default parameters.

For phylogenetic analyses based on the combined COI (512 nt), 16S (398 nt), and ITS (967 nt) data, 59 *Lineus sanguineus* specimens were selected to represent all European sampling sites except for Banyuls. Furthermore, COI and 16S data were combined with sequence data taken

Table 1 Information on all sampled specimens of *Lineus sanguineus*

Locality	Year	Amount	Labcode	Region	COI	16S	ITS
Concarneau, France	2016	1	NE140	NE Atlantic	ON097164	ON103401	–
Bergen, Norway	2014	12	NE323 –334	North Atlantic	ON097165-ON097176	ON103402-ON103412	ON108577-ON108583
Roscoff, France	2014	7	NE336 – 338, 341, 344 – 346	NE Atlantic	ON097177-ON097180, ON097182-ON097184	ON103413-ON103414, ON103416, ON103418-ON103420	ON108584, ON108585, ON108587, ON108588
Concarneau, France	2014	4	NE343, 347 – 349	NE Atlantic	ON097181, ON097185-ON097187	ON103417, ON103421-ON103423	ON108586, ON108589, ON108590
Wimereux, France	2013	7	NE351 – 357	North Sea	ON097188-ON097193	ON103424-ON103429	ON108591-ON108595
Sylt, Germany	2010	11	NE358, 360, 362 – 370	North Sea	ON097194-ON097202	ON103430-ON103439	ON108596-ON108602
Helgoland, Germany	2010	10	NE371 – 380	North Sea	ON097203-ON097212	ON103440-ON103448	ON108603-ON108608
Ile de Groix, France	2011	3	NE381 – 383	NE Atlantic	ON097213-ON097215	ON103449-ON103451	ON108609-ON108611
Concarneau, France	2012	3	NE384 – 386	NE Atlantic	ON097216-ON097218	ON103452-ON103454	ON108612-ON108613
Sylt, Germany	2011	5	NE390 – 394	North Sea	ON097219-ON097223	ON103455-ON103458	ON108614-ON108616
Bergen, Norway	2018	1	NE900	North Atlantic	ON097224	ON103459	ON108617
Concarneau, France	2019	11	NE917 – 920, 924 – 928, 935, 936	NE Atlantic	ON097225-ON097234	ON103460-ON103470	–
Concarneau, France	2020	13	NE1037, 1040—1051	NE Atlantic	ON097235-ON097245	–	–
Banyuls, France	2014	3	N1411, 1416, 1418	Mediterranean	ON097246-ON097248	–	–

Sampling locality, year and region as well as GenBank accession numbers for COI, 16S, and ITS gene fragments are provided

from GenBank (for Accession numbers see Supplementary Table 1), whereas ITS data was analyzed separately. For all four approaches the model for phylogenetic reconstruction was selected by MrModeltest2 version 2.3 based on the Akaike information criterion (Nylander 2004). General-time-reversible model and a gamma distribution with a proportion of invariant sites (GTR + G + I) were selected as best-fitting substitution model for the COI and 16S dataset (Nei and Kumar 2000), whereas Hasegawa-Kishino-Yano model and a gamma distribution of invariant sited (HKY + G + I) were selected for the ITS and the combined dataset (Hasegawa et al. 1985). Phylogenetic trees were reconstructed in MEGA version 6.06 using a maximum likelihood (ML) as optimality criterion (Tamura et al. 2013). Branch support was estimated using 500 bootstrap replicates. *Lineus ruber*, *Lineus viridis*, and *Lineus clandestinus* were used as outgroups.

Haplotype networks for the COI, 16S, and ITS datasets were estimated using statistical parsimony implemented in TCS version 1.21 (Clement et al. 2000) with the connection limit set to 95% (Templeton et al. 1992). Haplotype and nucleotide diversity were calculated using DnaSP version 6.12.03 (Rozas et al. 2017). Analysis of Molecular Variance (AMOVA) was utilized to examine genetic variation within and among populations (Excoffier et al. 1992). The analyses were performed with GenAlEx version 6.503 (Peakall and Smouse 2006, 2012), using 999 permutations to test the significance. A Mantel test was also performed using GenAlEx to determine the correlation between genetic and geographic distances of populations (Mantel 1967).

Fragmentation/sexual maturity experiments

Specimens of *L. sanguineus* from Bergen (Norway) were collected in 2014. Until the start of the experiments in 2018, the worms were kept at constant temperature (18 °C) and 16 h of light in plastic boxes with approx. one liter of standing sea water. The water was changed every other week. The animals were fed with *Tubifex tubifex* (Müller, 1774) once a week. To gain information on the influence of temperature and light on fragmentation and sexual maturation, two experimental setups were designed: two sets of 20 worms each, were kept together in plastic boxes with 0.5 L of aerated artificial sea water under the contrasting influence of winter conditions (9 °C, 8 h light) and summer conditions (18 °C, 16 h light). All specimens had the same haplotypes of the markers used herein as identified by sequence analysis. During the 6-month duration of the experiment, the worms were fed once a week and the water was changed once every week. At the onset of the experiments and afterward each month, the complete worms, defined as possessing a cephalic lobe (i.e. head) and the fragments were counted and measured, and the sexual development was recorded. For these procedures, the animals were immobilized by immersion in 3,5% $MgCl_2$ in seawater (mixed from equal volumes of 7% $MgCl_2$-solution and artificial sea water) for a minimum of 15 min. That causes the entire musculature to relax and thus allows for standardized measurements. Specimens were regarded as sexually mature when gonads and gonopores were well visible. Detailed results of the counting and measurement are provided in Supplementary Tables 2 and 3.

The results were tested for normal distribution in R version 3.6.1 (R Core Team 2019). None of the datasets were normally distributed. Non-parametric Mann–Whitney U test was performed to compare animal length under the different conditions. Results were regarded as significant when $p < 0.05$. Boxplots were computed using the ggplot2 package (Wickham 2016).

Microscopy of larvae

In April 2013, larvae were found during a water change in the container with specimens collected in Roscoff, France kept at 18 °C with 16 h of light. Due to the small number and size of the larvae, they were not fixed for DNA extraction, but documented with light microscopic methods. After immobilization of the musculature by immersion in 3,5% $MgCl_2$ in seawater, the larvae were mounted in the same mixture on a glass slide. To prevent them from swimming around with ciliary action, the larvae were slightly compressed with the cover slip mounted on clay feet. The mounted larvae were examined with a BX 51 everse microscope (Olympus) using differential interference contrast with a 40×, NA 0.75 dry objective. Images were taken with a ColorView IIIu CCD camera (Soft Imaging System). Images were further processed and mounted with Adobe Photoshop CS5 and Illustrator CS5 (Adobe).

Results

Phylogenetic analysis

No resolution of different geographic *Lineus sanguineus* populations was visible in the ML analyses of any of the single genes or the combined gene set. All included specimens form one well-supported clade. Because of this, we only show the ML analysis based on the combined COI, 16S, and ITS sequences (Fig. 2). The Norwegian population from Bergen (represented by one specimen) that has been used in the fragmentation/sexual maturity experiments forms one clade with three specimens from Sylt and three specimens from Roscoff. Besides this, specimens from all localities are mixed.

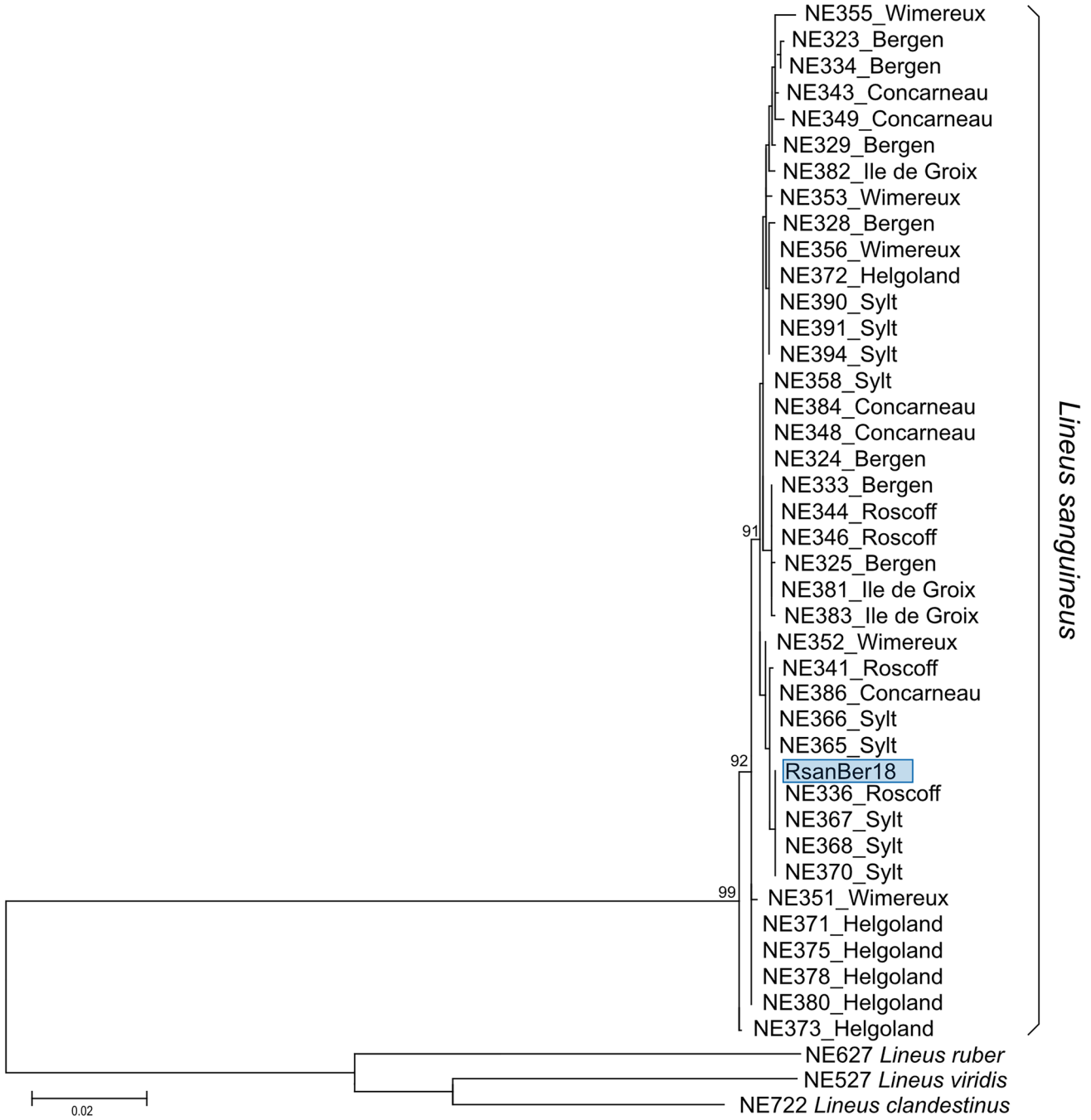


Fig. 2 Maximum likelihood tree (HKY + G + I) of selected *Lineus sanguineus* specimens based on concatenated COI mtDNA, 16S rRNA, and ITS rRNA sequences. *Lineus ruber*, *Lineus viridis*, and *Lineus clandestinus* were used for outgroup rooting. Numbers above nodes indicate bootstrap support from 500 replicates for each clade. Only values above 90 are shown. Specimens from both further examined populations are highlighted (blue = Bergen, Norway)

Population analysis

We obtained COI sequences for 104 specimens from eight European localities which resulted in an alignment of 512nt in length. These sequences were combined with 183 *Lineus sanguineus* specimens deposited at GenBank. These GenBank specimens originated from different localities in Europe, the Pacific coast of Canada, China, Argentina, and Chile. The whole dataset comprised 15 polymorphic sites. Haplotype diversity was moderately high (Hd = 0.732; SD = 0.011), but nucleotide diversity was very low ($\pi = 0.00315$; SD = 0.00016).

The haplotype network based on the COI dataset yielded 15 haplotypes (Fig. 3A). Approximately 90% of all individuals possess one of three highly frequent haplotypes (72, 86, and 89 specimens, respectively). Specimens with these haplotypes originate from all sites sampled for this study, as well as from other locations in Europe, Pacific Canada, China, Argentina, and Chile. There is no separation of haplotypes by locality although all specimens from the Mediterranean share the same haplotype (H4). The specimens used in the fragmentation/sexual maturity-experiments from Bergen possess haplotype H1.

The analysis of the 398-nt-long alignment of 16S was based on 86 specimens sampled for this study and 16 sequences taken from GenBank. The data deposited at GenBank originates from individuals sampled in Buenos Aires, Oregon, and Wales. In this dataset, 11 polymorphic sites were present. Haplotype diversity was moderate (Hd = 0.535; SD = 0.037), but nucleotide diversity was very low ($\pi = 0.00204$; SD = 0.00037).

The haplotype network based on all 105 specimens comprised seven haplotypes (Fig. 3B). Approximately 80% of all individuals have one of the two highly frequent haplotypes (H1: 57 specimens, H2: 30 specimens; the latter also found in specimens from Bergen used in the fragmentation/sexual maturity-experiments). At least some specimens of each population have one of the two most frequent haplotypes. The remaining seven haplotypes occur in only one to four individuals, respectively. As in the network obtained from COI data, haplotypes are not clustered based on locality.

We obtained ITS sequences for 60 specimens from seven European localities that resulted in an alignment of 967nt in length. In total, 26 polymorphic sites were present. Haplotype diversity was high (Hd = 0.885; SD = 0.024), whereas nucleotide diversity was low ($\pi = 0.00527$; SD = 0.00032).

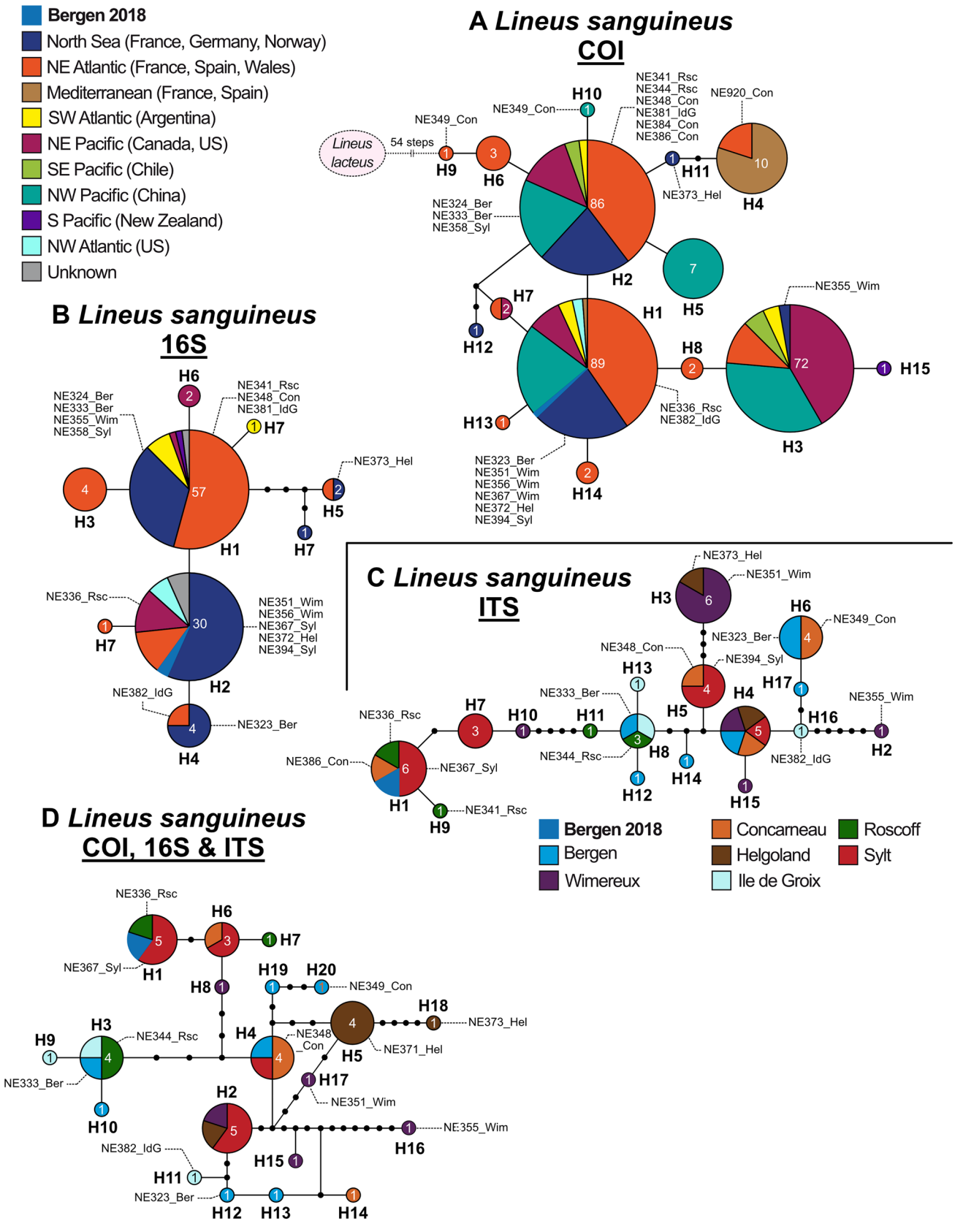
The statistical parsimony analysis of the ITS dataset yielded one haplotype network represented by 17 haplotypes (Fig. 3C). There are no highly frequent haplotypes, but seven medium frequency haplotypes found in three to six individuals, respectively. Six of these are shared by specimens from at least two different locations. The remaining ten haplotypes are represented by one specimen each. Interconnections to the closest haplotype vary between one and six substitutions. In comparison to the haplotype networks generated by the COI and 16S datasets, haplotype diversity and structuring is most pronounced in the ITS dataset.

A fourth statistical parsimony analysis was based on a concatenated dataset of all three genetic markers comprising 58 specimens from seven European localities. The analysis yielded one network represented by 22 haplotypes (Fig. 3D). The two most frequent haplotypes (H1: 14 specimens, H2: 9 specimens) includes specimens of the further investigated population from Bergen. Both are separated by 25 nucleotide substitutions. Four of the five medium-frequency haplotypes found in three, four and five specimens, respectively, are shared by specimens from at least two different locations. The fifth haplotype (H5) is only found in 4 specimens collected in Helgoland. The remaining 15 haplotypes are only found in one specimen each. As for the ITS dataset, the combined dataset also shows more pronounced haplotype structuring compared to COI and 16S.

Based on the AMOVA analysis of the COI dataset there is a higher level of differentiation among populations ($\Phi_{PT} = 0.327$; $p < 0.05$) than among regions ($\Phi_{RT} = 0.109$; $p < 0.05$). This result hints at gene flow among the regions, although structuring is to some extent present among populations. Only 11% of all variation is found among regions, 29% is found among populations, and 60% within populations indicating that variation between individuals is higher than variation between populations. Pairwise Φ_{PT} values were obtained by combining close geographical populations into 8 regions (Table 2). The three different northern Atlantic regions showed low levels of differentiation from each other indicating a high amount of gene flow between these populations ($\Phi_{PT} = 0.016–0.085$, $p < 0.05$). Comparably low levels of differentiation are inferred between Chile and Pacific Canada, Chile and China, and Argentina and the north eastern Atlantic region ($\Phi_{PT} = 0.000–0.034$, $p > 0.05$) although these results are not significant due to the small number of specimens present for Chile and Argentina. Low to moderate differentiation is present between Chinese and northern Atlantic and Canadian Pacific populations ($\Phi_{PT} = 0.03–0.153$, $p < 0.05$). Moderate differentiation was inferred between Canadian Pacific populations and the three northern Atlantic regions ($\Phi_{PT} = 0.230–0.422$, $p < 0.05$). Significantly high levels of differentiation can be observed between populations from Norway and north western Pacific coasts, and between Chilean and northern Atlantic populations ($\Phi_{PT} = 0.415–0.98$; $p < 0.05$). Populations from the Mediterranean on the other hand are highly differentiated from all other regions indicating reduced gene flow between the Mediterranean and the other regions ($\Phi_{PT} = 0.618–0.785$, $p < 0.05$). This high level of differentiation between the Mediterranean and all other population might account for the relatively high differentiation among all included populations ($\Phi_{PT} = 0.327$; $p < 0.05$). In the Mantel test, there was no significant relationship between geographic and genetic distance ($R_{xy} = 0.047$, $p > 0.05$), thus excluding isolation by distance.

Fragmentation

In the Bergen population, fragmentation starts earlier under winter conditions and also yields more fragments than under summer conditions (Fig. 4A). The number of specimens that regenerated a head under winter conditions does not significantly increase. Summer conditions, on the other hand, delay


Bergen 2018
North Sea (France, Germany, Norway)
NE Atlantic (France, Spain, Wales)
Mediterranean (France, Spain)
SW Atlantic (Argentina)
NE Pacific (Canada, US)
SE Pacific (Chile)
NW Pacific (China)
S Pacific (New Zealand)
NW Atlantic (US)
Unknown
A Lineus sanguineus COI
Lineus lacteus
54 steps
NE349_Con
NE341_Rsc
NE344_Rsc
NE348_Con
NE381_IdG
NE384_Con
NE386_Con
NE920_Con
NE324_Ber
NE333_Ber
NE358_Syl
NE373_Hel
NE323_Ber
NE351_Wim
NE356_Wim
NE367_Wim
NE372_Hel
NE394_Syl
NE336_Rsc
NE382_IdG
NE355_Wim
B Lineus sanguineus 16S
NE324_Ber
NE333_Ber
NE355_Wim
NE358_Syl
NE341_Rsc
NE348_Con
NE381_IdG
NE373_Hel
NE336_Rsc
NE351_Wim
NE356_Wim
NE367_Syl
NE372_Hel
NE394_Syl
NE382_IdG
NE323_Ber
C Lineus sanguineus ITS
NE373_Hel
NE351_Wim
NE323_Ber
NE349_Con
NE348_Con
NE394_Syl
NE333_Ber
NE336_Rsc
NE386_Con
NE367_Syl
NE344_Rsc
NE382_IdG
NE355_Wim
NE341_Rsc
Bergen 2018
Bergen
Wimereux
Concarneau
Helgoland
Ile de Groix
Roscoff
Sylt
D Lineus sanguineus COI, 16S & ITS
NE336_Rsc
NE367_Syl
NE349_Con
NE373_Hel
NE344_Rsc
NE348_Con
NE371_Hel
NE333_Ber
NE351_Wim
NE355_Wim
NE382_IdG
NE323_Ber

◂**Fig. 3** Statistical parsimony haplotype networks of all sequenced *Lineus sanguineus* specimens with a connection limit of 95% based on mitochondrial cytochrome *c* oxidase subunit I gene (**A**), mitochondrial 16S rRNA (**B**), nuclear ITS rRNA (**C**), and a concatenated dataset including all three gene fragments (**D**). **A** and **B** include additional sequence data taken from GenBank. Geographic distribution by maritime zones is represented by color. Specimens from Bergen, Norway used in fragmentation/sexual maturity-experiments are highlighted (blue). Geographic distribution in **C** is based on the sampling sites. Numbers within pie charts represent number of specimens with respective haplotype. Empty lines indicate a single substitution; each black dot indicates one additional mutational step

fragmentation until month 3 and the total amount of generated fragments is always lower than under winter conditions (Fig. 4A). Nevertheless, the total amount of individuals that successfully regenerated a head is significantly higher under summer conditions (Month 6: 32 summer vs. 20 winter). At the beginning of the experiments all specimens used for the different setups where approximately the same size (Mann–Whitney *U* test, $W = 160.5$, $p = 0.3$). At the end of experiments specimens kept at winter conditions are significantly longer than specimens kept at summer conditions (Mann–Whitney *U* test, $W = 182$, $p = 0.007$). Growing in length commences later at winter conditions than at summer conditions but at summer condition there is an overall strong decrease in length over time with the majority of specimens ranging below 50 mm in length after three months (Fig. 4B).

Sexual maturation and spawning

In the specimens from Bergen, gonads were already present in approximately 2/3 of all specimens at the start of the experiment (Fig. 5A). During the course of the experiment though, the number of specimens bearing gonads at summer conditions drastically declined. In contrast to this, nearly half of the specimens at winter conditions had gonads during the time of the experiment (Fig. 5A). In month six, spawned oocytes were found during a water change on one occasion. Moreover, specimens bearing gonads at winter conditions fragmented in a few cases, which did not happen at summer conditions. At winter conditions, there is no significant difference in length between specimens bearing gonads (sexually mature) and those without visible gonads (immature) (Mann–Whitney *U* test, $W = 1451$, $p = 0.05$), whereas mature specimens are significantly longer at summer conditions than immature ones (Mann–Whitney *U* test, $W = 563.5$, $p < 0.05$) (Fig. 5B, C).

So far, no larva has been recorded for *L. sanguineus*. Here, we give the first incidental report on a pilidium larva in this species. Few larvae of *L. sanguineus* were found in the container with animals that had been sampled in Roscoff, France. The larvae appear to be typical heteronemertean pilidium larvae resembling the larvae of *Riseriellus occultus* (Beckers et al. 2015). The outer appearance is roughly similar to a 3 to 4-day old larva of *R. occulus* with an apical plate and an apical tuft, two lateral lappets with ciliated bands and a developed, blind-ending midgut (Fig. 6). The size of midgut and esophagus indicate beginning malnutrition as no appropriate food (such as unicellular planktonic algae) had been available.

Discussion

A rare case of cosmopolitan distribution in *Lineus sanguineus*

The results of our phylogenetic analysis clearly show that all specimens of *L. sanguineus* included in this study belong to only one species with a wide geographic range. Thus, as already previously shown, a division into four species based on geographic locality can again be refuted (Bierne et al. 1993; Riser 1993; Kang et al. 2015). Analogously to polychaete species, in which it has been stated that most species naturally have a relatively limited range of distribution (Hutchings and Kupriyanova 2018), a cosmopolitan distribution in a nemertean species currently represents an exception. On the contrary, cryptic speciation, i.e. a separation into several species, even within smaller areas, is a common phenomenon in Nemertea (e.g. Leasi et al. 2016 for *Ototyphlonemertes*, Krämer et al. 2017 for *Lineus viridis* and *Lineus clandestinus*, or Sagorny et al. 2019 for European *Cephalothrix* species). Nevertheless, a recent study in the fissiparous polychaete *Proscoloplos cygnochaetus* Day, 1954 showed that most likely three nominal species from different, distant localities actually represent one species that was assumed to have been transmitted from its native area of distribution in Europe to Australia and South Africa in the fouling community of ship hulls (Kelaher and Rouse 2003; Meyer et al. 2008). The situation in *P. cygnochaetus* superficially resembles that in *L. sanguineus*.

Like *P. cygnochaetus*, *L. sanguineus* is a species that is also known to show strong regenerative abilities enabling asexual reproduction by fissiparity. Since only little is known about sexual reproduction in this species, fissiparity was assumed to be the main mode of reproduction (Gontcharoff 1951; Bierne 1970; Gibson 1972). Therefore, it has been suggested that the cosmopolitan distribution of *L. sanguineus* is mainly the result of dispersal via anthropogenic means. (Riser 1993, 1994; Caplins and Turbeville 2011; Kang et al. 2015). Whereas nemertean species have already been found rafting on macroalgae (Thiel and Gutow 2005), the century-old importance and extent of cosmopolitan shipping traffic makes dispersal on ship hulls appear to be the more important vector for adult specimens (Kang et al. 2015). Due to their ability to establish new colonies from only one individual, population expansion after anthropogenic dispersal

Table 2 COI AMOVA values based on regions

	N Atlantic	NE Atlantic	North Sea	Mediter-ranean	SW Atlantic	NE Pacific	NW Pacific	SE Pacific
N Atlantic	–	0.030	0.033	0.001	0.023	0.001	0.005	0.001
NE Atlantic	0.048	–	0.016	0.001	0.397	0.001	0.013	0.018
North Sea	0.085	0.016	–	0.001	0.029	0.001	0.003	0.003
Mediter-ranean	**0.785**	**0.618**	**0.728**	–	0.001	0.001	0.001	0.001
SW Atlantic	0.201	0.000	0.165	**0.692**	–	0.231	0.372	0.225
NE Pacific	0.422	0.230	0.391	**0.730**	0.034	–	0.005	0.330
NW Pacific	0.153	0.037	0.111	**0.634**	0.000	0.099	–	0.262
SE Pacific	0.498	0.188	0.415	**0.725**	0.000	0.000	0.034	–

Pairwise Φ_{PT} values are given below diagonal; *p*-values are given above diagonal. Pairwise Φ_{PT} values between Mediterranean and all other populations are given in bold. N Atlantic (=13) contains Norway; NE Atlantic (=96) contains France (=46), Spain (=13), and Wales (=37); North Sea (=31) contains France (=7) and Germany (=24); Mediterranean (=8) contains France (=3) and Spain (=5); SW Atlantic (=8) contains Argentina; NE Pacific (=45) contains Canada (=42) and US west coast (=3); NW Pacific (=70) contains China; SE Pacific (=6) contains Chile

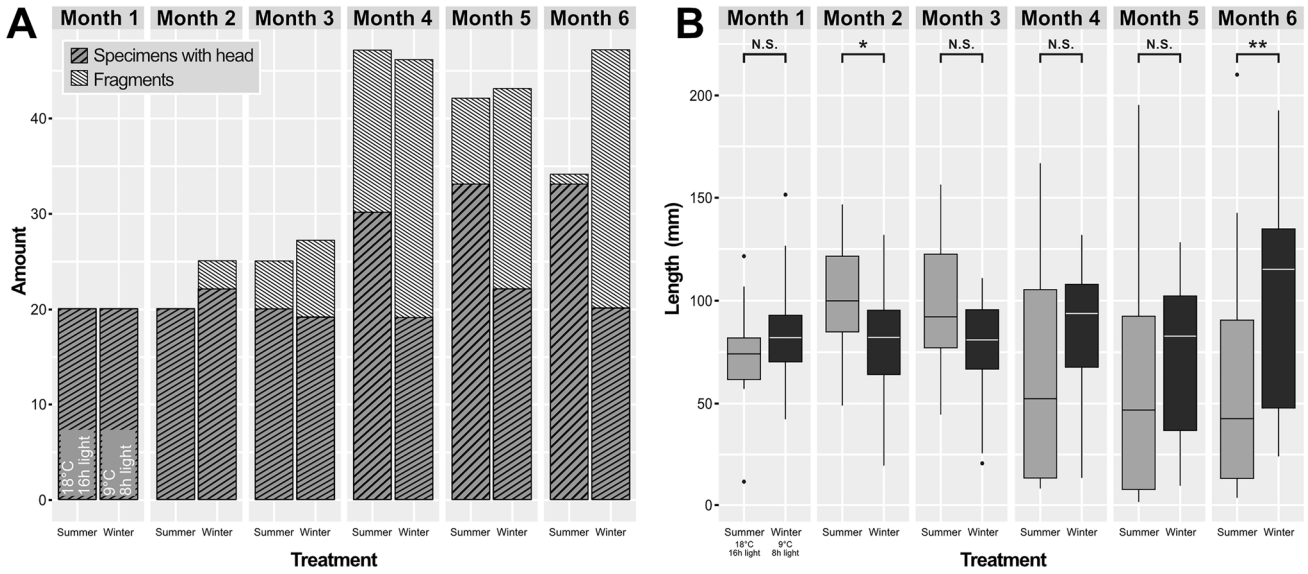


Fig. 4 Fragmentation and growth of *Lineus sanguineus* specimens from the Bergen, Norway population at summer (18 °C, 16 h light) and winter conditions (9 °C, 8 h light). Experimental procedures commenced with 20 individuals for each of the two setups. Complete animals and fragments were counted and measured for six months. **A** Amount of fragments and specimens with head of *L. sanguineus*. **B** Length variation of specimens with head over a period of 6 months. Significance was tested based on the Wilcoxon rank sum test (*p < 0.05, **p < 0.01)

is likely more successful in asexually reproducing species (Waters and Roy 2003; Lázaro et al. 2009). For that reason, dispersal of adult specimens or fragments by ships, leading to multiple foreign introductions, has been regarded as so far best explanation for the cosmopolitan distribution of *L. sanguineus* (Riser 1994; Kang et al. 2015). Based on the frequency and extent of global shipping transport and the biology of *Lineus sanguineus* this mode of dispersal probably accounts for at least some of the global distribution of the species (Riser 1994; Runnels 2013). The formation of cysts, fragmentation and a habitat preference of fouling community might facilitate dispersal in the fouling community of ships (Riser 1994; Runnels 2013; Kang et al. 2015). Since several shipping routes, especially between northern Europe, the Atlantic coasts of North America, and the Indian Ocean have already been extensively traveled since the seventeenth century, gene flow based on anthropogenic dispersal could have been happening for centuries (Runnels 2013; Druzin 2016). Nevertheless, as already stated by Kang et al. (2015) some patterns of population distribution cannot be explained by frequent dispersal by ship.

If specimens of *L. sanguineus* were dispersed by anthropogenic means, one would expect more genetic variation in the native population whereas only a fraction of the native haplotypes should be found in the introduced population as shown for *Cephalothrix simula*, the currently most

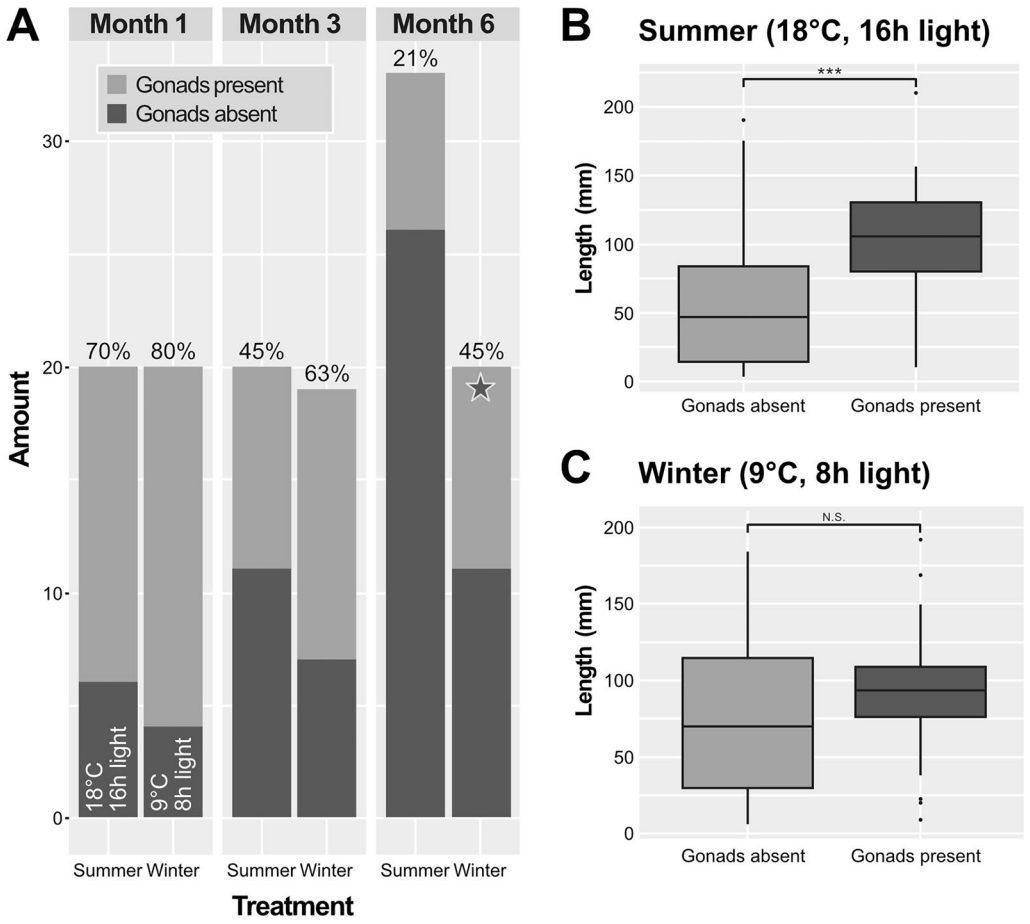


Fig. 5 Sexual maturation of *Lineus sanguineus* in the Bergen, Norway population. **A** Amount of specimens bearing gonads at summer (18 °C, 16 h light) and winter conditions (9 °C, 8 h light), examined at the beginning, halfway through, and at the end of the experiments. Number of specimens bearing gonads given in percent. Asterisk denotes presence of egg clutches. **B** At summer conditions there is a significant length difference of sexually mature and immature specimens. **C** At winter conditions, no significant length difference between sexually mature and immature specimens is present. Significance was tested based on the Wilcoxon rank sum test (***$p < 0.001$)

prominently documented nemertean example for anthropogenic introduction (Fernández-Álvarez and Machordom 2013; Kajihara et al. 2013; Faasse and Turbeville 2015; Sagorny et al. 2019). A pattern like this could not be detected in our datasets. Therefore, we argue that anthropogenic means most likely contribute to the cosmopolitan distribution, but other factors like rafting or dispersal of larvae cannot be ruled out by the results presented in this study. Furthermore, the genetic difference found in Mediterranean specimens of *L. sanguineus* is especially pronounced. This difference might lend evidence against anthropogenic dispersal on ship hulls, since the Mediterranean is traversed by one of the most-frequented freight routes worldwide. To elucidate the species distribution patterns further specimens from different localities need to be sampled and the biodiversity of the ship fouling communities should be investigated (Runnels 2013; Kang et al. 2015).

No restriction of gene-flow was found between populations of *Lineus sanguineus*

Whereas anthropogenic introduction and establishment of populations by asexual reproduction has been convincingly shown in *P. cygnochaetus*, the situation seems to be different in *L. sanguineus*. In truly asexual species one would expect only little genetic variation within populations because all specimens derive from the same founder individual and no recombination takes place (Johnson and Threlfall 1987; Bürger 1999). Additionally, high levels of genetic differentiation are expected between different populations as mutations happening in one population are maintained in this population but not shared with other populations (Palumbi 1992; Bürger 1999). However, this study shows that there is a high level of genetic variation within the species, but not between the populations. According to the haplotype networks generated based on the two different mitochondrial

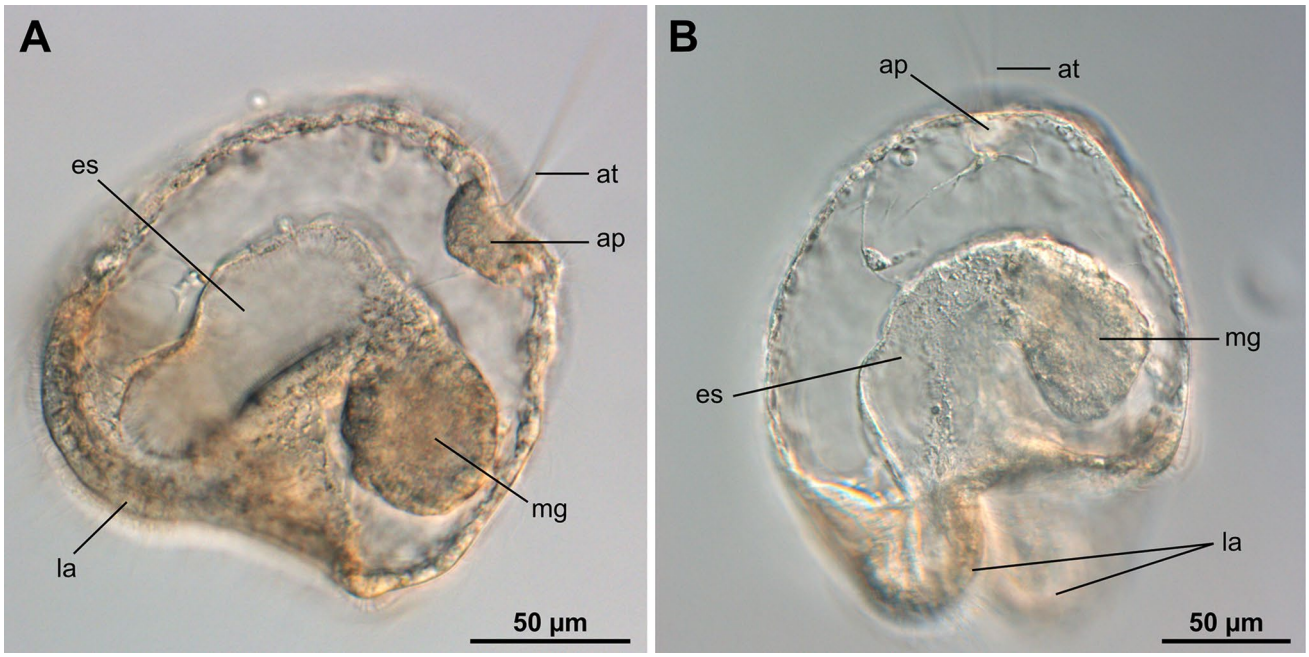


Fig. 6 Two young pilidium larvae (three to four days post fertilization) of *Lineus sanguineus*, differential interference contrast (**A** & **B**). The larval umbrella and the lateral lappets (*la*) are differentiating. An apical tuft (*at*) arises from the apical plate (*ap*). Esophagus (*es*) and sac-like midgut (*mg*) are developed, but the larvae did not take up any food. Note that the larvae resemble pilidium larvae of the closely related lineid species *Riseriellus occultus* (*cf.* Beckers et al. 2015)

gene fragments (COI and 16S) haplotypes are shared by numerous individuals that originate from distant populations. This may be the consequence of using mitochondrial markers as these are often not sufficient to reliably differentiate between recently diverged populations. Therefore, we included a nuclear, more variable gene region in the form of the internal transcribed spacer (ITS). The haplotype network based on the ITS gene fragment shows more structuring, i.e. more haplotypes shared by less individuals. Nevertheless, there is no clear distinction between populations from different European collection sites. Most haplotypes that are shared by more than one specimen are found in specimens from more than one sampling site. Inferring our result from only three gene regions may limit its informative value. Nevertheless, the low levels of population divergence found especially in the ITS region are supported by the results of the genome-wide study by Ament-Velásquez et al. (2016). The observed haplotype distributions indicate that genetic exchange has to take place between distant populations, a conclusion that is also supported by the results of the AMOVA analysis of the mitochondrial gene fragments. Most variation is found between individuals within the same populations and there is only little structuring among regions, indicating that isolation by distance may not play a major part. This result is in contrast to the results of Runnels (2013), that imply isolation by distance, especially between different north-west Atlantic populations. Unfortunately, there are only few sequences available for some of the regional populations. To clarify the apparent contradiction between the published results and the data herein, more marker-sequences, especially from nuclear genes, should be included in future analyses. Herein, the only region that differs significantly from all other regions is the Mediterranean. The presence of the relatively narrow Strait of Gibraltar could account for a limited dispersal and genetic exchange between populations from the Mediterranean and Atlantic populations but more data is needed to resolve this question.

The observed population structuring contradicts the pattern expected for truly asexual species, therefore sexual reproduction has to occur to some extent. This structuring supports the hypothesis that fissiparity is the main mode of reproduction in *L. sanguineus*. Since only few sexually derived individuals per generation are enough to establish a genetic population structure comparable to fully sexual species (Bengtsson 2003; Hartfield et al. 2016), it could be possible that sexual reproduction is very rare in *L. sanguineus* and has therefore not been documented previously. As mentioned above, anthropogenic dispersal might also account for some of the observed haplotype structuring. The high differentiation between Norwegian and Canadian, and between Chilean and northern Atlantic populations could be the result of only infrequent dispersal by ship as these localities are not on main shipping routes (Rodrigue 2016).

To fully answer this question, more data is needed on the correlation between population structuring and main shipping routes.

In contrast to the polychaete *P. cygnochaetus* (Meyer et al. 2008), neither recent anthropogenic introductions by hull fouling, nor subsequent establishment of populations exclusively by asexual reproduction could be substantiated in *L. sanguineus* by our data. Instead, our results indicate sexual reproduction resulting in some gene-flow between different populations. A population structure resulting from panmixia is also indicated by the analysis of genomic data of *L. sanguineus*, where no geographic structuring but normal levels of heterozygosity were observed (Ament-Velásquez et al. (2016). Therefore, we assume that at least the distribution and genetic variability of European populations are the result of at least infrequent sexual reproduction and thus larval dispersal leading to one large European metapopulation.

Although all data hint at successful sexual reproduction resulting in viable offspring, it has never been documented in *L. sanguineus*. In the French population studied by Gontcharoff (1951) only 1/3 of all specimens reached sexual maturity. In this study, we were able to obtain a few young pilidium larvae of *L. sanguineus*. As typical of heteronemertean larvae between three and four days, an apical tuft and lateral lappets were developed, as well as the midgut (Maslakova 2010; Beckers et al. 2015). The larvae closely resembled larvae of the heteronemertean species *Riseriellus occultus* Rogers, Junoy, Gibson and Thorpe 1993 (Beckers et al. 2015). Interestingly, these larvae occurred in a French population, in which oogenesis has been reported to be abortive (Gontcharoff 1951) The occurrence of larvae in *L. sanguineus* raised the question which factors influence gonad maturation and sexual reproduction. Although the larvae were not subjected to COI-barcoding, and thus direct evidence for their specific identity is absent, there is circumstantial evidence indicating a high likelihood of the larvae belonging to *L. sanguineus*. Only one other species that possesses superficially similar-looking larvae and has adults that could be taken for *L. sanguineus* is known to be present in the same habitat. However, this species, *R. occultus* has so far not been recorded from Roscoff. It is therefore unlikely that specimens of *R. occultus* had been misidentified as *L. sanguineus*. The COI-fragments taken from adult specimens collected at that occasion identify them as *L. sanguineus*. Furthermore, the reproductive period of *R. occultus* ranges from September to October, with gonopores being visible no earlier than late July (Beckers et al. 2015). Although gametogenesis starts as early as late March, the short time (5 weeks) the animals had spent at an elevated temperature (18 °C) in captivity seems to not be long enough, even for accelerated gonad maturity due to elevated temperatures. Therefore, even if specimens of *R. occultus* were erroneously present in the container from which the larvae were extracted, it would be very improbable that they had developed fertile gametes at the time when the larvae were found. Although we are confident about the identity of the larvae as being larvae of *L. sanguineus*, we admit that confirmation by DNA barcoding on a future occasion would be preferable, as it provides more straightforward, direct evidence.

Fissiparity as the prevalent reproductive mode in *L. sanguineus*

According to literature, the main factor influencing fissiparity in *L. sanguineus* is temperature (Coe 1930a). Former studies have shown that high temperatures promote fragmentation and subsequent regeneration. Therefore, asexual reproduction mainly takes place during the summer months (Coe 1929, 1930a; Gontcharoff 1951; Reutter 1967). In general, lower temperatures decelerate the rate of fragmentation down to almost complete inhibition at temperatures between 5 and 10 °C, whereas temperatures around 21 °C yield the highest number of fragments and fastest regeneration (Coe 1930a; Reutter 1967). For this reason, we chose temperatures that range at the respective ends of this spectrum (low: 9 °C; high: 18 °C) for our experiments. In contrast to published data, the specimens from the Bergen population fragmented to a higher degree at winter temperatures. Unexpectedly, even some sexually mature specimens fragmented under winter conditions although fragmentation was reported to be strongly reduced and regeneration to be less complete in sexually mature specimens (Coe 1930a; Gontcharoff 1951; Reutter 1967).

With regard to the influence of photoperiod on fragmentation, our results do not contradict the findings of Coe (1930a) and Reutter (1967) that temperature seems to have a stronger effect on fragmentation than light. However, photoperiod seems to have an influence on regeneration and growth of the animals. Our results might indirectly support the findings of Arnould and Vernet (1995) that regeneration is negatively correlated with melatonin production. Long hours without light usually lead to the production of melatonin in the closely related species *Lineus lacteus* (Arnoult et al. 1994). Melatonin retards or even completely inhibits regeneration in *Lineus sanguineus*, depending on the concentration, possibly by reducing mitotic activity (Arnoult and Vernet 1995). These assumptions suggest that longer hours of light should in turn lead to more regenerating and also longer individuals. This expectation is met for the Bergen specimens where summer conditions resulted in more regenerating fragments as well as faster growing specimens. However, elucidating the exact physiological mechanisms underlying fragmentation and regeneration in *L. sanguineus*

exposed to changing light and/or temperature needs further investigation.

Sexual reproduction—more frequent than previously assumed

The effects of light and temperature on sexual maturation on *L. sanguineus* are not well-studied. Winter conditions have for long been assumed to trigger egg maturation and sexual reproduction in the fissiparous species *Lineus sanguineus* (Coe 1899; Gibson 1972; Gontcharoff 1951; McIntosh 1873–1874; Riser 1994). According to Gontcharoff (1951) oogenesis is abortive in *L. sanguineus* in the majority of female specimens from Brittany, whereas in males, normal spermatogenesis occurs (Riser 1994). In October, testes and ovaries are formed between the midgut diverticula (McIntosh 1873–1874; Gontcharoff 1951; Bierne 1983). From January to March the testes are filled with small, awl-handle shaped, mobile spermatozoa that resemble spermatozoa of *Lineus lacteus* and *Riseriellus occultus* (McIntosh 1873–1874; Gontcharoff 1951; Döhren et al. 2010; Beckers et al. 2015). At the same time of the year, numerous small, mature oocytes can be observed in female specimens (McIntosh 1873–1874; Riser 1994). Oocytes have been described to undergo autolysis or are resorbed into the intestine (Gontcharoff 1951; Riser 1994). Moreover, females of this species produce only very few oocytes per individual leading to rather small clutches of eggs that have been found rarely. Those eggs are shed directly into the water, lacking a gelatinous sheath, and have been observed to develop into swimming gastrulae (Coe 1899; Gontcharoff 1951; Bierne et al. 1993). Aside from this, nothing is known about the development of *Lineus sanguineus* although Coe (1899, 1943) states that *L. sanguineus* is well-suited for embryonic studies.

In the closely related species *Lineus lacteus* and *Lineus ruber*, more gonads develop under colder temperature than under warmer temperature regimes with the more southwardly distributed species *Lineus lacteus* developing more gonads at 12 °C than the more northwardly distributed species *Lineus ruber* (Vernet and Bierne 1988). In these species, a gonad-inhibiting hormone of unknown nature (GIH) produced in the cerebral ganglia was made responsible for gonad maturation (Vernet and Bierne 1988, 1993; Arnoult and Vernet 1996). During the summer months, synthesis of this factor is supposedly increased, leading to a sexual rest (Vernet and Bierne 1993; Arnoult and Vernet 1996). The amount of light apparently has no influence on gonadogenesis in *L. lacteus* and *L. ruber* (Vernet and Bierne 1988, 1993).

For the reasons stated above, we expected low temperatures to promote sexual maturity in *L. sanguineus*. In the specimens from Norway, some influence of temperature could be shown as winter conditions led to a slower decrease in the number of mature specimens (measured by the presence of gonopores) and even the deposition of eggs. Thus, maintenance of a sexually mature state was seen in more specimens at the lower temperature (9 °C) than at the higher temperature (18 °C). This potentially indicates a connection between temperature and sexual reproduction in *L. sanguineus*. The reason why the number of specimens to show gonads didn't rise at lower temperatures can only be speculated about. Since the majority of specimens from the Norway population already had gonads at the onset of the experimental procedure, it is conceivable that spawning in several specimens might not have been noticed and subsequently the gonads were resorbed. Unfortunately, the potential influence of light on gonad maturation could not be directly assessed since the number of Norwegian specimens had been too small from the beginning to set up conditions that uncouple temperature and light exposition (i.e. long photoperiod with low temperature versus short photoperiod and higher temperature) with a reasonable sample size. Successful gonad maturation was also observed in specimens from Uruguay (Sivaradjam and Bierne 1981; Bierne 1983; Tarpin and Bierne 1995). Therefore, it might be possible that populations from colder habitats like Norway and Uruguay are more likely to develop gonads and reproduce sexually than populations from warmer habitats. To confirm this hypothesis, specimens from localities with warmer water temperatures will need to be experimentally tested.

Conclusions and outlook

Our results show that *Lineus sanguineus* is a cosmopolitan species. Although it has been reported that it mainly relies on asexual reproduction by fissiparity, *L. sanguineus* is able to reproduce sexually to promote genetic exchange. One question that prevails is whether the main reason for the cosmopolitan distribution is a result of larval dispersal, adult rafting or anthropogenic dispersal, e.g. along international freight routes. Of focal interest in this regard is the genetically deviating population found in the Mediterranean.

In the specimens from a population from Norway, sexual maturation seems to be result of low temperatures and/or short light period, but is only reached in approximately half of the specimens. Seasonal changes in day-length and water temperature therefore are apparently not the only factors influencing sexual reproduction in *L. sanguineus*. It has to be investigated whether these different reactions are lineage specific or influenced by population density, food provisioning, local adaptation or epigenetic factors. Another aspect of future research regards the interplay between sexual and asexual reproduction and its ecological consequences for

L. sanguineus as an upcoming spiralian model species to study the different (sexual vs. asexual) reproductive modes.

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Author contributions Both authors contributed to the study conception and design. Collection of genetic data, formal analysis and visualization were performed by CS. Experimental procedures were executed by JvD. The first draft of the manuscript was written by CS with additional remarks by JvD. All authors read, revised and approved the final manuscript.

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Data availability All sequence data have been deposited in the GenBank database (accession numbers provided in Table 1). Detailed results of the experimental setups are provided in the electronic supplementary files.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All applicable international, national, and institutional guidelines for sampling of organisms for this study have been followed.

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