RESEARCH ARTICLE

Population genetics and geometric morphometrics of the freshwater snail *Segmentina nitida* reveal cryptic sympatric species of conservation value in Europe

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Abstract

Segmentina nitida Müller 1774 is a rare European freshwater snail of drainage ditches and marshland, which has seen a marked decrease in range (~80%) over the last 100 years in the UK. This has been attributed to over-dredging of drainage ditches for land management, conversion of grazing marshes to arable farmland, as well as eutrophication. *Segmentina nitida* is identified as a priority species in the UK Biodiversity Action Plan (UKBAP) that recommends further research to inform reintroduction and translocation for its conservation. We used nuclear markers (microsatellites and ITS2) and a mitochondrial (COI) marker to investigate population structure in *S. nitida* individuals sampled from Poland, Germany, Sweden, and the UK to identify differences within and between populations. Data based on 2D landmark-based geometric morphometrics of *S. nitida* shells was used to determine if phenotypic variation followed genetic differentiation. Two distinct genetic lineages of *S. nitida* were identified in ITS and COI phylogenies as well as cluster analysis of microsatellite markers, one of these lineages was present in eastern Europe (Poland, Sweden- Lineage 2), and one in western Europe (UK, Germany- Lineage 1), with lineages co-occurring in German populations. No genetic admixture was observed in German populations containing both lineages. These two lineages were also distinct in shape, with lineage 2 individuals having significantly wider shells and taller and wider apertures than those in Lineage 1. ~85% of shells assigned to the predicted lineage in a discriminant analysis of Procrustes shape coordinates. We infer that *S. nitida* includes at least one sympatric cryptic species. We discuss the implications of these findings on the conservation status of *S. nitida* in the UK and Europe.

Keywords Microsatellites · Gastropod · Segmentina · Cryptic species · Geometric morphometrics

Introduction

Freshwater ecosystems and their associated biodiversity provide essential ecological (Covich et al. 2004; Macadam and Stockan 2015), economic (Jones et al. 2006), and cultural (Miller et al. 2015) benefits. Accurate understanding of freshwater biodiversity is critical for evaluating impacts and slowing the global decline of freshwater habitats and associated species (Strong et al. 2008; Régnier et al. 2009; Strayer and Dudgeon, 2010). Unfortunately, the diversity of freshwater invertebrate taxa is still poorly understood (Wallace and Webster 1996; Cardinale et al. 2002; Jonsson and Malmqvist 2003; Dangles et al. 2004). Gaps in our knowledge of the range, genetic structure and consequent ecology of species may reduce the efficacy of monitoring programmes and the accuracy of biodiversity assessments (Macher et al. 2016). Additionally, misidentification of species can undermine conservation efforts. For example, two species of predatory land snail *Euglandina* spp. were inadvertently released instead of one in Hawaii in a failed bio-control effort of the giant African land snail, which severely impacted native snail species (Meyer et al. 2017). In freshwater ecosystems, taxonomic and genetic work on

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apple snails has provided greater understanding of invasion patterns and native ranges of species, informing appropriate management decisions for conservation and control (Hayes et al. 2008, 2012).

Segmentina nitida Müller 1774, is a small (< 10 mm shell diameter) freshwater snail found throughout the temperate regions of Europe, including southern Scandinavia. It has been identified as a freshwater conservation surrogate, whose presence can indicate a high biodiversity and conservation value of a site (Ormerod et al. 2010). There has been a significant decline in the distribution of *S. nitida* in the United Kingdom, most notably in the last 50 years (Kerney 1991). This is attributed to frequent mechanical ditch clearance, eutrophication from fertiliser run-off and draining of grazing marshes to create land for arable farming (Wells and Chatfield 1992; Hill-Cottingham 2004). In continental Europe, *S. nitida* is regarded as widespread but declining due to threats to its already fragmented habitat (JNCC 2010). While *S. nitida* was listed as endangered in the UK in the IUCN Red Data Book 3 (Kerney 1991), a change to the IUCN criteria in 1994 led to it being excluded from subsequent editions of the Red List. As is the case for many invertebrate species, insufficient data for assessing its status according to current criteria have left *S. nitida* without an updated IUCN conservation category (Régnier et al. 2009, 2015). *Segmentina nitida* is, however, included on the UK Biodiversity Action Plan (UKBAP) as a priority species for conservation action (JNCC 2010).

Segmentina nitida has numerous synonyms in the literature across Europe that reflect a history of taxonomic confusion. This has contributed to the difficulty of producing accurate range maps of the species that are necessary for assessing its need for local conservation. Kennard and Woodward (1926) list 43 instances of synonyms for *S. nitida* in literature published between 1774 and 1884 alone. They also note that *Hippeutis complanatus,* a sister taxon, was used as a junior synonym for *S. nitida* multiple times. Additionally, confusion persists over a form of *S. nitida* (*S. nitida* f. *distinguenda* Gredler 1859) that some have suggested represents a separate species (Piechocki 1979; Stadnychenko 1990; Piechocki and Wawrzyniak-Wydrowska 2016). The status of this form and synonyms of *S. nitida* (e.g. *Segmentina clessini* Westerlund 1873) have, to date, remained unresolved due to a lack of genetic data.

In addition to identifying population structure, population genetics can identify populations with rare or bottlenecked genetic profiles that may be particularly vulnerable and therefore priority targets for conservation (Toro and Caballero 2005). Augmentation of declining populations through reintroduction and translocation programmes can support such conservation efforts (Jourdan et al. 2019). The success and efficacy of species conservation therefore usually depends on thorough knowledge of the genetic

makeup of donor and recipient populations and identifying evolutionarily significant units within them (Jourdan et al. 2019). Translocations and reintroductions should aim to maintain or, ideally, enhance the genetic diversity of populations (Frankham 2010; Weeks et al. 2011). Where genetics indicate the presence of cryptic species (two or more species described as one), this might 'create' two rare species (with smaller populations than previously assumed) where previously there was only thought to be one or reveal a species under threat of extinction that was previously 'hidden' among a much larger population that was considered of less concern (Bickford et al. 2007).

Often in conjunction with genetic data, the statistical analysis of shape has been used to reveal cryptic species (Simison and Lindberg 1999; Villemant et al. 2007; Arribas et al. 2013; Canal et al. 2015). For example, geometric morphometrics, a landmark-based method of shape analysis (Adams et al. 2004; Zelditch et al. 2004) can reveal small but significant shape differences between morphologically highly similar populations to help delineate subpopulations and/or cryptic species (e.g. Fontoura and Morais 2011; Karanovic et al. 2016). Combining multiple complementary lines of evidence, such as genetics and morphometrics in an integrative framework aids robust delineation of species and investigating of evolutionary processes (Klingenberg 2010). Consequently, these integrative methods are increasingly being used in conservation to resolve uncertainties arising from phenotypic plasticity and aiding in the identification of evolutionary significant units (Chiari et al. 2009; McKendrick et al. 2017).

To date, genetic data available for *S. nitida* have been relatively limited and do not provide clarity on the evolutionarily significant units contained within its current range. Seven *S. nitida* sequences amplified with four commonly used barcoding markers (three COI, two 16S and one 18S, and one Histone H3), are currently deposited in GenBank (Jørgensen et al. 2004; Albrecht et al. 2007; Saito et al. 2018; as of 22nd February 2021). One other study of the population genetics of *S. nitida* (Mensch 2009) used COI and 12S ribosomal DNA markers as well as Amplified Fragment Length Polymorphisms (AFLPs) to detect genetic differences between populations of *S. nitida* in Germany, Poland, and the UK. The mitochondrial marker (COI) indicated low genetic diversity in the UK, and it was estimated that this loss of diversity had occurred in the last 40 years, based on comparisons with DNA samples collected in the UK in 1969 (Mensch 2009). COI sequences for Polish and UK samples differed from each other, though AFLP results did not clearly delineate lineages (Mensch 2009). The study highlighted the need for further investigation using additional markers to assess the extent of genetic variation in *S. nitida*

and the differentiation between geographically separated populations (Mensch 2009), but there has been no further work on *S. nitida* in this regard since.

To provide a better understanding of *S. nitida* in Europe that can inform potential reintroduction or translocation of the species in the UK under its Biodiversity Action Plan (JNCC 2010), this study combines population genetics and geometric morphometrics to analyse newly sampled European populations of *S. nitida* in the UK, Germany, Poland and Sweden. Novel *S. nitida* microsatellite markers were developed and used in addition to using established ITS and COI markers to assess genetic structure and clustering of populations. Morphological differences between countries and genetic lineages of *S. nitida* were explored using 2D landmark-based geometric morphometrics.

Materials and methods

Sample collection

A total of 367 *Segmentina nitida* individuals were collected in the summer of 2016 from 3 to8 sites each in Germany, Poland, Sweden, and the UK (Fig. 1; GPS coordinates for sampling locations and details of use in genetic analysis provided in Online Resource 1) following the washing method described by Hobbs and Harvey (2020). Samples were stored in 2–5 mL absolute ethanol (Analytical Reagent Grade; Fisher, Loughborough, UK) or 70% isopropyl alcohol (German snails only; Hetterich, Fürth, Germany) in rubber-sealed screw-topped tubes at room temperature. The German samples were transferred to analytical reagent grade absolute ethanol (Fisher, Loughborough, UK) upon return to the laboratory (within 14 days of collection) and stored at room temperature.
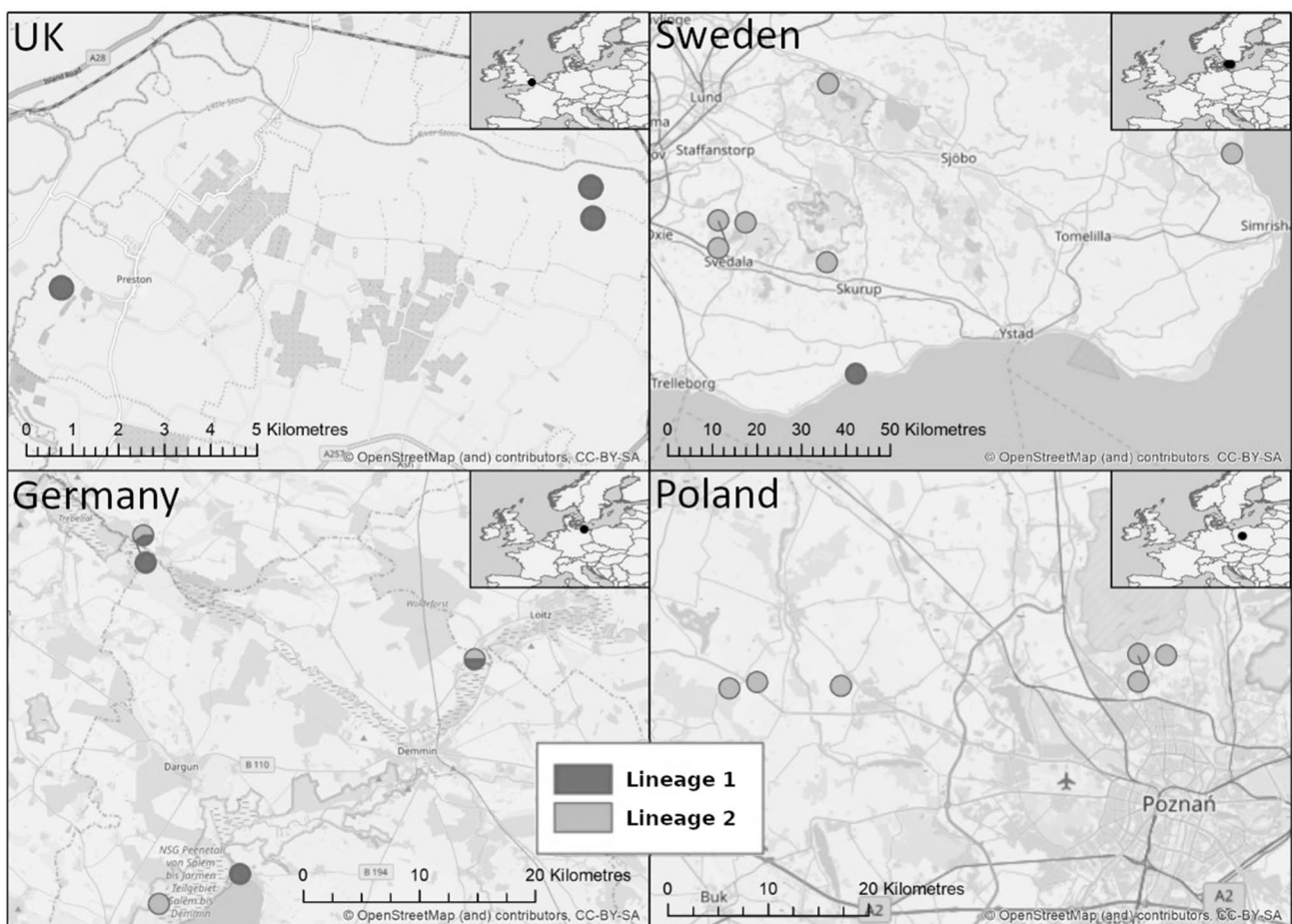

Fig. 1 Location of the sites in the UK, Germany, Sweden and Poland where the *Segmentina nitida* were sampled. Proportions of circles are light or dark grey according to the proportion of individuals belonging to each of the genetic lineages later identified

DNA extraction

DNA was extracted from 367 whole snails across the four sampled countries by homogenizing each snail including the shell in extraction buffer using genomic DNA purification kits according to manufacturer's instructions (DNeasy; QIAGEN, Manchester, UK; GeneJET; Thermo Scientific, Waltham, USA). Extracted genomic DNA was eluted twice into a single tube, each time in 25 uL volume of elution buffer.

Amplification, sequencing and analysis of mitochondrial and nuclear barcode markers

For a subset of snails from across all sites sampled (Online Resources 2 and 3), a mitochondrial cytochrome c oxidase subunit I sequence (COI, n = 98) and a nuclear ribosomal internal transcribed spacer 2 sequence (ITS2, n = 100) was amplified and sequenced. The COI sequence was amplified using the modified LCO1490 and HCO2198 primers (Albrecht et al. 2007). Mitochondrial DNA can be inherited paternally in some bivalves, resulting in mtDNA heteroplasmy and separately inherited and evolving mitochondrial lineages as 'doubly uniparental inheritance' (DUI) (Ladoukakis and Zouros 2017). There is currently no evidence of heteroplasmy or DUI in gastropods (Gusman et al. 2017). The ITS2 ribosomal sequence was amplified using the ITS2F and ETTS2 primers for genus *Biomphalaria* (another genus within the Planorbidae) (Vidigal et al. 2000). PCR reaction mixtures consisted of QIAGEN Multiplex PCR Master Mix, 5 μM forward primer, 5 μM reverse primer, ddH$_2$O and 1μL sample DNA with a final reaction volume of 10 μL. Reactions were cycled in a DNA Engine Peltier Thermo Cycler (BioRad, Watford, UK). Cycling conditions for COI were 95 °C for 15 min for one cycle, 40 cycles of 94 °C for 30 s, 48 °C for 1 min, and 72 °C for 1 min, and a final extension of 72 °C for 5 min. Cycling conditions for ITS2 were 95 °C for 15 min for one cycle, 32 cycles of 95 °C for 45 s, 54 °C for 1 min, and 72 °C for 2 min, followed a final extension of 72 °C for 5 min. Amplified PCR products were cleaned with EXO-Sap (ThermoFisher Scientific, Waltham, USA). Cycle sequencing reactions were performed using the BigDye V3.1 cycle sequencing kit (Applied Biosystems, Waltham, USA), with BigDye at 1/8th volume (0.5 μL per reaction). Automated sequencing was performed by capillary electrophoresis on an ABI3730 sequencer (Applied Biosystems, Waltham, USA).

Sequences were manually checked for calling errors and edited with CodonCode Aligner v. 8.0.2. (CodonCode Corporation; Centerville, USA). Sequence products were aligned with ClustalW using MEGAX (Kumar et al. 2016). Sequences for unique haplotypes of both COI and ITS2 were deposited in GenBank (Online Resource 4, GenBank Accession Numbers: COI- MW829457-MW829487; ITS2- MW566752-MW566754). Protein coding (COI) alignments were checked against amino acid translations. The number of polymorphisms, haplotypes, nucleotide and haplotype diversity, average number of nucleotide differences between individuals, and phylogenetically informative sites for all European populations, as well as within each country, were calculated in DnaSP v6 (Rozas et al. 2017). Population expansion in the dataset as a whole and within each country was also tested using DnaSP using Fu's Fs test.

For COI, aligned sequences were reduced to unique haplotypes in DnaSP (n = 31). A minimum spanning haplotype network was built using POPART (Leigh and Bryant 2015) with an epsilon value of 0. Maximum Likelihood (ML) phylogenies using unique haplotypes were inferred for the haplotypes using MEGAX. Appropriate models for ML trees were selected by evaluating 24 ML nucleotide substitution models using BIC and Akaike Information Criterion (AIC) in MEGAX. For COI the General Time Reversible model of nucleotide substitution was used, with gamma distribution rate = 5. Strength of support of tree nodes was assessed via bootstrapping (n = 1000). For COI, *Hippeutis complanatus,* a sister taxon in the same tribe (Segmentinini) as *S. nitida* was used as the outgroup (GenBank Accession Number: EF012170; Albrecht et al., 2007). For additional validation, phylogenetic trees were also generated following Neighbour-joining (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The sequence alignment for both trees was constructed using Clustal Omega version 1.2.2 (Sievers and Higgins 2014). In each case, *Hippeutis complanatus* was used as an outgroup using Jukes-Cantor genetic distance and a bootstrap value of 500. The constructed trees were inspected using FigTree version v1.4.4 (Rambaut and Drummond 2017) and CLC Genomics workbench API version 21.0.2 (http://www.clcbio.com).

The COI sequence from the *S. nitida* type locality in Denmark could not be used in the construction of the tree as the fragment of COI used in the previous study for an individual from Denmark (Jørgensen et al. 2004) did not overlap with the sequences obtained in this study. However, COI sequences from Lodz, Poland (GenBank Accession Number: LC429396; Saito et al. 2018) and Brandenburg, Germany (GenBank Accession Number: EF012178.1; Albrecht et al. 2007) were included in phylogenetic analyses. For ITS2, due to no closely related outgroup being available for this marker and limited genetic diversity in the sequences obtained, instead of a phylogenetic tree a minimum spanning haplotype network was built using POPART (Leigh and Bryant 2015) with an epsilon value of 0.

Microsatellite genotyping and analysis

All individuals ($n = 367$) were genotyped at seven newly isolated *S. nitida* microsatellite loci (GenBank Accession Numbers MW960425-MW96031, Online Resource 5), a number in line with that sufficient for investigating genetic structure and identify cryptic species in terrestrial and freshwater invertebrates (e.g. Patel et al. 2015; McKendrick et al. 2017). Microsatellites were isolated using the enrichment hybridization method of Armour et al. (1994) using modifications suggested by Gibbs et al. (1997) and Glenn and Schable (2005) and sequenced on an Illumina MiSeq Sequencer. For loci P5943, P25580, and P12998 primer sets were designed from a sequence isolated from a Polish individual sampled for the present study (Online Resource 1; individual P3.19). Microsatellite loci UK21826, UK15523, UK16382, and UK19417 were isolated from a UK individual sampled for the present study (Online Resource 1; individual UK1C). Forward primers were 5′-labeled with HEX or 6FAM fluorescent dyes (Table S1). To reduce the cost and time required during the microsatellite genotyping multiplex PCR sets were designed based on the different expected products sizes and by selecting contrasting fluorescent labels using Multiplex Manager v. 1.2. (Holleley and Geerts 2009). The size of PCR products was assigned by comparison against an internal size marker (ROX-500). Amplifications were performed in 2 μL volumes with 2–10 ng genomic DNA, a final concentration of 0.2 μM of each primer and Qiagen MasterMix (Kenta et al. 2008). All multiplex PCR sets included negative controls containing no genomic DNA. Thermal cycling was performed with a DNA Engine Peltier Thermo Cycler (BioRad) under the following conditions: 94 °C for 15 min for a single cycle, 44 cycles of 94 °C for 30 s, annealing at 56 °C for 1 min 30 s, 72 °C for 1 min 30 s, with a final extension at 72 °C for 30 min, followed by 4 °C for 15 min. Amplicons were separated on an ABI3730 48-well capillary sequencer (Applied Biosystems, Waltham, USA).

Microsatellite alleles were scored using GeneMapper version 3.7 (Applied Biosystems, Waltham, USA). Randomized, resampled genotyping ($n = 72$) of *S. nitida* populations from different European countries consistently showed the same allele profile for each of the seven loci. In repeat PCRs of the samples, 95.8% ($n = 69$) of individuals produced the same allele genotype. Five of the microsatellite PCR products from three individual snails across all samples and all loci produced different profiles between runs, showing false alleles and some PCR artefacts. These samples were excluded from analysis. Probability of null alleles (alleles where only one of the two alleles amplified) in the data was estimated using CERVUS v3.0 software (Kalinowski et al. 2007) and probability of allele dropout calculated with MICROCHECKER (van Oosterhaut et al. 2004).

The number of alleles per locus and observed and expected heterozygosity (H_O and H_E, respectively) at each microsatellite locus were calculated by country using CERVUS (Kalinowski et al. 2007). Genotypic linkage disequilibrium, inbreeding and departures from Hardy–Weinberg equilibrium (HWE) at the seven microsatellite loci were assessed using Fisher's exact test. Analysis of molecular variance (AMOVA), pairwise genotypic distances (F_{ST}) and inbreeding coefficients (F_{IS}) were obtained using ARLEQUIN V.3.5.2.2 (Excoffier and Lischer 2010). To assess the levels of genetic diversity of *S. nitida* lineages identified, the allelic richness (i.e. number of alleles, Na), observed heterozygosity (H_O), and measured heterozygosity (He; Nei's gene diversity), inbreeding coefficient (F_{IS}) and private alleles (Np) per lineage were estimated in GenAlEx version 6.5 (Peakall and Smouse 2012). Genetic distances among individuals were estimated (Smouse and Peakall 1999), and the distance matrix was converted to a covariance matrix to perform a Principal Coordinate Analysis (PCoA) in GenAlEx.

A Bayesian approach in STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to detect the most likely number of genetic clusters among *S. nitida* populations doing 10 replications with number of clusters K = 1–10 with 100,000 burn-in, 1,000,000 MCMC iterations after burn-in, and admixture model (using sampling locations as prior information) with correlated allele frequencies (Falush et al. 2003). The most likely number of K clusters was examined in StructureSelector (Li and Liu 2018) using log likelihood scores [mean LnP(K)] and ΔK values (Evanno et al. 2005). As these methods often underestimate clusters due to uneven sample sizes (Janes et al. 2017), we obtained estimates of K based on Puechmaille's method by subsampling the independent clusters previously identified, a technique which accounts for uneven sample size across groups (Puechmaille 2016). Likelihood scores and clusters were obtained using the CLUMPAK (Kopelman et al. 2015) function in StructureSelector and individual probability plots were generated using Structure Plot (Ramasamy et al. 2014). To further investigate genetic structure within identified clusters, these analyses were repeated separately for each identified cluster.

Geometric morphometrics of identified genetic lineages

Of the 367 snails for which microsatellite data were generated, 339 shells were imaged for morphometric analysis, excluding damaged shells. Shells were imaged to ensure consistency in orientation and allow all six landmarks to be visible (Fig. 2). Shell photographs were loaded into tpsUtil, version 1.78 (Rohlf 2019) to build a file for landmark
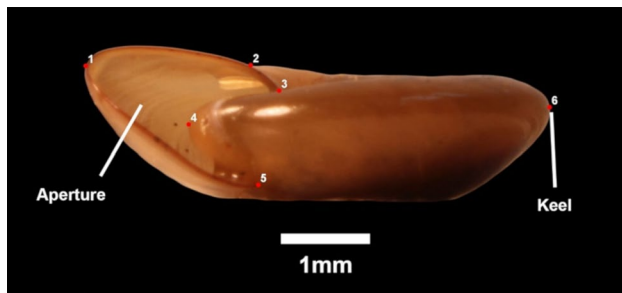
Fig. 2 Landmark placement for geometric morphometric analysis of *Segmentina nitida* shells. Red dots represent landmarks, next to corresponding landmark number

placement and digitisation was performed in tpsDig2 version 2.31 (Rohlf 2017) with a 1 mm scale set for all images. Shell width, aperture height, and aperture width for each specimen was measured using the distances between landmarks 1 and 6, 2 and 5, and 1 and 4, respectively. Summary statistics (minimum, maximum, mean, and range) were calculated for each genetic lineage previously detected. Mann–Whitney *U* tests were used to compare median values for shell width, aperture height, and aperture width.

Scale, orientation, and position were removed from landmark data using Procrustes superimposition to obtain Procrustes coordinates and centroid size for each sample. A Mann–Whitney *U* test was performed to compare median landmark centroid sizes between snails grouped by country of origin in Statistics Package for the Social Sciences (SPSS) version 24 (IMB 2018) as the data had unequal variance (Levine's test; α = 0.05). A Principal Components Analysis (PCA) of the Procrustes shape coordinates obtained from the Procrustes superimposition was performed to determine the linear combinations of the coordinates that maximise the variation in the data. Warp grids that showed the deviation of landmarks from the mean shape of all samples at the minimum and maximum of each principal component were generated. Pairwise Kruskal–Wallis tests were performed in SPSS on the principal components that described over 80% of the total shape variation in the sample (n = 3), with shells grouped by country as the data had unequal variance (Levene's test, α < 0.05) and were not normally distributed (Shapiro–Wilk test, P < 0.05). The Procrustes shape coordinates were then used for a discriminant analysis in Past version 3.21 (Hammer et al. 2001) to calculate probabilities of individual shells being assigned to the correct country of origin by shell shape. Data were then grouped by genetic lineages identified in microsatellite analysis and all analyses detailed above run with specimens grouped by lineage. Additionally, the specimens that most closely represented the mean shape for each lineage were identified and compared to each other using thin plate splines to produce deformation grids to determine key areas of difference in shell

shape between countries. All analyses were performed in with the R package 'geomorph' (Adams et al. 2019), unless otherwise stated.

Results

Nuclear and mitochondrial markers

COI sequences were generated for 97 individuals, a 513 bp length was aligned for each individual and sequences compared. Seven individuals, including six Polish and one German individual had a single 9 bp deletion in their sequence, which did not affect the reading frame. Alignment of sequences without the deletion revealed 106 polymorphic sites (20.1%), of which 88 were phylogenetically informative, representing 31 unique haplotypes. Nucleotide diversity across all populations was 0.07, with a haplotype diversity of 0.927. Populations in the UK and Poland had comparatively low nucleotide diversity (0.02), and populations from Germany had the highest nucleotide diversity (0.08). Germany had the highest haplotype diversity (0.930), followed by Poland and then Sweden (0.869 and 0.839, respectively), and the UK had the lowest haplotype diversity (0.363). The seven individuals containing the 9 bp deletion contained 70 polymorphic sites (13.9%), none of which were phylogenetically informative, with two unique haplotypes. All six Polish individuals with the 9 bp deletion represented one haplotype and the German individual with the deletion represented the other. The haplotype network analysis revealed two distinct clusters of haplotypes with a 67 bp gap between the two clusters (Fig. 3a).

The Maximum Likelihood tree for COI contained two well supported clades (bootstrap values = 99.8 and 100) representing some German populations and UK populations ('Lineage 1') and some German populations, Polish populations and one Swedish population ('Lineage 2') (Fig. 4). The German (Brandenburg; Albrecht et al. 2007) and Polish (Lodz; Saito et al. 2018) *S. nitida* sequences obtained from GenBank grouped with Lineage 1. These clades were also represented in the trees constructed using Neighbour-Joining and Unweighted Pair Group Method with Arithmetic Mean (Online Resources 6 and 7).

Among the 100 individuals sequenced at the ITS2 region, only three haplotypes with four polymorphisms and 3 indel (insertion/ deletion) polymorphisms were detected: a 412 bp haplotype for 66 individuals (A), a 412 bp haplotype with a 1 bp difference for 17 individuals (B), and a 413 bp haplotype with a 1 bp insertion for 17 individuals (C). Haplotype A comprised all individuals from Poland, eight individuals from Germany and all Swedish individuals except those of one population (S2). Haplotype B comprised the remaining individuals from

Fig. 3 Minimum spanning hap-lotype networks (epsilon = 0) of *Segmentina nitida* ITS (**a**) and COI (**b**) haplotypes. Each hatch mark represents a 1 bp difference, and each circle represents a unique haplotype. The size of each circle is proportional to the number of individuals with that haplotype
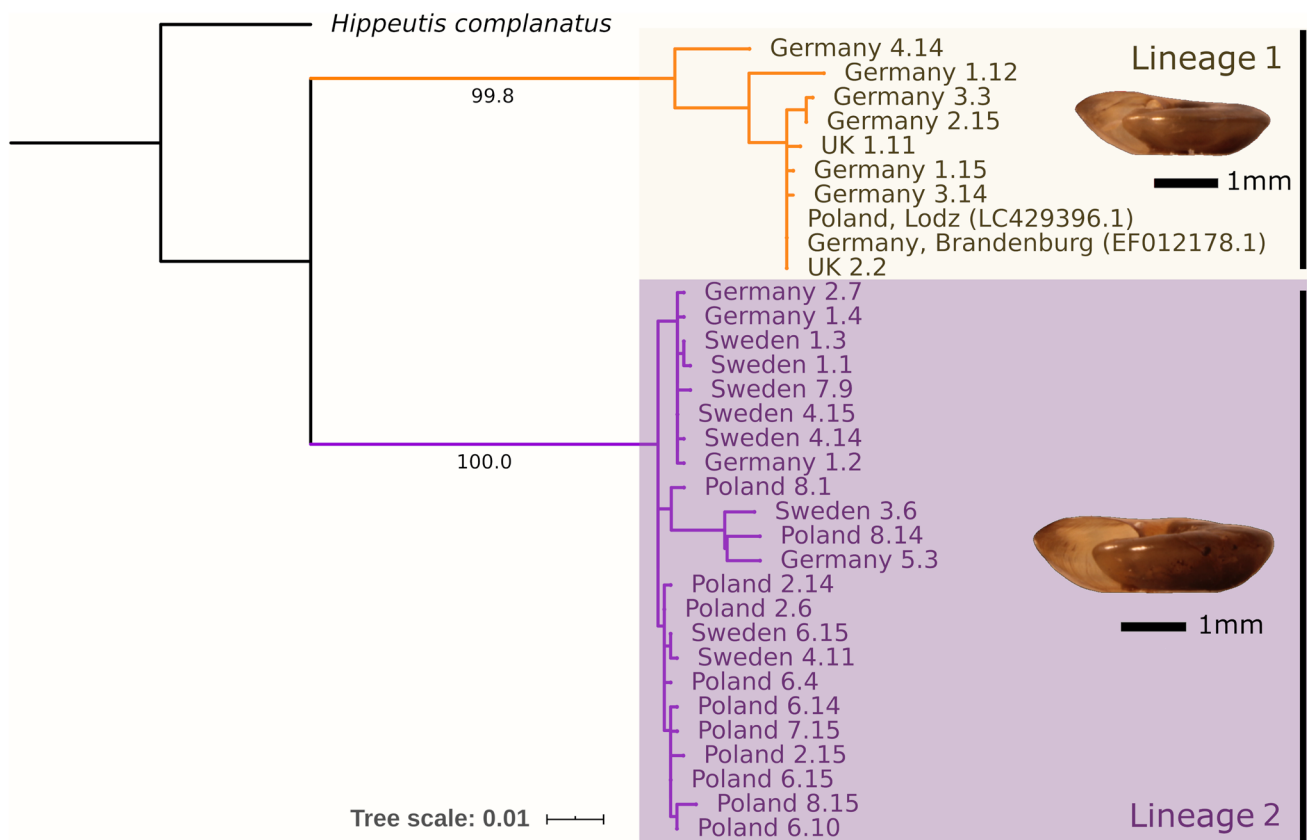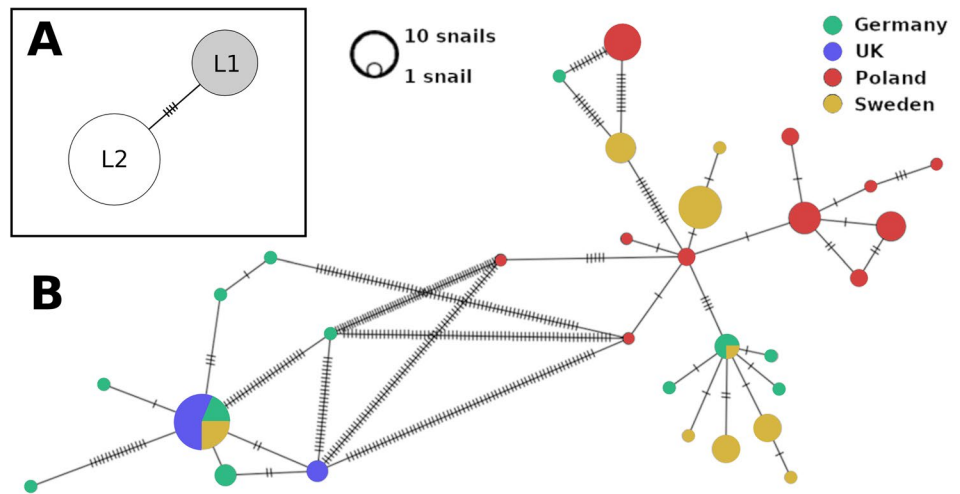

Fig. 4 Maximum Likelihood tree for *Segmentina nitida* cytochrome c oxidase subunit sequences from the UK, Germany, Sweden and Poland (numbers indicate source population and individual for each sequence). N = 31 haplotypes. Rooted using Hippeutis complanatus as an outgroup. Includes previously published sequences from Lodz, Germany (n = 13) and population S2. Haplotype C comprised all UK individuals. The nucleotide diversity across all populations was 0.004. The haplotype network analysis

Poland and Brandenburg, Germany, with GenBank accession numbers indicated. Values shown on branches of the two major clades indicate bootstrap values (n = 1000) representing branch support. Representative photographs of shell morphology for each of the two major clades identified are included with 1 mm scale

for ITS2 for *S. nitida* grouped Haplotype B and C together as a single haplotype separated from Haplotype A by 4 bp (Fig. 3b).

Table 1 Mean number of alleles (N_{ALL}), observed heterozygosity (H_O) and expected heterozygosity (H_E), inbreeding coefficient (F_{IS}) with significant values in bold, number of usable loci, and number of polymorphic loci across all microsatellite markers for each European *Segmentina nitida* population

Population	Number of individuals	N_{ALL}	H_O	H_E	F_{IS}	Number of informative/polymorphic loci
Germany	77	5.86	0.26	0.60	**0.53**	5/5
UK	31	1.57	0.16	0.14	− 0.17	7/3
Poland	83	3.43	0.28	0.35	0.08	7/6
Sweden	176	4.57	0.25	0.50	**0.42**	5/5
Lineage 1	97	3.57	0.18	0.31	**0.36**	5/2
Lineage 2	270	4.43	0.28	0.43	**0.29**	6/6

F_{IS} significance: Probability that random F_{IS} > observed F_{IS}, indicated by bold numbers in column. Usable loci: number of loci with fewer than 5% missing data. Number of polymorphic loci: number of usable loci with polymorphisms present in the population

Table 2 Pairwise genetic distance (F_{ST}) estimates with significance levels for the four European populations of *Segmentina nitida*

	Germany	UK	Poland	Sweden
UK	0.31***			
Poland	0.45***	0.77***		
Sweden	0.30***	0.62***	0.15***	

***$P < 0.001$

Table 3 Analysis of molecular variance (AMOVA) of microsatellite data for different groups of *Segmentina nitida,* grouped by country

Source of variation	Df	Sum of squares	Variance components	Percentage of variation	P
Among populations	3	340.26	0.69	39.39	***
Among individuals within populations	363	532.04	0.41	23.64	***
Within individuals	367	236	0.64	36.96	***
Total	733	1108.30	1.74		

***$p < 0.001$

Microsatellite genotyping, diversity and population structure

A total of 46 unique microsatellite genotypes were identified at the 7 loci typed across all snails analysed (n = 367 individuals, Table 1). No more than two alleles were found at any locus in any individual snail except for one UK snail, which had a tetraploid profile (possibly because it contained fertilized eggs) and this single sample was consequently discounted from further analyses. The mean number of alleles per population across all loci ranged from 1.57 for the UK to 5.86 for Germany. The loci across all populations contained between 3 (locus UK16382) and 11 alleles (loci UK15523 and UK21826) (Online Resource 8).

There was no evidence of allele dropout at any locus. The F_{IS} values revealed that the highest levels of inbreeding existed in Germany and Sweden, with comparatively low levels in the UK and Poland (Table 1). The negative value of F_{IS} for the UK population indicates a higher level of heterozygosity then expected in a randomly mating population.

Of the 28 possible population/locus combinations, five were monomorphic, however no locus was monomorphic across all countries. The UK had the most monomorphic loci (n = 4), with Poland being the only other country with a monomorphic locus (n = 1). At all loci, Germany had the highest observed heterozygosity (H_O), with Sweden showing the next greatest H_O.

All pairwise genetic distances (F_{ST}) by country were significant (< 0.001) over 110 permutations. The F_{ST} across all loci gave a value of 0.40. Pairwise F_{ST} values ranged between 0.15 and 0.77 (Table 2), with the UK population in comparison to both Polish and Swedish populations showing values greater than 0.50.

The partitioning of the genetic variation in the dataset by AMOVA showed that the amount of genetic variation between populations (39.39%) was similar to that when individuals from all populations were pooled (36.96%). Differences between individuals within populations accounted for 23.64% of variation (Table 3).

STRUCTURE analysis identified K = 2 and K = 4 as the most likely number of lineages that would explain the data (Fig. 5). For K = 2, STRUCTURE delineated Lineages 1 and 2 as also identified in the COI analysis. Only one individual in Swedish population S1 showed evidence of admixture, with a probability of 83.3% of being assigned to the Lineage 2 (Fig. 5). Lineage 2 (n = 270) was comprised of all individuals in Poland, all individuals in Sweden (apart from those in population S2) and some individuals from German populations Lineage 1 (n = 97) included all UK individuals, some of the German individuals as well as one of the Swedish populations. There was a significant difference between the two lineages ($F_{ST} = 0.64$, $P = < 0.005$) over
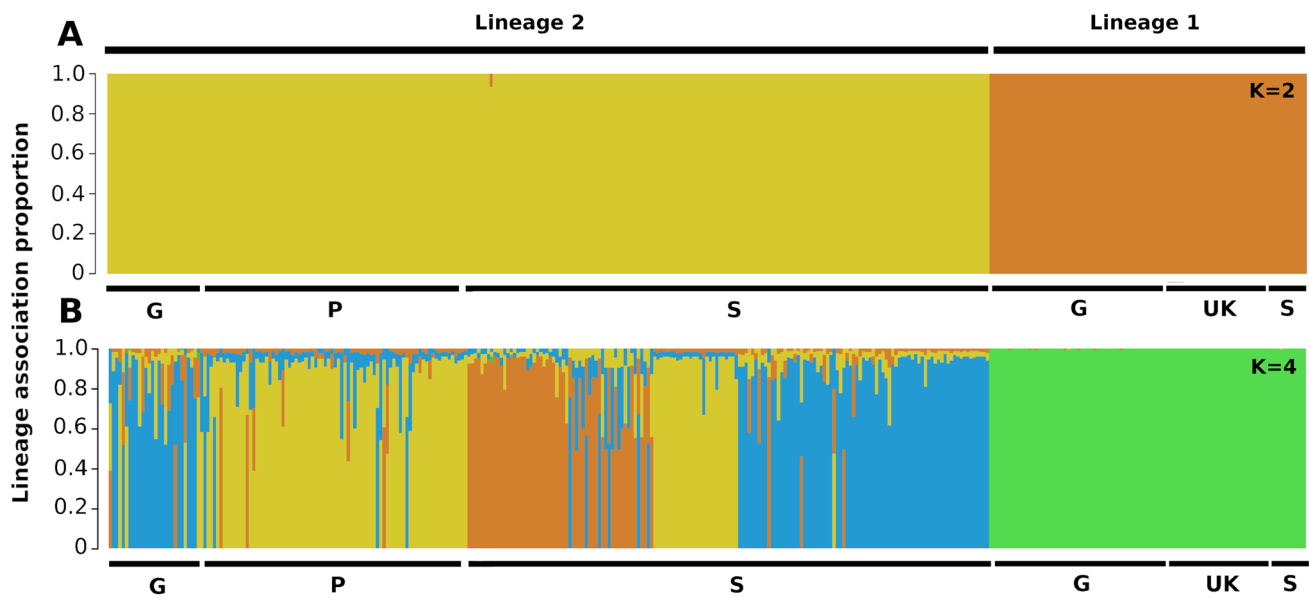
Fig. 5 Genetic lineages and sub-lineages of *Segmentina nitida* inferred from the STRUCTURE cluster analysis, using prior population information included in the model. Each vertical line represents one individual snail and shades of grey represent their association with hypothesized lineages. STRUCTURE identified K=2 (**a**) and K=4 (**b**) as the most likely number of lineages. Both models delineated Lineage 1 and Lineage 2 without significant admixture of alleles. In the K=4 model, additional sub-lineages were identified within Lineage 2, but there was no indication of further structure in Lineage 1

110 permutations. Both lineages had significant F_{IS} index scores (inbreeding coefficient) ($P = <0.005$, 1023 permutations; Lineage 2: $F_{IS}=0.26$, Lineage 1: $F_{IS}=0.35$). The genetic distance (F_{ST}) between Lineage 1 and Lineage 2 was significant over 110 permutations ($F_{ST}=0.64$, $P < 0.0001$). For K=4, Lineage 1 and Lineage 2 were again delineated without significant admixture, but three sub-lineages were located within the Lineage 2, with evidence of admixture between these sub-lineages (Fig. 5). When each of the identified lineages was subjected separately to STRUCTURE analysis, no further structure was detected in Lineage 1 (Online Resource 9). In Lineage 2, Evanno's ΔK method indicated the presence of four sub-clusters, with Polish snails representing a sub-cluster showing little admixture from three sub-clusters represented by Swedish and German snails (Online Resource 10).

In the GeneAlex analysis, snails in Lineage 1 and Lineage 2 were delineated sharply with no overlap along the first principal coordinate that explained 44.18% of variation in the similarity matrix (Fig. 6a). There was no evidence of further differentiation along the second principal coordinate (Fig. 6a). Based on their microsatellite alleles, all individuals were assigned to their putative lineage of origin (Lineage 1 or 2), which also produced a sharp delineation of snails from the two lineages (Fig. 6b). In addition, GeneAlex identified 25 private alleles in Lineage 1 and 19 in Lineage 2 (mean across all loci: 3.57 for Lineage 1, 2.71 for Lineage 2).

Geometric morphometrics of *Segmentina nitida*

Shell centroid size was significantly different between countries except between the UK and Germany, and the UK and Sweden (Table 4). When grouped by genetic lineage, the centroid size of snails in Lineage 2 was significantly greater than that of snails in Lineage 1 (Mann–Whitney *U* test; U=7420, P = <0.0001).

Lineage 2 individuals had significantly wider shells and taller and wider apertures than those in Lineage 1 (Mann–Whitney *U* tests: shell width: U=7766, $P = <0.0001$; aperture height: U=4988.5, $P = <0.0001$; aperture width: U=6531, $P = <0.0001$). Both lineages had similar minimum values for all three variables, however Lineage 2 had greater maximum values and mean values for all variables (Table 5).

In the principal component analysis of Procrustes shape coordinates, Principal Component (PC) 1 and PC2 accounted for 84% of variation in shape (60.6% and 23.4% respectively, Fig. 7). When grouped by country there was a significant pairwise difference in PC1 score between all countries except Sweden and Poland, and a significant pairwise difference in PC2 score between all countries except the UK and Sweden, and Germany and Sweden (Table 4). There was a significant difference in both PC1 score and PC2 score between individuals from the two lineages (Mann–Whitney *U* Test; PC1: U=4583, $P = <0.0001$; PC2: U=7627, $P = <0.0001$).
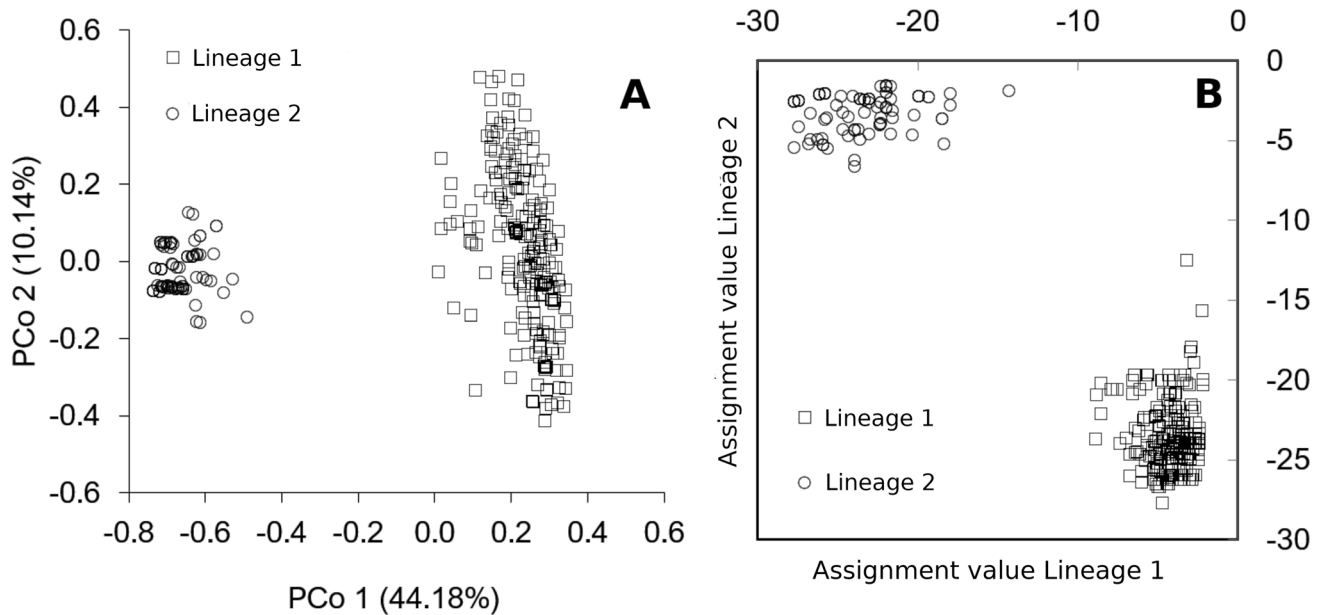
Fig. 6 **a** GeneAlex principal coordinates analysis (PCoA) based on a genetic distance matrix of individuals within the two putative genetic lineages of *S. nitida* (GeneAlex). **b** Snails grouped by assignment value for Lineage 1 and Lineage 2 in GeneAlex. In this analysis snails are assigned to a lineage based on the smaller of two assignment values calculated based on the log likelihood of their alleles occurring in that lineage

Table 4 Pairwise comparisons of centroid size and first two Principal Components (PCs) of Procrustes shape coordinates between *Segmentina nitida* individuals from European countries

Pairwise comparison of Countries	Centroid Chi squared	Centroid significance	PC1 U	PC1 significance	PC2 U	PC2 significance
UK-Germany	5.21	1.00	453	***	425	***
UK-Sweden	46.40	0.18	645	***	4850	0.06
UK-Poland	142.70	***	257	***	1098	***
Germany-Sweden	− 41.19	***	3383	***	4850	0.24
Germany-Poland	− 137.49	***	1559	***	1098	***
Sweden-Poland	13.34	***	6514	0.93	2216	***

Statistical tests: Centroid: Kruskal-Wallace. PCs: Mann–Whitney U. Bonferroni corrected *P* values. ***$p < 0.001$

Table 5 Summary statistics of traditional morphometrics of *Segmentina nitida* shells grouped by genetic lineage determined at least one genetic analysis

Statistic	Shell Width		Aperture Height		Aperture Width	
	Lineage 1	Lineage 2	Lineage 1	Lineage 2	Lineage 1	Lineage 2
Minimum	2.57	2.43	0.70	0.68	0.61	0.57
Maximum	4.95	7.46	1.50	2.32	1.18	1.84
Mean	3.56	4.02	1.05	1.32	0.87	1.02
Range	2.38	5.03	0.80	1.64	0.57	1.27

All measurements are given in mm

When grouped by country, the first two discriminant functions (DFs) of a discriminant analysis of the twelve Procrustes shape coordinates accounted for 87.3% of the total shape variation in the dataset (DF1- 56.3%, DF2- 31.0%) and the discriminant functions correctly assigned 49.3% of the shells in the analysis to their correct country of origin (jackknifed). When grouped by genetic lineage (Lineage 1 or Lineage 2), however, the discriminant analysis produced
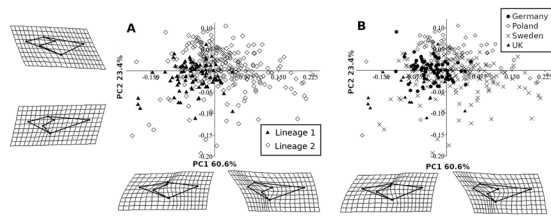
Fig. 7 Principal component plots of Procrustes shape coordinates for *Segmentina nitida* individuals. **a** Grouped by country. **b** Grouped by genetic lineage (Lineage 1 and Lineage 2). Deformation grids indicate the shape of shells at the extremes of each Principal Component, in relation to the mean shape of all individuals

Table 6 Matrix of classifications of *Segmentina nitida* shells grouped by genetic lineage in discriminant analysis model of Procrustes shape coordinates

	Lineage assigned to snail by discriminant shape analysis		
	Lineage 1	Lineage 2	Total
Genetic lineage of snail from Structure analysis			
Lineage 1	81 (94.2)	5 (5.8)	86
Lineage 2	46 (18.2)	207 (81.8)	253
Total	127	212	339

Numbers represent the number of shells classified as belonging to each lineage, with percentage in parentheses. Rows: genetic lineage assigned by structure analysis. Columns: Predicted lineage based on shape
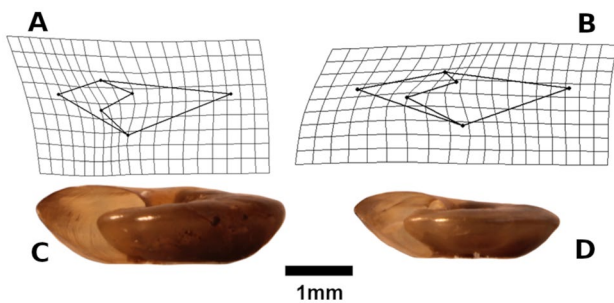
Fig. 8 Deformation grids of the two genetic lineages and photographs of the two specimens most closely representing the mean shape of each species. **a** Lineage 2 mean shape plotted to the mean shape of Lineage 1, magnification of deformation = 2; **b** Lineage 1 mean shape plotted to the mean shape of Lineage 2 mean shape, magnification of deformation = 2; **c** shell most closely representing the mean shape of Lineage 2 (Germany, population 3, snail 12); **d** shell most closely representing the mean shape of Lineage 1 (Germany, population 2, snail 15)

a single discriminant function, which accounted for 100% of the shape variation. The discriminant function correctly assigned 85% of shells to their genetic lineage (jackknifed) (Table 6).

The individuals that most closely represented the mean shape of each lineage were both from German populations (Fig. 8). The deformation grids of the mean shape of each lineage warped to the other lineage shows the aperture is the key area of differentiation between the two, with LMs 2, 3, 4 and, 5 showing greatest deformation of grid lines, with less deformation around LMs 1 and 2, representing the keel (Fig. 8a and b).

Discussion

Mitochondrial and nuclear markers revealed two genetically lineages of European *Segmentina nitida* collected for this study with high bootstrap support: one lineage represented by Polish and Swedish individuals (Lineage 2), and one represented by UK individuals, and one Swedish population (Lineage 1) (Figs. 3, 4, 5). German populations contained individuals from both lineages (Figs. 3 and 5). STRUCTURE analysis of the seven microsatellite loci investigated strongly supported these two main lineages (Fig. 6). Moreover, the F_{ST} results from the microsatellite data show the *S. nitida* populations studied here cannot be considered as being drawn from the same gametic pool (overall $F_{ST} = 0.40$; $F_{ST} > 0.15$ indicates significant level of genetic differentiation between populations (Hartl and Clark 2007; Frankham et al. 2010)). This is not the first time significant genetic differentiation between European populations of *S. nitida* has been reported. Mensch (2009) posited the presence of three genetic lineages of *S. nitida* in Europe based on a genome-wide genotyping method (Amplified Fragment Length Polymorphism, AFLP). One of these was comprised of UK and German individuals, one of only Polish individuals, and one of German, Polish and a UK individual. AFLP analysis can incorporate hundreds of polymorphisms across a whole genome, but it suffers from relatively high genotyping error (Zhang and Hare 2012) that can significantly affect analysis of population structure and make it impossible to determine the 'true' number of populations among a set of individuals. Mensch (2009) reports shifting AFLP peak profiles between individuals included in her study, which may have resulted in incorrect genotyping and thus the greater number of genetic lineages detected (Vašek et al. 2017). It is not clear if the lineages described here coincide with any of those identified by Mensch (2009) with the genetic data from that study unavailable, but the weight of genetic evidence suggests the existence of (at least) two morphologically cryptic but reproductively isolated species within *S. nitida*.

Reproductive isolation of Lineage 1 and Lineage 2 within *S. nitida* is supported by the co-existence of snails from the two linages—without evidence of allele admixture—along the river Peene in Germany (Figs. 3 and 5). An alternative

explanation for the lack of admixture would be that sampled locations were colonized only very recently by members of one of the two lineages, leaving insufficient time for mating and admixture. This seems highly unlikely, however. *Segmentina nitida* has a relatively short reproductive cycle, with multiple breeding events throughout a year (Książkiewicz and Gołdyn 2008). Moreover, *S. nitida* is a hermaphroditic species that is able to outcross (Mavárez ety al. 2002; Lamy et al. 2012). Consequently, if the two lineages are not reproductively isolated, some evidence of genetic admixture between them would be expected, even within a single breeding season.

In the populations sampled for this study, the two lineages were geographically discrete outside of Germany, except for one population in Sweden that belonged to Lineage 1 (Fig. 4). In addition, a GenBank COI sequence from a snail collected in Lodz, Poland, nested within Lineage 1, unlike the Polish snails collected around Poznan for this study (all within Lineage 2; Fig. 4). This may indicate that the overlap in the geographical distribution of the two lineages is more extensive than was captured by the geographical sampling range for the present study. There was also evidence that Lineage 2 contains three or four distinct genetic sublineages, based on STRUCTURE analysis (Fig. 5, Online Resource 10). There was no evidence of further structure within Lineage 1 (Online Resource 9). Analysis of the structure within Lineage 2 indicated that one sub-lineage was exclusive to Poland, while there was evidence of admixture in individuals from German and Swedish sub-lineages (Online Resource 10). Geneflow between sub-lineages and the presence of geographically isolated populations of one lineage of *S. nitida* within the range of the other is likely the result of long-distance dispersal of propagules by waterbirds (van Leeuwen et al. 2012). Figuerola et al. (2005) report that matrices of bird movement probabilities related to genetic differences between invertebrate populations in the same areas and explained significant variations in the mtDNA relationships between populations of freshwater microinvertebrates *Daphnia ambigua, Daphnia laevis,* and *Cristatella mucedo* populations (Figuerola et al. 2005). Geneflow between the sub-lineages within Lineage 2 of *S. nitida* is most likely also facilitated by dispersal via waterbirds (van Leeuwen et al. 2012) and flooding events. Most sample source locations in both Germany and Sweden were part of extensive wetlands and near large water bodies that are likely to experience seasonal flooding (e.g. the River Peene in Germany and coastal marsh areas in Sweden). Migratory birds favour coastal marshes as stopover sites (Figuerola et al. 2003; Green and Figuerola 2005), this may explain the high levels of sub-lineage admixture observed in these populations.

Differences between the two lineages corresponding to genetic differences were reflected in shell phenotype. For the 339 individuals with genetic and morphological data available, grouping snails by shape and genetic lineage (Lineage 1 or Lineage 2) explained the data better than grouping snails by country of origin and there was a significant difference in shape, traditional morphometrics, and centroid size between snails from Lineage 1 and Lineage 2 as captured (Figs. 6 and 7, Tables 4, 5, 6). Shape differences were also observed between snails form different countries, but these may be a consequence of the varying prevalence of snails from Lineage 1 or 2 in different countries.

While the differences in shell shape between the two lineages may be attributable to underlying genetic differences, it could also be the result of phenotypic plasticity, i.e. the expression of alternative phenotypes by the same genotype, usually in response to environmental conditions (Stearns 1989). Phenotypic plasticity can complicate and confound classification of snails and obscure morphological differences between species (Minton 2002; Perez and Minton 2008). Plasticity in snail shell shape has been attributed to factors such as thermal stress (Hazel and Johnson 1990), population density (Kemp and Bertness 1984), and predation (Appleton and Palmer 1988; DeWitt 1998). Changes in shell morphology can occur suddenly, sometimes within the growth phase of a single generation (Johnson and Black 1999; Urabe 2000; Minton and Gunderson 2001). Phenotypic plasticity in conjunction with evolutionary pressures may ultimately drive genetically fixed variation in shell morphology (Kistner and Dybdahl 2013). Further study into the climatic conditions and habitat parameters of *S. nitida* from both lineages in European populations and their genetics is required to investigate the contributions of directional selection and phenotypic plasticity to shape variation.

Nevertheless, there was still a large amount of overlap between lineages in the principal components of the Procrustes shape coordinates (Fig. 7). It is therefore not surprising that the status of *S. nitida* has been the subject of so much debate and confusion (Kennard and Woodward 1926), especially given the importance of shell morphology in snail taxonomy. The presence of an additional morphologically distinct form of *Segmentina nitida* in Eastern Europe (*Segmentina nitida* f. *distinguenda*) has previously been posited (Piechocki 1979). It has been argued, however, that this form is a species separate from *S. nitida* (Stadnychenko 1990) synonymous with that previously described as *Segmentina clessini* Westerlund 1873. Piechocki and Wawrzyniak-Wydrowska (2016) have distinguished the shell of *Segmentina nitida* f. *distinguenda* from that of *S. nitida* referring to a keel displaced towards the centre of the shell, a narrow umbilicus, more strongly developed internal thickenings (compared to *S. nitida*) and having a lower shell height than *S. nitida*. This description more closely matches Lineage 1 identified here, which seems to extend into Poland as indicated by the placement of the COI sequence from Lodz,

Poland (Saito et al. 2018; Fig. 4). To resolve the confusion around the various synonyms and their geographic range, the next step should be to compare individuals from Lineage 1 and Lineage 2 revealed by genetic and shape analysis to individuals from Denmark, the type location, including the shells of the type specimens. This should reveal which of the lineages (1 or 2) represents *Segmentina nitida* Müller 1774, and whether one lineage should be described as a new species or resurrect a previously synonymised species name. Comparison to genetic data of *S. nitida* individuals from the species type locality (Denmark) was not possible here as the only genetic data available for this population (Jørgensen et al. 2004) is a fragment of the COI locus outside of the region amplified for the present study.

The data presented here indicate that *S. nitida* is most likely diploid (almost uniformly two alleles were recorded per microsatellite locus), as are other planorbids (e.g. Szabelska et al. 2015). *Segmentina nitida* is hermaphroditic and can self-fertilize, which will increase the likelihood of directly related individuals occurring in a population. The prevalence of such individuals in our samples is not likely to have been high enough to significantly affect the outcomes of analysis, however, for two reasons. First, egg clutches produced by *S. nitida* are small (typically up to 11 eggs, Bondesen 1950), reducing the number of directly related individuals per generation in a population. Second, samples in each country were collected at multiple locations that were separated from others physically (i.e. no direct connection of freshwater habitats) or geographically (i.e. distance of > 5 km between locations). Even if a small number of snails from any individual location were directly related, it is highly unlikely there was any direct relatedness between snails from different locations, due to the poor dispersal of *S. nitida* even at a local scale (Niggebrugge et al. 2007).

Regardless of the taxonomic identity of the two lineages of *Segmentina nitida* identified here, our results could have significant implications for the conservation of both lineages and *S. nitida* sensu lato, especially in the UK. If Lineage 2 indeed represents a reproductively isolated species, the lineage found in the UK (Lineage 1) may have a reduced geographical range and number of remaining populations than that of *S. nitida* sensu lato. However, without expanded sampling to cover the entirety of the range of *S. nitida* sensu lato, inferences as to the phylogeographic implications of this study, and relative population ranges of each species are limited. A collaborative, international effort to sample and genotype *S. nitida* sensu lato would allow more effective estimation of the distribution of each lineage across Europe, particularly in areas where their ranges of overlap. This would facilitate a more thorough evaluation of the genetic diversity, range and population size of each lineage and aid in the development of a collaborative management decisions. Potentially informative populations could be located

in Spain (coastal marshland associated with bird-mediated dispersal (Green and Figuerola 2005), France (as the closest country to the UK), the Netherlands (containing extensively networked wetland areas) and the Czech Republic. All UK snails included in this study were taken from a single marsh system in the southeast of England. Analysis of individuals from populations in Norfolk, Sussex and Yorkshire (Hill-Cottingham 2004; Watson and Ormerod 2004; Mensch 2009) would allow comparison of populations in light of the proposed Lineage 1 and a more in-depth analysis of local diversity and gene flow in the UK. Of the 367 European individuals genetically analysed, only 26.4% (n = 97) clustered with Lineage 1. In the UK Biodiversity Action Plan for *S. nitida* the current distribution of the species is stated as "widespread but declining" and the UK populations are "unlikely to be significant in global terms" (JNCC 2010). With the populations in the UK having the potential to represent a distinct species (or at least a regionally highly distinct genotype), these assessments may have to be revised, after expanded surveying. Existing populations may therefore require more attention and more extensive management and any breeding or translocation from outside of the UK can only originate from genetically compatible Lineage 1 populations (e.g. some German populations, currently). Moreover, the low genetic diversity in UK populations indicates that the sampled habitats have only recently been colonised by a limited number of individuals or that they have undergone a genetic bottleneck. This makes these populations more vulnerable to local extinction (Aguilar et al. 2008) and also means that translocation of snails within the UK may result in further loss of genetic diversity. These findings underscore the importance of having detailed knowledge of the genetic structure and providence of *S. nitida* and other freshwater invertebrates—especially those with a complex taxonomic history—that are the subject of ongoing management and conservation efforts.

Declarations

Conflict of interest There are no conflicts of interest or competing interests to declare.

Ethical approval Sequence and genotyping data and original DNA samples are available upon request (source DNA has been used up for some samples).

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