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# An Integrative Analysis of the Specific Distinctness of *Valvata* (*Cincinna*) *ambigua* Westerlund, 1873 and *Valvata* (*Cincinna*) *piscinalis* (Müller, 1774) (Gastropoda: Valvatidae)

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**Abstract:** *Valvata* (*Cincinna*) *piscinalis* (Müller, 1774) is a widespread and variable Palaearctic freshwater snail species. Some authors have separated more depressed forms with a wider umbilicus as a distinct species, *Valvata* (*Cincinna*) *ambigua* Westerlund, 1873. The latter species was described from Scandinavia and has also been reported from Siberia and Kazakhstan and more recently from Central Europe. We conducted an integrative study of the delimitation and relationships of *V. ambigua* and *V. piscinalis* using both morphometric and molecular genetic analyses. Analyses of the morphometric data did not reveal differentiation into distinct clusters. Rather, the shell characteristics used to distinguish *V. ambigua* and *V. piscinalis* showed continuous variation. There is little variability in mitochondrial DNA sequences in the *V. piscinalis* complex. A median-joining network based on cytochrome oxidase sequences showed that the morphological character states supposedly characteristic of *V. ambigua* and *V. piscinalis* did not correlate with the genetic relationships of the individuals studied. We therefore consider *V. ambigua* to be synonymous with *V. piscinalis*.

**Keywords:** Europe; integrative taxonomy; phylogeny; taxonomy; *Valvata*; Gastropoda



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## 1. Introduction

Valvatidae Gray, 1840 (Gastropoda: Heterobranchia) are a group of freshwater snails from the Northern Hemisphere. They are most diverse in the Palaearctic, with approximately 60 species, while 10 species are known from the Nearctic and only one from the Afrotropical region [1,2]. However, many of the species are in need of revision. Even for some European species, the delimitation is still unclear. While Western European malacologists distinguished only five nominal species of the genus *Valvata* Müller, 1773 in Northern and Central Europe [3,4], Russian and Ukrainian researchers recognized up to 28 *Valvata* species in the European part of Russia and neighbouring countries alone [5,6]. One of the problematic species complexes is the widespread and variable *Valvata* (*Cincinna*) *piscinalis* (Müller, 1774) (Figure 1a). Glöer [2] divided this species into five subspecies, four of which were later recognized as separate species by Glöer [7]. In addition, Glöer [7] listed *Valvata* (*Cincinna*) *ambigua* Westerlund, 1873, *Valvata* (*Cincinna*) *kliniensis* Milachevich, 1881, and *Valvata* (*Cincinna*) *lilljeborgi* Westerlund, 1897, which also belong to the *V. piscinalis* complex, as separate species following the opinion of Anistratenko [8] and Starobogatov et al. [9].

In this study, we investigated the distinctness of *V. ambigua* (Figure 1c). Westerlund [10] described this species based on specimens from Göteborg in Sweden. Later, Westerlund [11] classified the nominal taxon as a variety of *V. piscinalis* and listed it from Sweden and Finland. In contrast, Krivosheina and Starobogatov [12] resurrected *V. ambigua* as a distinct species and recorded it from Western Siberia. Anistratenko [8] and Starobogatov et al. [9] also treated *V. ambigua* as a valid species in their comprehensive works on freshwater molluscs of Ukraine and Russia and adjacent countries, respectively. Glöer and

Diercking [13] reported the species from Hamburg in Germany and discussed its ecology. Vinarski et al. [14] discussed the distinctness of *V. ambigua* and *V. piscinalis* and reported the species also from Kazakhstan and Norway. According to Glöer [7], the range of *V. ambigua* covers Northern, Central and Eastern Europe as well as the southern part of Western Siberia.

We used shell morphometric data as well as mitochondrial gene sequences to investigate the distinctness of *V. ambigua* and *V. piscinalis*. In particular, we studied samples from the Hamburg surroundings. This area is particularly suitable for testing the distinctness of the two nominal species because they are said to occur there syntopically [13] and because Vinarski et al. [14] also focused on material from the Hamburg region.


**Figure 1.** Shells of specimens of the *Valvata piscinalis* complex. (**a**) Specimen corresponding to *V. piscinalis* sensu Vinarski et al. [14] from the Reitbrooker Sammelgraben in Hamburg-Bergedorf (ZMH 159600). (**b**) Intermediate specimen from the Untenburger Schleusengraben in Hamburg-Harburg (ZMH 159583). (**c**) Specimen corresponding to *V. ambigua* sensu Vinarski et al. [14] from the Spadenländer Deichsielgraben in Hamburg-Harburg (ZMH 159598). Scale bar = 1 mm.

## 2. Materials and Methods

### *2.1. Materials*

*Valvata* specimens were collected from slowly flowing ditches and rivers in different locations in Hamburg in the summer of 2023. Geographical coordinates were recorded with a GPS and are given in WGS 84 format. The specimens were preserved in 100% isopropanol at −20 °C in the Zoological Museum Hamburg (ZMH) (Supplementary Table S1). Additional shell material from the ZMH was used for morphometric analyses.

### 2.2. Morphometric Analyses

The shell diameter (D), shell height (H), diameter of the second whorl (D2W), diameter of the umbilicus (U), diameter (da) and height (ha) of the aperture were measured using a Leica MZ9.5 stereomicroscope with an ocular micrometer (accurate to 0.05 mm). The number of whorls was counted with an accuracy of 0.25 whorls using the method described by Glöer [4].

The shells were photographed with a Passport Imaging System (Dun, Palmyra, VA, USA) with a Canon EOS 6D (Canon Inc., Tokyo, Japan) camera and processed with Zerene Stacker (Zerene Systems LLC, Richland, WA, USA) and Adobe Photoshop v. 25.9.1 (Adobe Systems Inc., San José, CA, USA) software.

We used normal mixture models (NMMs, [15]) to explore the existence of distinct morphologic clusters without a priori information about groups [16,17]. Following the approach proposed by Cadena et al. [18], we first reduced the dimensionality of the phenotypic space via principal component analysis (PCA) on the covariance matrix of log-transformed data (the seven shell measurements) with the statistical software R v. 4.2.0 [19]. We used the R package clustvarsel 2.3.4 [20] to select the set of principal components most useful for group discrimination. Multiple NMMs for 1–10 clusters were fitted to this multivariate dataset with the R package mclust 5.4.9 [21], and the best model was selected according to the Bayesian Information Criterion.

### 2.3. DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from tissue samples following the protocol proposed by Sokolov [22] with slight modifications as detailed in Scheel and Hausdorf [23]. Parts of the mitochondrial cytochrome c oxidase subunit I (COI) and 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the primer pairs LCO1490 and HCO2198 [24] and 16Sar-L and 16Sbr-H [25], respectively. PCR amplifications were carried out in 25 µL volumes containing 15.8 µL dd$H_2O$, 2.5 µL 10× amplification buffer B (biolabproducts, Bebensee, Germany), 2.5 µL $MgCl_2$ (25 mM), 1 µL dNTP mix (5 mM each, biolabproducts), 1 µL of each primer (10 µM), 0.2 µL Crystal Taq DNA polymerase (5 units/µL; biolabproducts) and 1 µL of template DNA under the following reaction conditions: an initial denaturation step at 95 °C for 2 min, 35 PCR cycles (95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s) and a final extension step at 72 °C for 7 min. Both strands of the amplified products were sequenced at Macrogen Europe Laboratory (Amsterdam, The Netherlands).

### 2.4. Alignment and Analyses of DNA Sequences

ChromasPro 2.1.10.1 (Technelysium, Tewantin, Australia) was used to assemble the forward and reverse sequence reads. The GenBank accession numbers of the new COI and 16S rDNA sequences, as well as sequences of additional *Valvata* and *Megalovalvata* Lindholm, 1906 specimens for which both the COI and 16S rRNA genes were available from the studies of Hauswald et al. [26], Dinapoli and Klussmann-Kolb [27], Clewing et al. [28] and Saito et al. [29], are listed in Supplementary Table S2.

The sequences were aligned with MAFFT v. 7.525 [30] using the Q-INS-i algorithm. Kimura 2-parameter (K2P) distances were calculated with MEGA11 [31]. We selected the optimal partitioning scheme and substitution models starting with the three codon positions of cytochrome oxidase and the 16S rRNA gene as initial partitions using ModelFinder [32] implemented in IQ-TREE v. 2.2.2.6 [33]. In the search for the optimal substitution models for the maximum likelihood analysis, we included the FreeRate heterogeneity model, while we restricted the search to models available in MrBayes for the Bayesian inference analysis.

Maximum likelihood phylogenetic analysis based on the concatenated sequences of the partial COI and 16S rRNA genes was performed using IQ-TREE v. 2.2.2.6 [33]. We evaluated branch support with 1000 standard nonparametric bootstrap replicates.

A Bayesian inference analysis of the phylogeny was performed using MrBayes version 3.2.2 [34]. Metropolis-coupled Monte Carlo Markov chain searches were run with four chains

in two separate runs with 10,000,000 generations. Diagnostics obtained from the MrBayes output were used to assess stationarity and convergence. The first 2,500,000 generations of each run were discarded as burn-in.

We constructed a median-joining network [35] based on COI sequences using the program POPART [36] with $\varepsilon = 0$. The constructed network was colour-coded according to the morphological trait ratios D/H and U/D. Vinarski et al. [14] distinguished *V. piscinalis* and *V. ambigua* based on their D/H (0.87–0.99 for *V. piscinalis* and 0.96–1.17 for *V. ambigua*) and U/D ratios. We divided the overlapping range of the D/H ratio at the centre (0.975 mm) and used this value as a threshold to indicate the potential classification of individuals in the network. The corresponding threshold of U/D is based on the D/H threshold, as Vinarski et al. [14] did not measure the umbilicus. We set the threshold midway between the lowest U/D value of specimens with D/H $\geq$ 0.975 mm and the highest U/D value of specimens with D/H < 0.975 mm (U/D = 0.11).

## 3. Results

### *3.1. Shell Morphology*

We measured shell parameters of 178 specimens from several sites around Hamburg and Ratzeburg in northern Germany (Supplementary Table S1). The range of the measurements covers the range of the parameters of *V. ambigua* and *V. piscinalis* reported by Vinarski et al. [14]. Plots of shell height against shell width (Figure 2a) and of relative umbilicus width (umbilicus width/shell width) against shell shape (shell width/height) (Figure 2b) show that these shell measurements and ratios vary continuously among the studied samples. This is also true if only the samples from the Reitbrooker Sammelgraben in Hamburg-Bergedorf, from which Glöer and Diercking [13] reported syntopic occurrences of *V. ambigua* and *V. piscinalis* without intermediates, were considered.

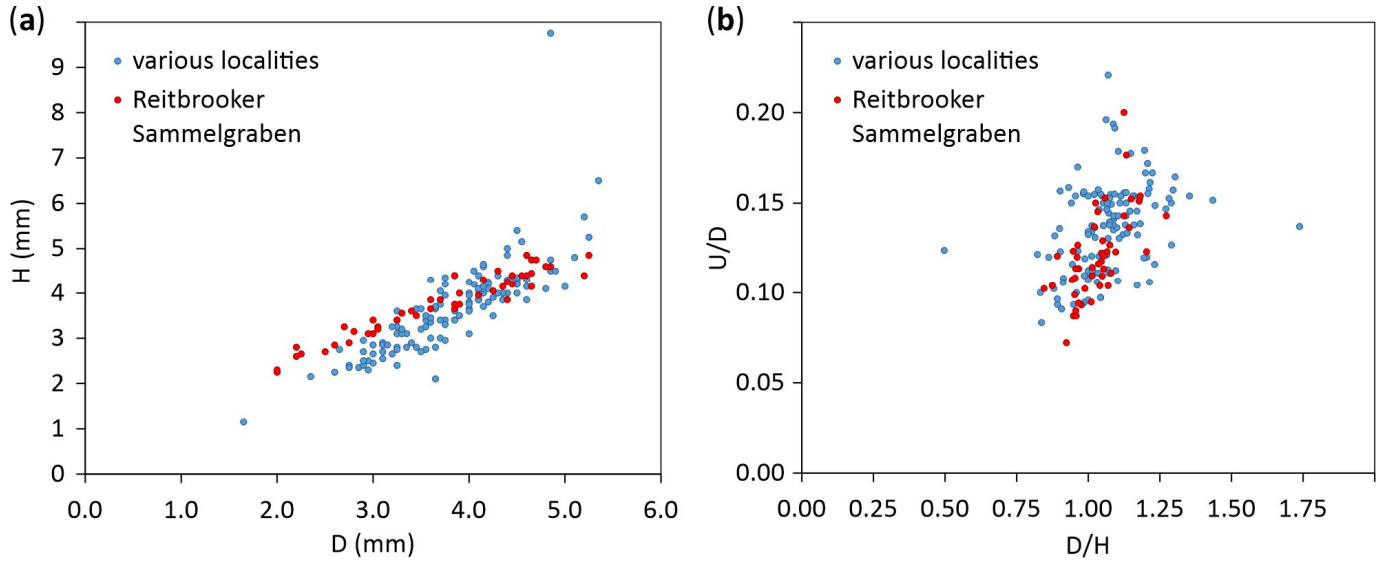


**Figure 2.** Plots of the shell parameters of specimens of the *Valvata piscinalis* complex from the Hamburg region and the Ratzeburger See. (**a**) Shell height (H) versus shell diameter (D). (**b**) Relative umbilicus width (U/D) versus shell shape (D/H). Specimens from the Reitbrooker Sammelgraben in Hamburg-Bergedorf, where both species are said to co-occur syntopically [13], are highlighted in red.

The highest empirical support based on the information in principal components 4, 5 and 6 was found for two NMMs (Figure 3). However, one NNM included 175 of the 178 measured specimens, whereas the other included only three specimens from three different localities, which did not form a homogeneous cluster (Figure 3). The three specimens included in the second NNM can be considered outliers rather than representatives of a second species. The first three components of a PCA based on the seven shell parameters

together explained 94% of the variance, whereas the following three components explained only 6% in addition. However, the varsel procedure recommended discarding the first three components because they are not very useful for group discrimination. A single NMM was favoured if we used the first three components of the PCA for fitting the NMMs. The results indicate that the variation in the dataset can best be described by a single NMM and that *V. ambigua* and *V. piscinalis* in the sense of Vinarski et al. [14] represent artificial subdivisions of the variability in a single species.

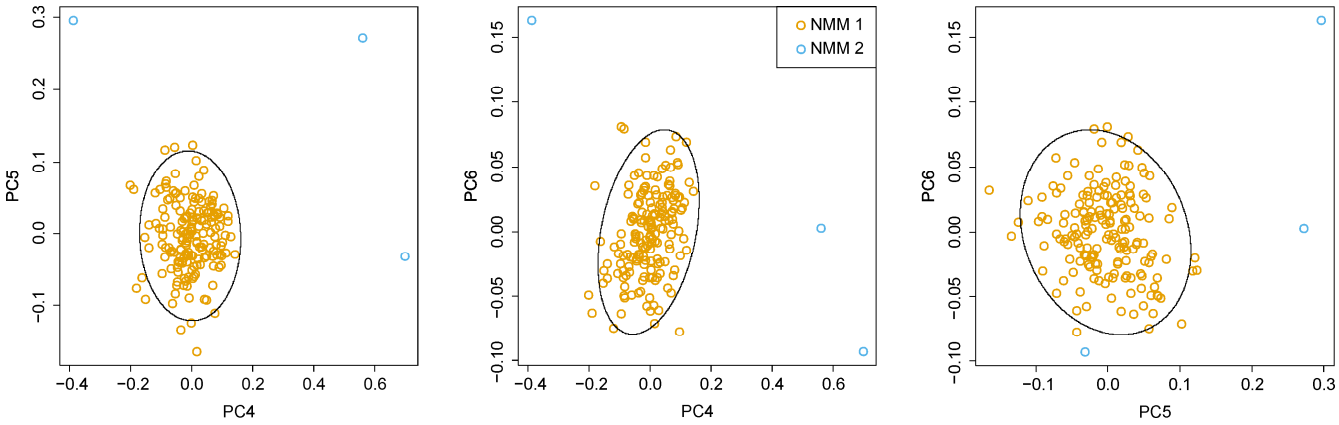


**Figure 3.** The two best supported NMMs in the space defined by the three principal components most useful for group discrimination (PC4−PC6). The ellipse shows the 95% high-density region for the normal distribution of morphological group 1.

### *3.2. Molecular Phylogeny*

The COI alignment was 649 base pairs (bp) long, and the 16S rDNA alignment was 453 bp, yielding a total of 1102 bp. The ModelFinder analysis suggested keeping the four initial partitions separate and using the following substitution models: TN+F+G4/GTR+F+G4 for the first codon positions of COI for the maximum likelihood analysis and Bayesian inference analysis, respectively; F81+F/F81+F for the second codon positions of COI; TIM2+F+G4/GTR+F+G4 for the third codon positions of COI; and TIM+F+G4/HKY+F +I+G4 for 16S rDNA.

In the maximum likelihood tree and the Bayesian inference tree based on the concatenated partial COI and 16S rDNA sequences (Figure 4), the newly sequenced specimens of the *V. piscinalis* complex from the Hamburg surroundings formed a clade with a sample identified as *V.* cf. *piscinalis* from Brandenburg in Germany. A *Valvata* cf. *piscinalis* from southern Russia represents the sister group to the specimens of the *V. piscinalis* complex from Germany in the Bayesian inference tree but not in the maximum likelihood tree. However, there is no statistical support for the relationships among the specimens of the *V. piscinalis* complex. A specimen identified as *V. piscinalis* by Dinapoli and Klussman-Kolb [27] from Lake Prespa in North Macedonia (EED-Phy-K388) forms a clade with *Valvata* (*Cincinna*) *stenotrema* Poliński, 1929 from Lake Ohrid and is likely closely related or conspecific with this species (Figure 4). There is neither statistical support for the relationships of the species within *Valvata* (*Cincinna*) Mörch, 1864 nor for the relationships between the (sub-)genera.

The genetic distances between the COI sequences of the *V. piscinalis* complex samples from Hamburg are low. The K2P distances between the COI sequences vary between 0.000 and 0.0252 with an average of 0.0106. For comparison, the average K2P distances between *V. piscinalis* and the most closely related *Valvata* species are 0.0405 to *Valvata* (*Cincinna*) *montenegrina* Glöer and Pešić, 2008, 0.0544 to a *Valvata* (*Cincinna*) sp. from Tibet (UGSB10876), 0.0544 to *Valvata* (*Costovalvata*) cf. *hirsutecostata* Poliński, 1929, 0.0562 to *Valvata* (*Costovalvata*) *rhabdota* Sturany, 1894 and 0.0605 to *V.* (*Cincinna*) *stenotrema*.

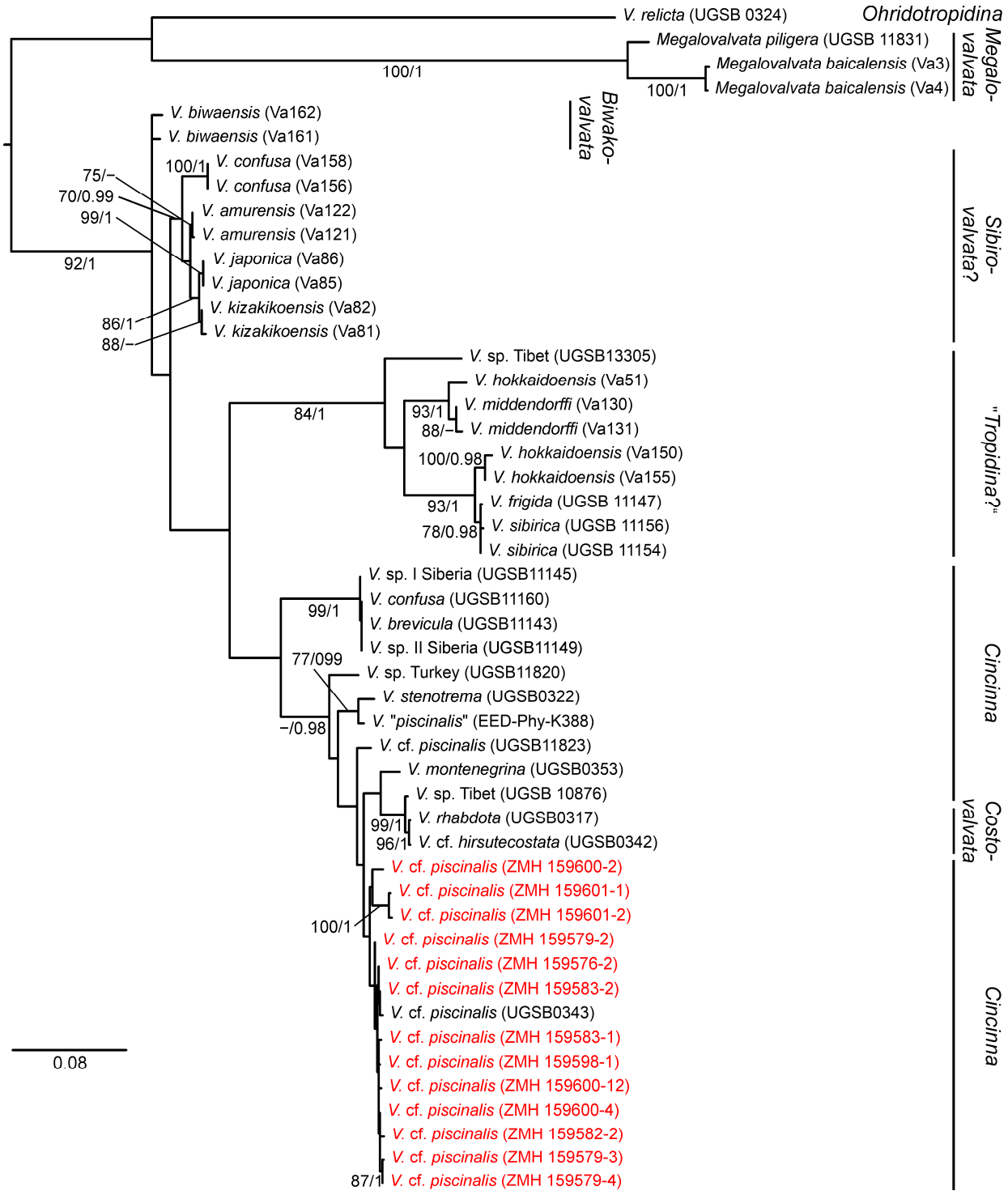


**Figure 4.** Maximum likelihood tree of Valvatidae based on the concatenated partial sequences of COI and 16S rDNA. Species names and extraction voucher numbers are given at the tips of the tree. Newly sequenced individuals are shown in red. Bootstrap support values from the maximum likelihood analysis ≥70 and posterior probabilities from the Bayesian inference analysis ≥0.95 are indicated at the branches.

Our focus is the relationships of specimens of the *V. piscinalis* complex and the question of whether these relationships are congruent with the supposed morphological differences between *V. ambigua* and *V. piscinalis*. We constructed a median-joining network based on the COI sequences of the *V. piscinalis* complex samples from Hamburg and plotted the states

of two ratios, shell shape (D/H) and relative umbilicus width (U/D), which distinguish *V. ambigua* and *V. piscinalis* according to Vinarski et al. [14], on the network (Figure 5). The distribution of the character states was not correlated with the genetic relationships of the individuals.

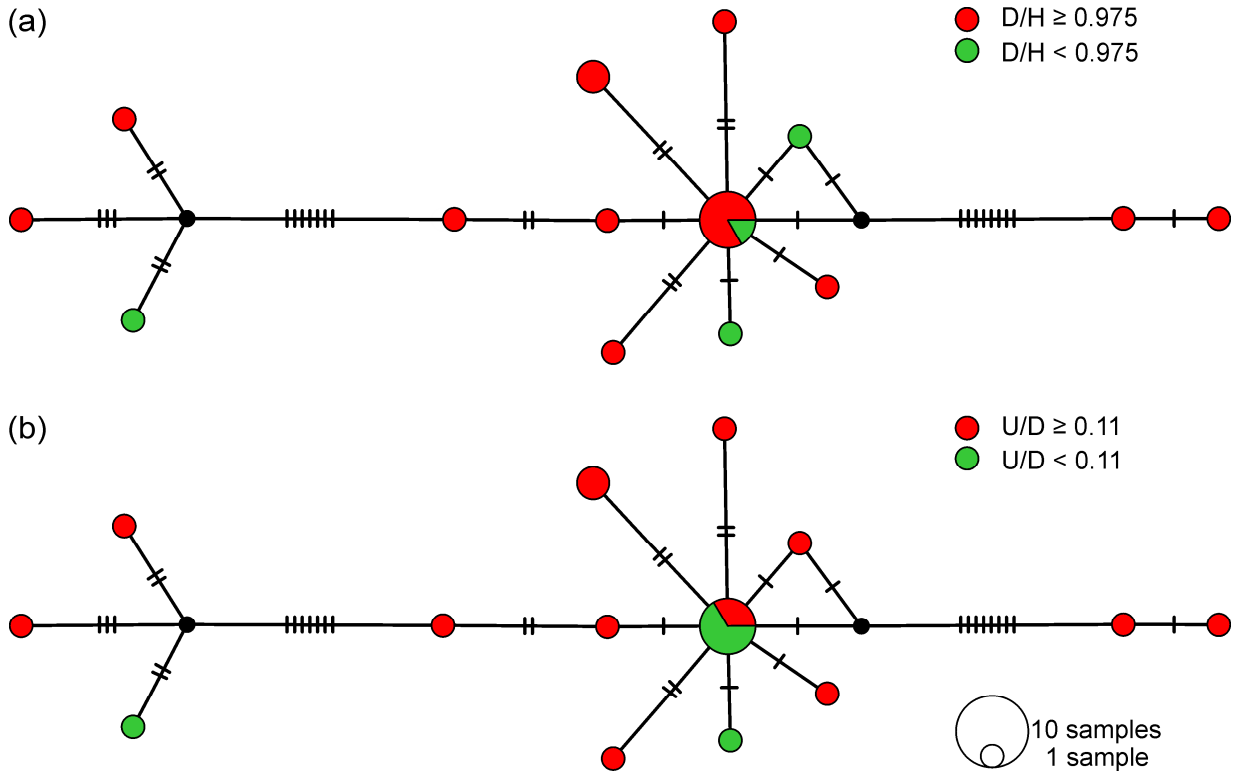


**Figure 5.** Median-joining network based on the partial COI sequences of specimens of the *Valvata piscinalis* complex from the Hamburg region (see Supplementary Table S2 for a list of specimens and GenBank accession numbers). (**a**) Specimens were classified according to shell shape (D/H). (**b**) Specimens were classified according to relative umbilicus width (U/D).

## 4. Discussion

### *4.1. Delimitation of Valvata (Cincinna) ambigua Westerlund, 1873 and Valvata (Cincinna) piscinalis (Müller, 1774)*

*Valvata* (*Cincinna*) *ambigua* Westerlund, 1873, a taxon of the *Valvata* (*Cincinna*) *piscinalis* complex, has recently been recorded in several regions of Central Europe [13,14,37,38]. It is difficult to identify *Valvata* specimens using the keys, which do not even provide the variability of shell measurements, and the simplified outline drawings of Anistratenko [8] and Starobogatov et al. [9]. Therefore, the morphometric data and photographs of *V. ambigua* and *V. piscinalis* provided by Vinarski et al. [14] were important advances. Vinarski et al. [14] specified that the shells of *V. ambigua* differ from those of *V. piscinalis* by a lower number of whorls, a larger shell width, a relatively lower spire, a concave tangential line of the spire (almost straight in *V. piscinalis*) and clearly wider umbilicus than in *V. piscinalis*. The morphometric analyses of Vinarski et al. [14] were based on specimens from the Hamburg region in Germany, where both taxa were previously reported to co-occur.

Our *Valvata* samples from the Hamburg region included forms representing *V. ambigua* and *V. piscinalis* according to the measurements provided by Vinarski et al. (Table 2) [14]. Plots of the shell measurements showed continuous variation in the shell diameter (D) and shell height (H) (Figure 2a), as well as in the shell shape (D/H) and relative width of the umbilicus (U/D) (Figure 2b). The results were similar when considering only specimens from the Reitbrooker Sammelgraben, where both taxa were previously reported to co-occur without intermediate forms [13]. The plots of the measurements provide no evidence that

the samples represent two distinct taxa. This is consistent with the principal component analysis shown by Vinarski et al. (Figure 4) [14] based on their shell measurements, in which the two taxa they distinguished also did not form distinct clusters. It is not an argument for the distinctness of artificially delimited subgroups of a continuously varying taxon that there are significant differences between these subgroups (as shown by Vinarski et al. (Table 2) [14]; see also [18]). A clustering approach based on NMMs using our morphometric dataset indicated that the dataset could be split into two NNMs. However, the second NNM includes only 3 of the 178 measured specimens (Figure 3), which do not form a morphologically homogeneous cluster but are better considered outliers. Therefore, the clustering approach indicated that *V. ambigua* and *V. piscinalis* are artificial subsets of a single NMM. The three outliers might be teratological specimens, with anomalies, e.g., caused by parasites.

In fact, 16S rDNA sequences from tissue samples of some specimens of the *V. piscinalis* complex from Reitbrooker Sammelgraben in Hamburg-Bergedorf and from the Untenburger Schleusengraben in Hamburg-Harburg showed that these specimens were infested by the oligochaet *Chaetogaster limnaei* von Baer, 1827 (Naididae). Smythe et al. [39] and Mack et al. [40] showed that *Chaetogaster limnaei* is a complex that includes several cryptic species. The *Chaetogaster limnaei* complex has been reported from a wide range of freshwater snails and mussels [41,42] but was not known from valvatids until now. The sequences we retrieved from the *V. piscinalis* complex (GenBank accession no. PP992211) belong to *Chaetogaster* sp. 23 in the sense of Mack et al. [40], corresponding to the European *Chaetogaster limnaei* in the strict sense. The *C. limnaei* clade has adapted to a mixed lifestyle. The specimens can live as ectosymbionts on molluscs and feed on a diet of invertebrates, ciliates and diatoms or as parasites, which exclusively subsist on host cells [39,40]. *Chaetogaster limnaei* apparently lives as a parasite in *Valvata*, as we obtained the *Chaetogaster* sequences from tissue samples of the snail hosts. The feeding of *Chaetogaster* may affect the growth and, therefore, shell size and structure of *Valvata*. However, the specimens in which we detected *Chaetogaster* were morphologically inconspicuous and belonged to NMM 1, as shown in Figure 3.

We used DNA sequences of mitochondrial genes to further test the hypothesis that the morphologically variable samples of the *V. piscinalis* complex from Hamburg represent two distinct species and to investigate their relationships with other *Valvata* taxa. The low genetic distances between the COI sequences of the *V. piscinalis* complex samples from Hamburg (maximum K2P distance 0.0252) do not indicate that these samples represent different species. Plotting the character states of shell shape (D/H) and relative umbilicus width (U/D), which distinguish *V. ambigua* and *V. piscinalis* according to Vinarski et al. [14], on a median-joining network based on COI sequences (Figure 5) showed that these character states are not correlated with the genetic relationships of the studied individuals.

The analyses of the morphometric data of the studied specimens of the *V. piscinalis* complex revealed no distinct clusters, although they included forms corresponding to *V. ambigua* and *V. piscinalis*, as defined by Vinarski et al. [14]. The morphometric characters used by Vinarski et al. [14] to distinguish *V. ambigua* and *V. piscinalis* showed continuous variation. The genetic analyses showed that these character states are not correlated with the genetic relationships of the studied individuals. Accordingly, we consider *Valvata* (*Cincinna*) *ambigua* Westerlund, 1873, as delimited by Vinarski et al. [14], to be synonymous with *Valvata* (*Cincinna*) *piscinalis* (Müller, 1774). Samples from other regions identified as *V. ambigua* or *V. piscinalis* should be analysed to confirm their conspecificity with *V. piscinalis* as delimited here. Samples from Gothenburg in Sweden, the type locality of *V. ambigua*, and from Frederiksdal in Denmark, the type locality of *V. piscinalis*, should be genetically analysed to ensure that the names have been correctly interpreted.

### *4.2. Phylogenetic Relationships*

We investigated the relationships of the newly sequenced samples of *V. piscinalis* with previously sequenced *Valvata* taxa [27–29], for which both COI and 16S rDNA sequences

were available. The specimens of the *V. piscinalis* complex from the Hamburg surroundings formed a clade with a specimen from Brandenburg in Germany, identified as *V.* cf. *piscinalis* by Clewing et al. [28] (Figure 4). A *Valvata* cf. *piscinalis* from southern Russia represents the sister group to the specimens of the *V. piscinalis* complex from Germany in the Bayesian inference tree but not in the maximum likelihood tree. A further specimen identified as *V. piscinalis* by Dinapoli and Klussman-Kolb [27] from Lake Prespa in North Macedonia forms a clade with *Valvata* (*Cincinna*) *stenotrema* Poliński, 1929 from Lake Ohrid and is likely closely related or conspecific with this species (Figure 4). This illustrates the difficulties in the identification of the *Valvata* species resulting from few diagnostic shell characters. The lack of statistical support for the relationships between the *Valvata* (*Cincinna*) species as well as for the relationships between the subgenera indicates that more sequence data and nuclear sequence data are necessary for a robust reconstruction of the phylogeny of the Valvatidae. The polyphyly of the samples identified as *V. confusa* Westerlund, 1897, by Clewing et al. [28] and Saito et al. [29], the polyphyly of the samples identified as *V. hokkaidoensis* Miyadi, 1935 by Saito et al. [29] in the maximum likelihood tree (Figure 4) and the very low genetic distances between samples identified as *V. confusa*, *V. brevicula* Kozhov, 1936 (*V. aliena* Westerlund, 1876 according to the GenBank entries), *Valvata* sp. I and *Valvata* sp. II by Clewing et al. [28] show that integrative taxonomic studies are also necessary to clarify the delimitation of other *Valvata* species.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d16070419/s1, Table S1. Shell measurements and ratios of specimens of the *Valvata piscinalis* complex from northern Germany; Table S2: Data of specimens used for molecular genetics.

**Author Contributions:** Conceptualization, B.H.; methodology, B.H.; formal analysis, B.H. and M.S.; field work, M.S.; laboratory work, M.S.; investigation, M.S.; resources, B.H.; data curation, M.S. and B.H.; writing—original draft preparation, B.H. and M.S.; writing—review and editing, B.H. and M.S.; visualization, B.H. and M.S.; supervision, B.H. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** New DNA sequences were uploaded to GenBank. GenBank accession number: PP992205–PP992211, PP994814–PP994829.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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