

# TAXONOMIC STATUS OF *STAGNICOLA PALUSTRIS* (O. F. MÜLLER, 1774) AND *S. TURRICULA* (HELD, 1836) (GASTROPODA: PULMONATA: LYMNAEIDAE) IN VIEW OF NEW MOLECULAR AND CHOROLOGICAL DATA

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**ABSTRACT:** Analyses of nucleotide sequences of 5'- and 3'- ends of mitochondrial cytochrome oxidase subunit I (5'*COI*, 3'*COI*) and fragments of internal transcribed spacer 2 (ITS2) of nuclear *rDNA* gene confirmed the status of *Stagnicola corvus* (Gmelin), *Lymnaea stagnalis* L. and *Ladislavella terebra* (Westerlund) as separate species. The same results showed that *Stagnicola palustris* (O. F. Müll.) and *S. turricula* (Held) could also be treated as separate species, but compared to the aforementioned lymnaeids, the differences in the analysed sequences between them were much smaller, although clearly recognisable. In each case they were also larger than the differences between these molecular features of specimens from different localities of *S. palustris* or *S. turricula*. New data on the distribution of *S. palustris* and *S. turricula* in Poland showed – in contrast to the earlier reports – that their ranges overlapped. This sympatric distribution together with the small but clearly marked differences in molecular features as well as with differences in the male genitalia between *S. palustris* and *S. turricula* strongly support the view that they are separate species and not two subspecies within *S. palustris*.

**KEY WORDS:** Poland, lymnaeids, *COI*, ITS2, sympatric/allopatric distribution, species/subspecies status

## INTRODUCTION

Three separate species, namely *Galba corvus* (Gmelin, 1791), *G. turricula* (Held, 1836) and *G. occulta* Jackiewicz, 1959, were distinguished by JACKIEWICZ (1959) within the species complex *Galba palustris* (O. F. Müller, 1774). Soon after, the name *Lymnaea (Stagnicola) palustris* (O. F. Müll.) was reintroduced (FALKNER 1984, JACKIEWICZ 1989) for the species recognised by JACKIEWICZ (1959) as *G. turricula*, but *L. (S.) turricula* (Held, 1836) was redescribed as a fourth separate species (FALKNER 1985). Moreover, a fifth species, *L. vulnerata* (Küster, 1862), was re-

described from the locus typicus (Cetina River near Omiš, former Yugoslavia) (JACKIEWICZ 1988a) and other localities (JACKIEWICZ & GERBER 1990) within the (sub)genus *Stagnicola* Jeffreys, 1830. FALKNER (1995) reintroduced the name *S. fuscus* (Pfeiffer, 1821) as the older available name for this species. To sum up, on the boundary of the 20th and 21st centuries five European species – despite some controversies on the existence of ecological varieties (see: KILIAS 1992 vs. JACKIEWICZ 1996) and generic or subgeneric status of particular species (see: JACKIEWICZ 1993, 1998a vs. FALKNER 1995) – were recognised within the genus *Stagnicola*: *S. palustris* (O. F. Müller, 1774), *S.*

*corvus* (Gmelin, 1791), *S. fuscus* (Pfeiffer, 1821), *S. occultus* (Jackiewicz, 1959) and *S. turricula* (Held, 1836) (FALKNER 1995, JACKIEWICZ 1998a, 2000, FALKNER et al. 2001, GLÖER 2002, GLÖER & MEIER-BROOK 2003).

Since the 1990s several different molecular methods have been intensively used to solve taxonomic problems among Lymnaeidae (REMIGIO & BLAIR 1997a, b, BARGUES & MAS-COMA 1997, 2005, BARGUES et al. 1997, 2007, 2011, 2012, REMIGIO 2002, STOTHARD et al. 2000, TROUVE et al. 2000, RYBSKA et al. 2000a, KNOTT et al. 2003, REMIGIO & HEBERT 2003, CARVALHO et al. 2004, DE BOER et al. 2004, PFENNINGER et al. 2006, PUSLEDNIK et al. 2009, CORREA et al. 2010, SCHNIEBS et al. 2011, 2012, VINARSKI et al. 2011, 2012, VINARSKI 2013). As a result, some changes have been also introduced in the above classification of the genus *Stagnicola*.

First, *S. occultus* (Jack.) was assigned to a newly established genus *Catascopia* Meier-Brook et BARGUES, 2002 on the basis of the nucleotide sequence length of ITS2 fragment in nuclear *rDNA* gene, characteristic of *S. occultus* and three North American stagnicoline species (MEIER-BROOK & BARGUES 2002). However, VINARSKI (2012) proved that there was an older available name *Ladislavella* B. Dybowski, 1913 which should be used for the species treated by MEIER-BROOK & BARGUES (2002) as “Nearctic advanced stagnicolines”. Moreover, VINARSKI (2003, VINARSKI & GLÖER 2008) found that although

JACKIEWICZ (1992a, 1997, 1998b) was aware of the occurrence of *S. occultus* in Siberia, she overlooked an older available name *Limnaea palustris* var. *terebra* Westerlund, 1885 for the taxon described by her in 1959. Therefore, at present, *Galba occulta* Jackiewicz, 1959 should be named *Ladislavella* (*Ladislavella*) *terebra* (Westerlund, 1885) with the name given by JACKIEWICZ (1959) placed among its younger synonyms (KHOKHUTKIN et al. 2009).

The second change concerned *S. palustris* (O. F. Müll.) and *S. turricula* (Held) (WELTER-SCHULTES 2012). Based on the nucleotide sequence similarities of ITS1 and ITS2 fragments in *rDNA* gene, BARGUES et al. (2001, 2003, 2006) maintained that they could not be treated as separate species. Nevertheless, taking into account the differences in their male genitalia (JACKIEWICZ 1993, 1998a, 2000), they should be regarded as two subspecies, namely *S. palustris palustris* and *S. p. turricula* (BARGUES et al. 2001, 2003). Our earlier studies (RYBSKA et al. 2000a, b, 2008, WESOŁOWSKA 2010) were not decisive and confirmed a close relationship between these two taxa. However, with our new molecular and chorological data (preliminarily presented in PIEŃKOWSKA et al. 2008a, b and BANASIAK 2011 as well as in RYBSKA & LESICKI 2005, respectively) we decided to verify the opposite view – that the two taxa are two distinct but closely related species, i.e. as they were treated by JACKIEWICZ (1959, 1989, 1993, 1998a, 2000).

## MATERIAL AND METHODS

Snails were collected in drainage ditches, small pools, ponds, lakes, streams or small rivers, with a sieve (frequently mounted on a two-metre long boom) or a dredge, and hand-collected from plants, stones, or tree branches pulled out of the water. The list of 25 localities in Poland where specimens for molecular studies were collected is presented in Appendix 1. The lymnaeids caught in the field were kept in containers with aerated water of room temperature (21–24°C). They were sporadically fed with lettuce leaves.

The lymnaeid species were indentified on the basis of morphological and anatomical features (JACKIEWICZ 1998a, 2000, GLÖER & MEIER-BROOK 2003, KRUGLOV 2005). DNA was extracted from the foot tissue of each specimen. The rest of the body (including reproductive system) of each specimen was preserved in 75% ethyl alcohol. Total genomic DNA was extracted with DNeasy Tissue Kit (Qiagen) according to manufacturer’s procedure. Amplifications of 3'-end of the cytochrome oxidase subunit I (3'*COI*) mitochondrial gene were performed, according to Qiagen procedure, in the reaction mixture composed of 0.2 unit of Taq polymerase, 0.5  $\mu$ M of each primer,

and 0.2 mM of each dNTP in a final volume of 10  $\mu$ l. Amplifications of 5'-end of the same gene (5'*COI*) were accomplished in 25  $\mu$ l volume following modified protocol prepared by Biodiversity Institute of Ontario for Consortium for the Barcode of Life ([http://barcoding.si.edu/PDF/Protocols\\_for\\_High\\_Volume\\_DNA\\_Barcode\\_Analysis.pdf](http://barcoding.si.edu/PDF/Protocols_for_High_Volume_DNA_Barcode_Analysis.pdf)). Amplifications of ITS2 sequence were carried out in a 10  $\mu$ l volume with 1.25 unit of Taq polymerase, 1  $\mu$ M of each primer, and 0.2 mM of each dNTP. The PCR reactions were performed with the use of the following primers: CO1PU (5'-TTTTTGGGCATCCTGAGGTTAT-3') and CO1PL (5'-TAAAGAAAGAACATAATGAAAATG-3') (BOWLES et al. 1992, STOTHARD & ROLLINSON 1997) for the 3'*COI*, bcsmF1 5'-AAYCATAAAGA YATTGGDACWTTDTA-3' and bcsmR1 5'-TAWACYT CWGGRTGACCAAAAAAYCA-3' (PROČKÓW et al. 2013, the nucleotides and ambiguity codes were determined according to IUPAC) for 5'*COI* as well as NEWS2 5'-TGTGTCGATGAAGAACGCAG and ITS2-RIXO 5'-TTCTATGCTTAAATTCAGGGG (ALMEYDA-ARTIGAS et al. 2000) for ITS2. The PCR conditions were as follows: 3'*COI* – initial denaturation step of 5 min at 95°C, followed by 30 cycles of 30 sec at 94°C



(denaturation), 30 sec at 54°C (hybridisation), 30 sec at 72°C (elongation), and final incubation of 5 min at 72°C; 5'COI – 3 min at 96°C followed by 6 cycles of 1 min at 96°C, 30 sec at 45°C, and 1 min 15 sec at 72°C followed by 36 cycles of 1 min at 96°C, 30 sec at 51°C, and 1 min 15 sec at 72°C, and final elongation of 15 min at 72°C; ITS2 – 2 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C followed by final elongation of 7 min at 72°C. The PCR products were visualised on 1% agarose gels and sequenced on Applied Biosystems Hitachi 3130xl Genetic Analyser automated sequencer.

Full-length sequences were edited by eye using the programme BioEdit, version 7.0.5. (HALL 1999) and aligned using Prank (LÖYTYNOJA & GOLDMAN 2008) for COI, or CLUSTAL W (THOMPSON et al. 1994) for ITS2. The COI sequences were aligned according to the translated amino acid sequences. The ends of all sequences were trimmed. The length of the sequences after cutting were 354 bp, 558 bp, 421-447 bp for 3'COI, 5'COI and ITS2, respectively. Gaps and ambiguous positions were removed from alignments. The sequences were collapsed to hap-

lotypes (3'COI and 5'COI) or consensus sequences (ITS2) prior to phylogenetic analysis.

The sequences were analysed using the Neighbour-Joining method implemented in MEGA4 (TAMURA et al. 2007) using the Kimura two-parameter model (K2P) (KIMURA 1980) for pairwise distance calculations for COI, or Maximum Composite Likelihood (MCL) method (TAMURA et al. 2004, 2007) for ITS2. NJ tree branches were supported by bootstrap analysis with 1,000 replicates (FELSENSTEIN 1985, SAITOU & NEI 1987).

All the sequences have been deposited in GenBank under accession numbers KP070769-KP070807. The following ITS2 sequences from GenBank were also used: AJ319616 for *L. stagnalis*, AJ319618, AJ319619, AJ457043 for *S. turricula*, AJ319620 for *S. palustris*, AJ319625 for *S. corvus*, and AJ457042 for *L. terebra* (BARGUES et al. 2001, 2006). The following *Planorbarius corneus* sequences from GenBank were used in NJ trees as outgroups: FR797858 and AY577512 for 5'COI and 3'COI, respectively (JØRGENSEN et al. 2004, VINARSKI et al. 2011).

## RESULTS

### MOLECULAR ANALYSES

Twenty seven different sequences of mitochondrial 5'COI gene fragments were deposited in GenBank (Table 1). Analysed by the NJ method, the 5'COI se-

quences were arranged in a dendrogram in five clusters representing five lymnaeid species (Fig. 1). Five haplotypes (5'COI 1 – 5'COI 5) were found among the specimens anatomically identified as *S. palustris*, three (5'COI 6 – 5'COI 8) – as *S. turricula*, three

Table 1. 5'COI haplotypes found in specimens of five lymnaeid species (locality numbers according to Appendix 1, number of specimens in parentheses)

5'COI haplotypes	GenBank ##	localities (specimens)	5'COI haplotypes	GenBank ##	localities (specimens)
<i>Stagnicola palustris</i>			<i>Lymnaea stagnalis</i>		
5'COI 1	KP070773	2 (1)	5'COI 12	KP070784	12 (1)
5'COI 2	KP070774	4 (2)	5'COI 13	KP070785	13 (1)
5'COI 3	KP070775	1 (2), 4 (1)	5'COI 14	KP070786	19 (1), 21 (1)
5'COI 4	KP070776	5 (1)	5'COI 15	KP070787	13 (1)
5'COI 5	KP070777	2 (4)	5'COI 16	KP070788	22 (4)
<i>Stagnicola turricula</i>			5'COI 17	KP070789	23 (1)
5'COI 6	KP070778	11 (2)	5'COI 18	KP070790	22 (1)
5'COI 7	KP070779	9 (1)	5'COI 19	KP070791	14 (1), 16 (1), 17 (1)
5'COI 8	KP070780	10 (2)	5'COI 20	KP070792	15 (1)
<i>Stagnicola corvus</i>			5'COI 21	KP070793	21 (1), 22 (2)
5'COI 9	KP070781	3 (1), 9 (4)	5'COI 22	KP070794	18 (1)
5'COI 10	KP070782	9 (1)	5'COI 23	KP070795	24 (3)
5'COI 11	KP070783	9 (1)	<i>Ladislavella terebra</i>		
			5'COI 24	KP070796	25 (2)
			5'COI 25	KP070797	25 (2)
			5'COI 26	KP070798	25 (1)
			5'COI 27	KP070799	25 (1)

(5'COI 9 – 5'COI 11) – as *S. corvus*, twelve (5'COI 12 – 5'COI 23) – as *L. stagnalis* and four (5'COI 24 – 5'COI 27) – as *L. terebra*.

The mean values of intraspecific variation between the sequences of 5'COI gene fragments characteristic of particular species, expressed as genetic K2P distances, were 0.3% for *S. corvus* and *L. terebra*, 0.7% for *S. turricula* and for *L. stagnalis*, and 0.9% for *S. palustris*,

whereas the genetic K2P distances between the species were higher (Table 2). The smallest distances between the sequences of 5'COI gene fragments distinguished *S. palustris* and *S. turricula* (mean 2.6%). The mean K2P distances between the sequences of *S. palustris* and the other species were 6.4%, 15.3%, and 21.1% for *S. corvus*, *L. stagnalis*, and *L. terebra*, respectively. Similarly, the mean distances which dis-

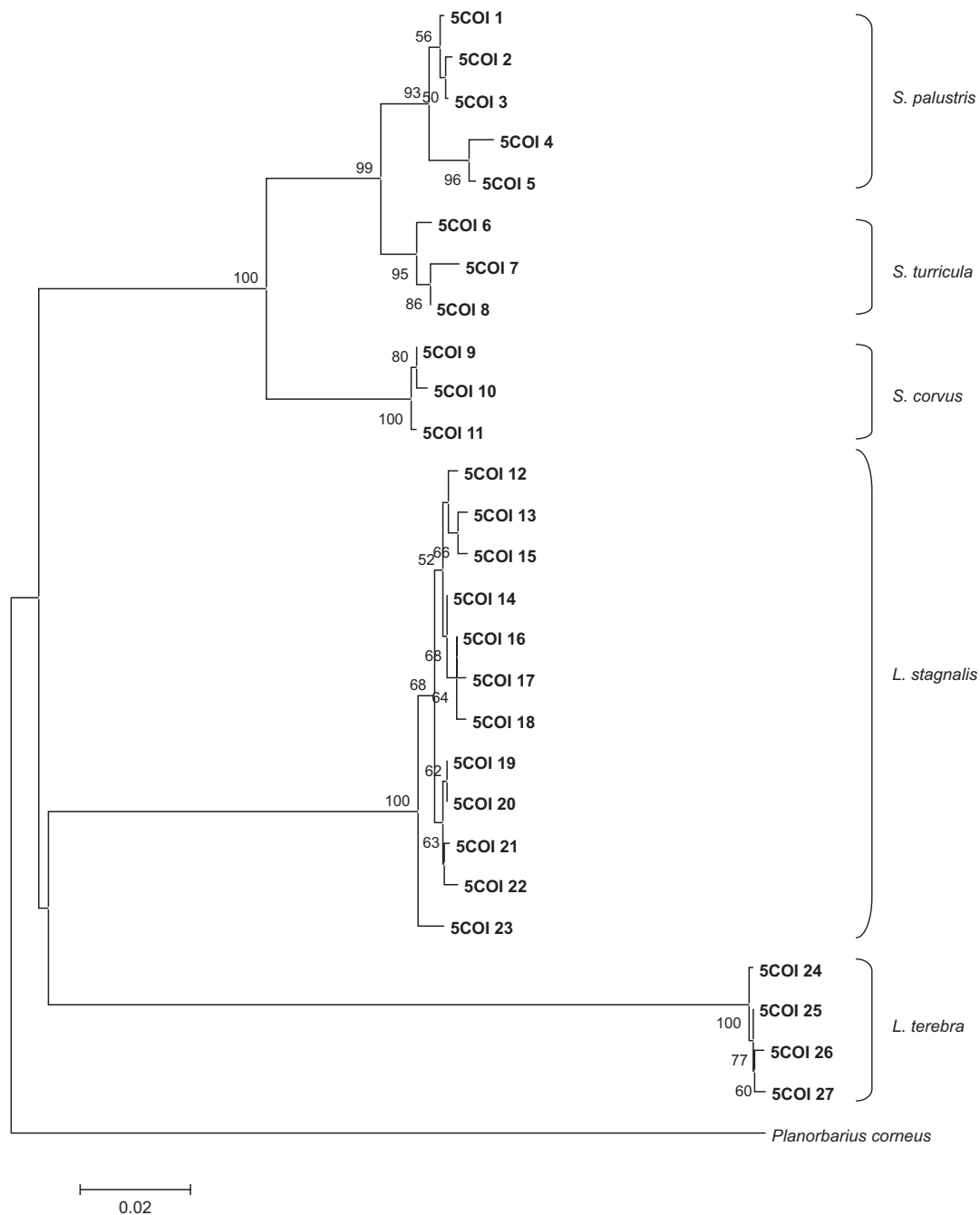


Fig. 1. Neighbour-Joining tree based on 558-nt-long fragment of 5'COI sequences of five lymnaeid species: *Stagnicola palustris*, *S. turricula*, *S. corvus*, *Lymnaea stagnalis* and *Ladislavella terebra*. 5'COI sequence of *Planorbarius corneus* (FR797858) was used as outgroup. Figures at the nodes indicate bootstrap support from 1,000 replicates. Bootstrap values below 50 not shown. Evolutionary distances computed using Kimura two-parameter method, expressed as number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from dataset (complete deletion option).





Table 2. Ranges of K2P genetic distances for 5'COI sequences (mean values in parentheses)

Comparison	5'-COI (%)
Within <i>S. palustris</i>	0.2–1.5 (0.93)
Within <i>S. turricula</i>	0.5–1.1 (0.70)
Within <i>S. corvus</i>	0.2–0.4 (0.27)
Within <i>L. stagnalis</i>	0.0–1.5 (0.72)
Within <i>L. terebra</i>	0.2–0.4 (0.30)
Between <i>S. palustris</i> and <i>S. turricula</i>	2.2–3.3 (2.60)
Between <i>S. palustris</i> and <i>S. corvus</i>	5.6–7.4 (6.39)
Between <i>S. palustris</i> and <i>L. stagnalis</i>	14.4–16.2 (15.30)
Between <i>S. palustris</i> and <i>L. terebra</i>	20.3–21.8 (21.10)
Between <i>S. turricula</i> and <i>S. corvus</i>	5.6–6.2 (5.87)
Between <i>S. turricula</i> and <i>L. stagnalis</i>	14.0–15.5 (14.71)
Between <i>S. turricula</i> and <i>L. terebra</i>	21.1–22.0 (21.60)
Between <i>S. corvus</i> and <i>L. stagnalis</i>	14.0–15.1 (14.52)
Between <i>S. corvus</i> and <i>L. terebra</i>	19.0–19.9 (19.49)
Between <i>L. stagnalis</i> and <i>L. terebra</i>	19.4–20.8 (20.24)

tinguished *S. turricula* from *S. corvus*, *L. stagnalis* and *L. terebra* were 5.9%, 14.7% and 21.6%, respectively. The mean values of K2P distances between *S. corvus* vs. *L. stagnalis* and *S. corvus* vs. *L. terebra* were 14.5% and 19.5%, respectively. The mean K2P distance between the 5'COI sequences for *L. stagnalis* and *L. terebra* was 20.2%.

Surprisingly, the sequences of 3'COI gene fragments did not provide resolution between species. A total of six 3'COI haplotypes were deposited in GenBank (Table 3): one (3'COI 5) was found in specimens anatomically identified as representing three different species: *S. corvus*, *L. stagnalis* and *L. terebra*, two other were unique for *S. corvus* and *L. terebra* (3'COI 4 and 3'COI 6, respectively) (Table 3). In general, these three sequences were very similar. Moreover, we found two unique sequences of the 3'COI gene fragment among specimens anatomically

Table 3. 3'COI haplotypes found in specimens of five lymnaeid species (locality numbers according to Appendix 1, number of specimens in parentheses)

3'COI haplotypes	GenBank ##	localities (specimens)
<i>Stagnicola palustris</i>		
3'COI 1	KP070800	6 (1), 7 (1), 8 (1)
3'COI 2	KP070801	1 (1), 2 (1), 3 (1)
<i>Stagnicola turricula</i>		
3'COI 3	KP070802	11 (1)
<i>Stagnicola corvus</i>		
3'COI 4	KP070803	9 (3)
<i>Stagnicola corvus</i> , <i>Lymnaea stagnalis</i> , <i>Ladislavella terebra</i>		
3'COI 5	KP070804	<i>S. c.</i> 3 (2), 9 (1)
	KP070805	<i>L. s.</i> 2 (1), 20 (2)
	KP070806	<i>L. t.</i> 25 (2)
<i>Ladislavella terebra</i>		
3'COI 6	KP070807	25 (1)

identified as *S. palustris* (3'COI 1 and 3'COI 2) and one characteristic of *S. turricula* only (3'COI 3). The dendrogram built with the use of NJ method included three groups of sequences – the first for the sequences of *S. corvus*, *L. stagnalis* and *L. terebra*, the second for the sequences of *S. palustris* and the third for the sequence of *S. turricula* (Fig. 2).

To confirm our identification of the species of *Stagnicola* s.l. we amplified their DNA to obtain sequences of the ITS2 fragment of nuclear *rDNA* gene. We obtained four sequences, deposited in GenBank, one for each: *S. palustris*, *S. turricula*, *S. corvus* and *L. terebra* (Table 4). The sequence identified for the specimens of *S. palustris* (ITS2 1) was identical (MCL distance equal to 0.0%) with the appropriate sequence AJ319620 deposited in GenBank for this species (BARGUES et al. 2001). The sequence found for *S. turricula* (ITS2 2) differed in one indel and one, two or

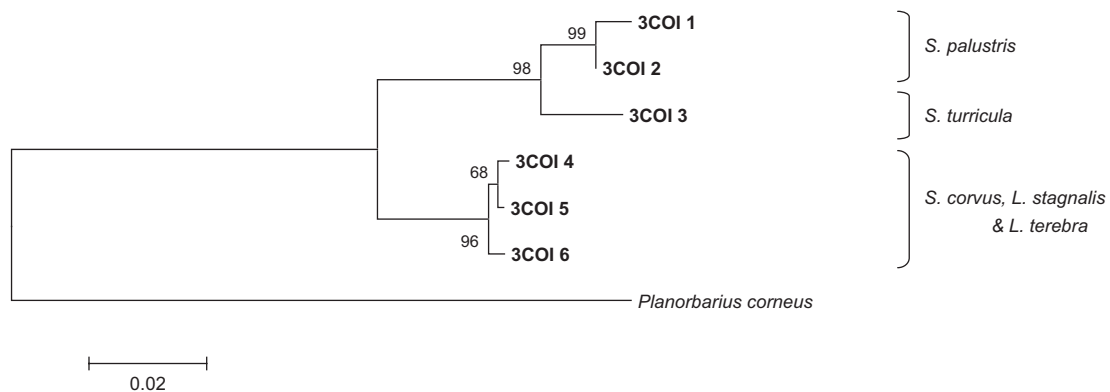


Fig. 2. Neighbour-Joining tree based on 354-nt-long fragment of 3'COI sequences of five lymnaeid species: *Stagnicola palustris*, *S. turricula*, *S. corvus*, *Lymnaea stagnalis* and *Ladislavella terebra*. 3'COI sequence of *Planorbarius corneus* (AY577512) was used as outgroup. Figures at the nodes indicate bootstrap support from 1,000 replicates. Bootstrap values below 50 not shown. Calculation parameters same as for Fig. 1.

Table 4. ITS2 sequences found in specimens of four lymnaeid species (locality numbers according to Appendix 1, number of specimens in parentheses)

ITS2 haplotypes	GenBank ##	localities (specimens)
<i>Stagnicola palustris</i>		
ITS2 1	KP070769	1 (3)
<i>Stagnicola turricula</i>		
ITS2 2	KP070770	9 (1)
<i>Stagnicola corvus</i>		
ITS2 3	KP070771	9 (2)
<i>Ladislavella terebra</i>		
ITS2 4	KP070772	25 (2)

three nucleotide positions, compared to the sequences AJ457043, AJ319618 and AJ319619 (deposited in GenBank by BARGUES et. al. 2001, 2003; MCL=0.0–0.7%), respectively. The sequence ITS2 3 identified for *S. corvus* differed in one indel from the sequence AJ319625 (MCL=0.0%) deposited in GenBank for this species (BARGUES et. al. 2001) and – finally – one indel was found between the sequence ITS2 4 of *L. terebra* and the sequence AJ457042 (of BARGUES et. al. 2003; MCL=0.0%). These similarities are also visible in the NJ dendrogram (Fig. 3). For comparison we added the sequence AJ319616 obtained for *L. stagnalis* from GenBank (BARGUES et. al. 2001). Our sequences grouped together with those found by BARGUES et al. (2001, 2003): ITS2 1 with AJ319620 (*S. palustris*), ITS2 2 with AJ457043, AJ319618 and AJ319619 (*S.*

*turricula*), ITS2 3 with AJ319625 (*S. corvus*), ITS2 4 with AJ457042 (*L. terebra*), leaving AJ319616 as the fifth independent group (*L. stagnalis*).

#### DISTRIBUTION OF *STAGNICOLA PALUSTRIS* AND *S. TURRICULA* IN POLAND

Chorological data on the distribution of *S. palustris* and *S. turricula* in Poland are presented in Fig. 4 (with additional data shown in Appendix 2). There are localities of *S. palustris* and *S. turricula* which were published in several papers and those found in our own studies. JACKIEWICZ's (1988b, 1992b, 1998a, 2000) older papers showed that *S. palustris* occurred in northern, central and western parts of Poland while *S. turricula* was found farther to south-east. KORALEWSKA-BATURA (1992) mentioned 241 localities of *S. palustris* in Wielkopolska (W Poland). This distribution was confirmed by more recent publications for *S. palustris* (LEWIN & SMOLIŃSKI 2006, NOWAK & ŻBIKOWSKA 2009, JAKUBIK & LEWANDOWSKI 2011, CICHY 2013, PIECHOCKI & SZLAUER-ŁUKASZEWSKA 2013) and *S. turricula* (LEWIN & CEBULA 2002, 2003) as well as by our own field studies in which we found *S. palustris* at several localities in western, south-western or central Poland (see: Fig. 4, Appendix 2: localities nos 3, 8–14). On the other hand, we also confirmed a locality of *S. turricula* in the Bieszczady Mts (locality no. 30). Unexpectedly, we also found six new localities of *S. palustris* in south-eastern Poland (Fig. 4,

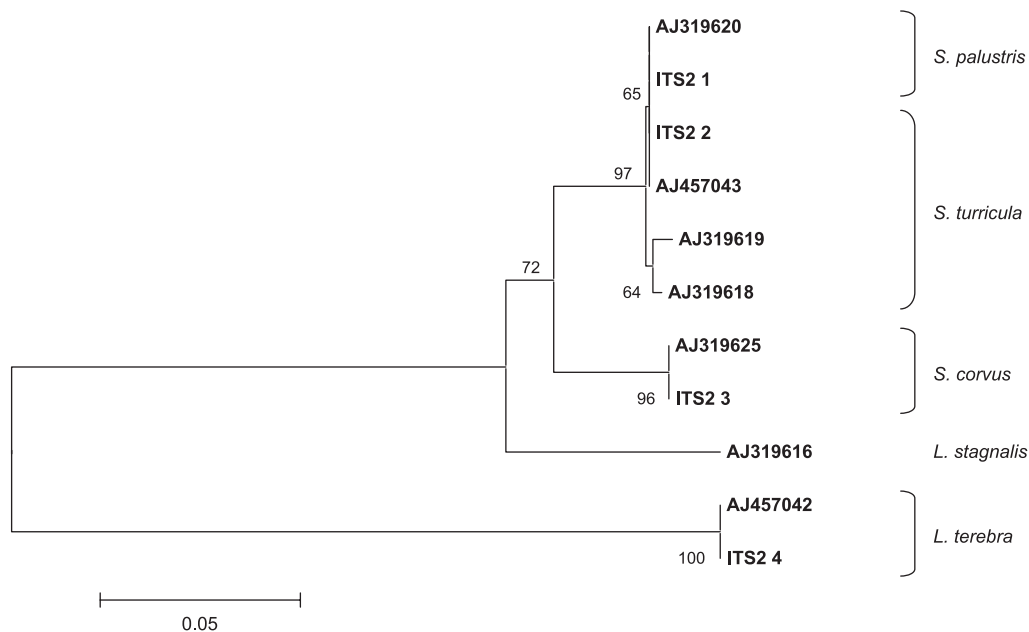


Fig. 3. Neighbour-Joining tree for five lymnaeid species using ITS2 fragment. Percentage of replicate trees in which the associated taxa clustered together in bootstrap test (1,000 replicates) shown next to the branches. Bootstrap values below 50 not shown. Evolutionary distances computed using Maximum Composite Likelihood method, expressed as number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

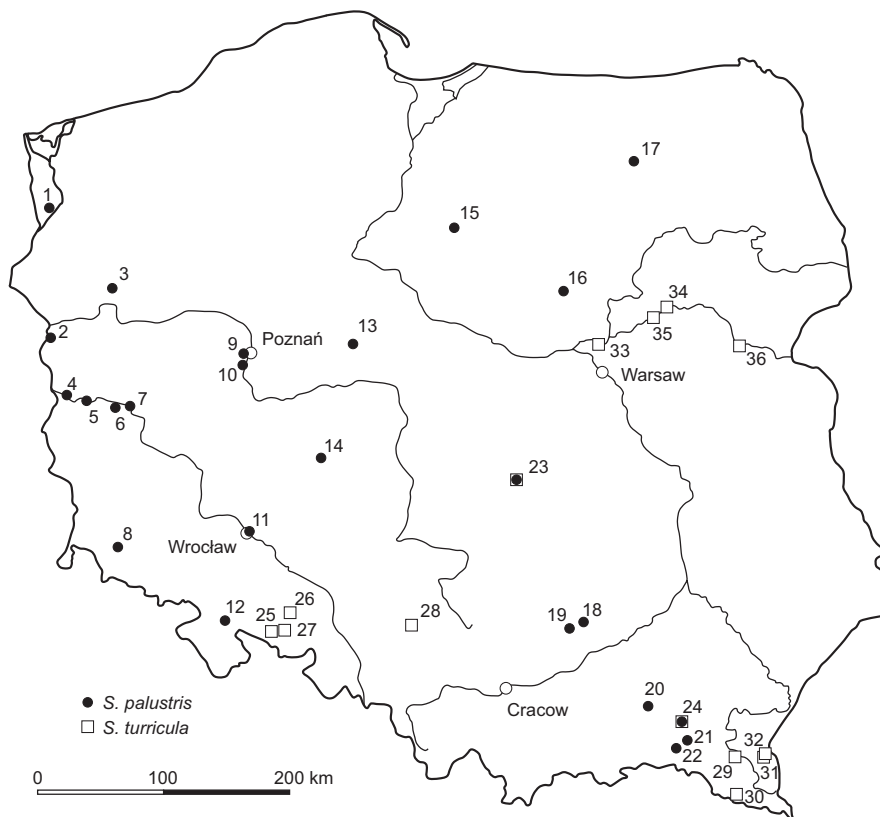


Fig. 4. Distribution of *Stagnicola palustris* and *S. turricula* in Poland. For locality details see [Appendix 2](#)

[Appendix 2](#): localities nos 18-22 and 24). Some new localities of *S. turricula* located much farther to the west and north were published recently ([PIECHOCKI 2000](#), [JURKIEWICZ-KARNKOWSKA 2004, 2006, 2008](#),

[SKOWROŃSKA-OCHMANN et al. 2012](#)). We can also add a new record of *S. turricula* at Niwnica (Silesia), i.e. in south-western Poland ([Fig. 4](#), [Appendix 2](#): locality no. 27).

## DISCUSSION

Distinctness of *L. stagnalis* (L.), *S. corvus* (Gmel.) and *L. terebra* (Westerlund) was well established on the basis of the conchological, other morphological and reproductive system features ([JACKIEWICZ 1993, 1998a, 2000](#), [FALKNER 1995, FALKNER et al. 2001, GLÖER 2002, GLÖER & MEIER-BROOK 2003](#)) as well as on the molecular features found in the nucleotide sequences of ITS1 and ITS2 fragments in *rDNA* gene ([BARGUES et al. 2001, 2003, 2006](#)) and obtained with the use of RAPD technique ([RYBSKA et al. 2000a, b, 2008](#)). The distinct species status of these three taxa is confirmed by the results of this study. In our analyses we used molecular markers frequently applied for taxonomic or phylogenetic studies, i.e. sequences of mitochondrial 3'COI ([LOAIZA et al. 2013, ZEIN-EDDINE et al. 2014](#)) and 5'COI ([REMIGIO & HEBERT 2003, RUNDELL et al. 2004, PEREZ et al. 2005, KANE et al. 2008, FALNIOWSKI et al. 2009, FALNIOWSKI & SZAROWSKA 2011, SZAROWSKA et al. 2013, 2014](#)) gene fragments as well as nuclear ITS2 fragment of *rDNA* gene ([BARGUES & MAS-COMA 2005, KÄLLERSJÖ et al. 2005, PUSLEDNIK et al. 2009, BARGUES et al.](#)

[2012, LOAIZA et al. 2013](#)). The nucleotide sequences of mitochondrial 5'COI gene ("barcode sequence") differentiate between the above three lymnaeids at the level of 14.0–20.8%. These genetic distances are much greater than the threshold established by [HEBERT et al. \(2003, 2004a, b\)](#) in whose opinion a difference exceeding 2% may be treated as the sign of separateness at the species level. Short 3'COI gene fragments are rather useless for the separation of these three species although they were useful in the study on cryptic species of *Anopheles* ([LOAIZA et al. 2013](#)) and species identification of some *Bulinus* ([ZEIN-EDDINE et al. 2013](#)). These sequences are very similar (0.0–0.6%) for *S. corvus*, *L. stagnalis* and *L. terebra*. On the other hand the three species differ in their nucleotide sequences of ITS2 fragment, as was previously shown by [BARGUES et al. \(2001, 2003\)](#).

Also *S. palustris* (O.F. Müll.) and *S. turricula* (Held) are the taxa well separated from the three above species, as the genetic distances of their nucleotide sequences of the 5'-end of COI gene are between 5.6 and 22.0%. The distinctness of *S. palustris/turricula* was

established in earlier molecular analyses (BARGUES et al. 2001, 2003, 2006, RYBSKA et al. 2000a, 2008). However, the separateness between *S. palustris* and *S. turricula* is under debate (BARGUES et al. 2011: table S1). They differ only in 3 or 2–3 nucleotides in the sequences of ITS1 (BARGUES et al. 2006) or ITS2 (BARGUES et al. 2001, 2003) fragments, respectively, while the difference in comparison with other representatives of *Stagnicola* (s.l.) are 19–42 nucleotides. Similar small differences were also found in our analyses of the ITS2 sequences of *S. palustris* and *S. turricula* (they differ in one indel only). In the RAPD fragments *S. palustris* and *S. turricula* differed at the level of 12–18% while each of these two taxa differed from the other Polish *Stagnicola* species at the level of 43–65% (RYBSKA et al. 2008). However, it must be stressed that interpopulation genetic variation within each of these species was by one order of magnitude smaller (3.1–4.4% between populations of *S. palustris*, 3.9–5.9% between populations of *S. turricula*) (RYBSKA et al. 2008). Our results on 5'COI sequences shed new light on the taxonomic status of *S. palustris* and *S. turricula*. The genetic distances between *S. palustris* and *S. turricula* in these sequences vary from 2.2 to 3.3%. Such differences are greater than interpopulation 5'COI differentiation (0.9% and 0.7% for *S. palustris* and *S. turricula*, respectively). Moreover, the sequences of 3'COI gene fragments of *S. palustris* and *S. turricula* are similar but distinctly different.

It should be stressed that the genetic difference between *S. palustris* and *S. turricula* in the “barcode sequence” is also greater than the above-mentioned 2% threshold of HEBERT et al. (2003, 2004a, b). Probably the low 5'COI divergence between *S. palustris* and *S. turricula*, which is much smaller than the divergences between each of these two taxa and each of the other three lymnaeid species studied in this paper, may suggest their recent origin. The conclusion that *S. palustris* and *S. turricula* are closely related is supported by the fact that the differences in the mitochondrial gene (5'COI, 3'COI) are larger than those in the nuclear (ITS2) gene fragment. Mitochondrial genome evolves much faster than the nuclear one (REMIGIO & HEBERT 2003). Therefore analyses of ITS2 sequences can be useless when distinguishing between closely related species which was demonstrated in the earlier studies on *Trochulus hispidus* species complex (KRUCKENHAUSER et al. 2014) and *Monacha claustralis* vs. *M. cartusiana* identification (PIEŃKOWSKA et al. 2015). On the other hand, ITS2 sequences are very useful for analyses of phylogenetic relationships between more distant taxa (BARGUES & MAS-COMA 2005, KÄLLERSJÖ et al. 2005, BARGUES et al. 2012, LOAIZA et al. 2013).

We are aware that taxonomic decisions based on molecular features only are precarious as they are based on arbitrarily adopted rules. Therefore we

tried to use also characters used in classical taxonomy which is often practiced by other researchers (e.g. SZAROWSKA 2006, HAASE et al. 2007, FALNIOWSKI et al. 2009, PUSLEDNIK et al. 2009, FALNIOWSKI & SZAROWSKA 2011, SZAROWSKA et al. 2013, 2014). Each specimen taken for molecular studies was identified on the basis of its genital anatomy (according to JACKIEWICZ 1998a, 2000 and GLÖER & MEIER-BROOK 2003).

JACKIEWICZ (1993, 1998a, 2000) showed distinct differences in the structure of male genitalia. The praepitium : penis sheath length ratio was 1 : 1 for *S. palustris* and 1 : 3 to 1 : 5 in *S. turricula* (see e.g. JACKIEWICZ 1998a: fig. 68 for *S. palustris* and fig. 70 for *S. turricula*). Consequently, JACKIEWICZ (1993, 1998a, 2000) treated *S. palustris* and *S. turricula* as two distinct species. However, GARBAR (2001) and KRUGLOV (2005) did not confirm this difference in the male genitalia between *S. palustris* and *S. turricula*. They showed several other differences in their anatomy, and placed them in two different sections of *Stagnicola* – *S. palustris* in *Stagnicola* s. str., *S. turricula* in *Fenziana* Servain, 1881. According to VINARSKI et al. (2011) “*S. palustris* and *S. turricula* were obviously distinct by their morphological traits, but were shown to be almost indistinguishable with the genetic markers”. The differences in male genitalia prompted BARGUES et al. (2001, 2003, 2006) to treat them as two subspecies, *S. palustris palustris* and *S. p. turricula*.

GARBAR et al. (2004), studying karyotypes of Ukrainian representatives of Lymnaeidae, found that *S. palustris* and *S. turricula* had the same number of chromosomes ( $n=36$ ) however they differed in the chromosome types (14 matecentric, 10 submetacentric, 12 subtelocentric for *S. palustris* vs. 12 metacentric, 8 submetacentric, 16 subtelocentric for *S. turricula*). Although we cannot confirm by molecular features that *S. turricula* from Ukraine is conspecific with *S. turricula* from Poland, their localities (especially those in the Bieszczady Mts, Poland) are not very far apart.

According to JACKIEWICZ (1988b, 1998a, 2000) *S. palustris* and *S. turricula* were differently distributed in Poland: the former was a northern species common in the lowland (west-northern) part of Poland while the latter lived in the south-eastern part of the country (Bieszczady Mts). Similarly in Germany – *S. palustris* lives rather in the northern part, *S. turricula* – in the Danube region of southern Germany (GLÖER & MEIER-BROOK 2003). JACKIEWICZ (1998a, 2000) stated that she did not find *S. palustris* in the former Czechoslovakia, similarly older Czech papers (JUŘIČKOVÁ et al. 2001, BERAN 2002) listed only *S. turricula* for the Czech Republic, but not *S. palustris*. Recently ČEJKA et al. (2007) and BERAN (2008) listed *S. palustris* only for Slovakia and the Czech Republic, respectively, however they stated that their reports concerned *S. turricula* sensu BARGUES et al. (2001).





HORSÁK et al. (2013) listed *S. palustris* among Czech and Slovak molluscs only, but they synonymised it with *S. turricula*, listing features treated by JACKIEWICZ (1993, 1998a) as characteristic of *S. turricula* both in the text and in the figures (see: HORSÁK et al. 2013: p. 49, fig. 9; the figure taken from JACKIEWICZ'S (1988b, 1993, 1998a, 2000) papers). JACKIEWICZ (1998a, 2000) reported the occurrence of *S. turricula* in Hungary and Bulgaria. The above allopatric distribution supported the subspecies status of *S. palustris* and *S. turricula* established by BARGUES et al. (2001, 2003, 2006). However we found some localities of *S. palustris* in south-eastern Poland and some localities of *S. turricula* much farther west- and north. It should be stressed that PIECHOCKI (1972, 2000) found both species at one locality in the Niebieskie Źródła Nature Reserve near Tomaszów Mazowiecki (Central Poland), identifying individuals based on their anatomical features. We also found *S. palustris* near Odrzykoń where *S. turricula* was reported earlier (identified anatomically, see LEWIN & CEBULA 2003: fig. 3). It proves that *S. palustris* and *S. turricula* occur sympatrically. They co-occur in Hungary (KILIAS 1992, JACKIEWICZ 1996), Ukraine (GARBAR 2001, GARBAR et al. 2004, KRUGLOV 2005), Eastern Europe (NEKHAEV 2011) and Bulgaria (GEORGIEV & HUBENOV 2013).

Sympatric occurrence of two taxa excludes their subspecific status within one species. Subspecies of the same species are by definition allopatric. Therefore, taking into account the well established

differences in the male genitalia and the very small but clearly recognisable differences in the molecular features, combined with the sympatric distribution, we conclude that *S. palustris* and *S. turricula* should be treated as two distinct species.

Finally, it is noteworthy that our results of the comparison of the 5'COI gene fragment sequences support FALKNER'S (1995) opinion that *S. corvus* should be included with *S. palustris* and *S. turricula* in the genus *Stagnicola* Jeffreys, 1830, leaving *L. stagnalis* within the genus *Lymnaea* Lamarck, 1799. The genetic distances between *L. terebra* and all other species (19.0–22.0%) also suggest that it was reasonable to create a new genus *Catascopia* Meier-Brook et BARGUES, 2002 (= *Ladislavella* B. Dybowski, 1913) to accommodate *G. occulta* Jackiewicz, 1959 (MEIER-BROOK & BARGUES 2002, VINARSKI 2012).

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## Appendix 1. Localities where lymnaeids for molecular studies were collected

Locality No.	Locality coordinates	Species	Remarks
1.	lake Mrowinko, Santoczno near Gorzów Wlkp., W. Poland 52°51'15"N, 15°19'38"E	<i>S. palustris</i>	<i>S. p.</i> 3 specimens, leg. E. RYBSKA
2.	ponds at Morasko near Poznań, W. Poland 52°28'14"N, 16°55'42"E	<i>S. palustris</i> <i>L. stagnalis</i>	<i>S. p.</i> 23 specimens, leg. A. LESICKI; 1 specimen, leg. E. RYBSKA; 4 specimens, leg. J. PIEŃKOWSKA <i>L. s.</i> 1 specimen, leg. E. RYBSKA
3.	water bodies near railroad close to Kocie doły, Luboń, W. Poland 52°19'49"N, 16°53'30"E	<i>S. palustris</i> <i>S. corvus</i>	<i>S. p.</i> 3 specimens, leg. E. RYBSKA <i>S. c.</i> 7 specimens, leg. E. RYBSKA
4.	lake Wilczyńskie, near Konin, Central Poland 52°29'03"N, 18°07'24"E	<i>S. palustris</i>	<i>S. p.</i> 3 specimens, leg. E. RYBSKA
5.	lake Wysokie Brodno, near Brodnica, Central Poland 53°18'08"N, 19°21'59"E	<i>S. palustris</i>	<i>S. p.</i> 1 specimen, leg. E. ŻBIKOWSKA
6.	pond at Busko Zdrój, S.E. Poland 50°28'32"N, 20°43'27"E	<i>S. palustris</i>	<i>S. p.</i> 1 specimen, leg. E. RYBSKA
7.	backwaters of Nida, Chroberz, S.E. Poland 50°26'00"N, 20°33'56"E	<i>S. palustris</i>	<i>S. p.</i> 1 specimen, leg. E. RYBSKA
8.	water bodies near Odrzykoń, S.E. Poland 49°44'44"N, 21°45'19"E	<i>S. palustris</i>	<i>S. p.</i> 1 specimen, leg. E. RYBSKA
9.	stream Niwnica, around Nysa, S.W. Poland 50°26'50"N, 17°23'43"E	<i>S. turricula</i> <i>S. corvus</i>	<i>S. t.</i> 1 specimen, leg. E. RYBSKA <i>S. c.</i> 2 specimens, leg. E. RYBSKA
10.	anthropogenic reservoirs, near Pniowiec River, Tarnowskie Góry, S.W. Poland 50°29'06"N, 18°48'40"E	<i>S. turricula</i>	<i>S. t.</i> 2 specimens, leg. K. SKOWROŃSKA-OCHMANN
11.	private pond, near Krościenko-Ustrzyki Dolne road, S.E. Poland 49°28'15"N, 22°38'53"E	<i>S. turricula</i>	<i>S. t.</i> 2 specimens, leg. E. RYBSKA
12.	Bystrzyca River, Spiczyn, S.E. Poland 51°20'29"N, 22°44'51"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. A. RYBAK
13.	oxbow, Szczekarków, S.E. Poland 51°14'45"N, 21°51'40"E	<i>L. stagnalis</i>	<i>L. s.</i> 2 specimens, leg. A. RYBAK
14.	Wieprz River, Nowogród, S.E. Poland 51°19'21"N, 22°48'26"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. A. RYBAK
15.	Wieprz River, Kijany, near Lublin, S.E. Poland 51°20'20"N, 22°46'17"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. A. RYBAK
16.	Wieprz River, Ziółków, S.E. Poland 51°19'40"N, 22°47'52"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. E. RYBSKA
17.	Rydzówka Lake, Masurian district, N.E. Poland 54°14'20"N, 21°34'13"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. A. ABRASZEWSKA
18.	Lake Niskie Brodno, near Brodnica, Central Poland 53°17'27"N, 19°22'28"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. E. ŻBIKOWSKA
19.	drainage ditch at Malenin, Central Poland 52°25'27"N, 17°45'02"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. J. R. PIEŃKOWSKA
20.	lake Rusałka, Poznań, Central Poland 52°25'33"N, 16°53'00"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. E. RYBSKA
21.	lake Bnińskie, Bnin near Poznań, Central Poland 52°13'21"N, 17°05'56"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. J. R. PIEŃKOWSKA
22.	lake Kórnickie, Kórnik near Poznań, Central Poland 52°14'05"N, 17°05'15"E	<i>L. stagnalis</i>	<i>L. s.</i> 5 specimens, leg. J. R. PIEŃKOWSKA
23.	Głuszyna River, Czaporzy near Poznań, Central Poland 52°19'12"N, 16°55'02"E	<i>L. stagnalis</i>	<i>L. s.</i> 1 specimen, leg. J. R. PIEŃKOWSKA
24.	pond, Niemcza, S.W. Poland 50°43'30"N, 16°49'32"E	<i>L. stagnalis</i>	<i>L. s.</i> 3 specimens, leg. J. R. PIEŃKOWSKA
25.	drainage ditch at Gorzykowo, near Września-Gniezno road, Central Poland 52°24'51"N, 17°43'15"E	<i>L. terebra</i>	<i>L. t.</i> 7 specimens, leg. E. RYBSKA

Appendix 2. Distribution of *Stagnicola palustris* and *S. turricula* in Poland, based on data from references and our studies

Locality No.	Locality coordinates	Species	References/remarks
1.	water bodies near Szczecin, N.W. Poland 53°24'N, 14°33'E (1)	<i>S. palustris</i>	JACKIEWICZ 1992b, 1998a, 2000
2.	Odra River, near Górzycza, W. Poland 52°29.2'N, 14°37.9'E (1)	<i>S. palustris</i>	PIECHOCKI & SZLAUER-ŁUKASZEWSKA 2013
3.	lake Mrowinko, Santoczno near Gorzów Wlkp., W. Poland 52°51'15"N, 15°19'38"E (2)	<i>S. palustris</i> (*)	new locality of <i>S. palustris</i>
4.	Odra River, near Połęcko, W. Poland 52°03.1'N, 14°53.5'E (1)	<i>S. palustris</i>	PIECHOCKI & SZLAUER-ŁUKASZEWSKA 2013
5.	Odra River, near Krosno Odrzańskie, W. Poland 52°03.1'N, 15°05.0'E (1)	<i>S. palustris</i>	PIECHOCKI & SZLAUER-ŁUKASZEWSKA 2013
6.	Odra River, near Brody, W. Poland 52°03.5'N, 15°25.6'E (1)	<i>S. palustris</i>	PIECHOCKI & SZLAUER-ŁUKASZEWSKA 2013
7.	Odra River, near Cigacice, W. Poland 52°01.8'N, 15°36.7'E (1)	<i>S. palustris</i>	PIECHOCKI & SZLAUER-ŁUKASZEWSKA 2013
8.	lake in Lubomierz, S.W. Poland 51°00'50"N, 15°30'19"E (2)	<i>S. palustris</i>	new locality of <i>S. palustris</i>
9.	ponds at Morasko near Poznań, W. Poland 52°28'14"N, 16°55'42"E (2)	<i>S. palustris</i> (*)	JACKIEWICZ 1992b, 1998a, 2000 locality confirmed in our studies
10.	Luboń, water bodies near railroad, close to Kocie Doły, W. Poland 52°19'49"N, 16°53'30"E (2)	<i>S. palustris</i> (*)	JACKIEWICZ 1992b, 1998a, locality confirmed in our studies
11.	Wrocław, water bodies in Pilczycki Forest, S.W. Poland 51°09'20"N, 16°58'11"E (2)	<i>S. palustris</i>	JACKIEWICZ 1998a, 2000 locality confirmed in our studies
12.	backwater around Bardo Śląskie, S.W. Poland 50°30'32"N, 16°43'28"E (2)	<i>S. palustris</i>	new locality of <i>S. palustris</i>
13.	lake Wilczyńskie, near Konin, Central Poland 52°29'03"N, 18°07'24"E (2)	<i>S. palustris</i> (*)	new locality of <i>S. palustris</i>
14.	pond near Ostrów Wlkp. Franklinów, Central Poland 51°40'36.2"N, 17°47'06.7"E (2)	<i>S. palustris</i>	JACKIEWICZ 1992b, 1998a, 2000 locality confirmed in our studies
15.	lake Wysokie Brodno, Central Poland 53°18'08.6"N, 19°21'59.0"E (2)	<i>S. palustris</i> (*)	NOWAK & ŻBIKOWSKA 2009 locality confirmed in our studies
16.	clay pit, 2 km from the centre of Ciechanów, Central Poland 52°50'N, 20°38'E (1)	<i>S. palustris</i>	LEWIN & SMOLIŃSKI 2006
17.	Krutynia River near Będany Lake (Bobrowko), N.E. Poland 53°44.5'N, 21°32.2'E (1)	<i>S. palustris</i>	JAKUBIK & LEWANDOWSKI 2011
18.	pond at Busko Zdrój, S.E. Poland 50°28'32"N, 20°43'27"E (2)	<i>S. palustris</i> (*)	new locality of <i>S. palustris</i>
19.	backwaters of Nida, Chroberz, S.E. Poland 50°26'00"N, 20°33'56"E (2)	<i>S. palustris</i> (*)	new locality of <i>S. palustris</i>
20.	pond, Klecie near Dębica, S.E. Poland 49°51'31"N, 21°24'21"E (2)	<i>S. palustris</i>	new locality of <i>S. palustris</i>
21.	pond in Iwonicz Zdrój, S.E. Poland 49°36'09"N, 21°48'03"E (2)	<i>S. palustris</i>	new locality of <i>S. palustris</i>
22.	reservoir near Jasiółka, Dukla, S.E. Poland 49°32'52"N, 21°41'14"E (2)	<i>S. palustris</i>	new locality of <i>S. palustris</i>
23.	water bodies of Niebieskie Źródła reserve, near Tomaszów Mazowiecki, Central Poland 51°30.7'N, 20°01.7'E (1)	<i>S. palustris</i> <i>S. turricula</i>	PIECHOCKI 1972, 2000
24.	water bodies near Odrzykoń, S.E. Poland 49°44'44"N, 21°45'19"E (2)	<i>S. palustris</i> (*) <i>S. turricula</i>	new locality of <i>S. palustris</i> <i>S. turricula</i> in LEWIN & CEBULA 2002, 2003

Locality No.	Locality coordinates	Species	References/remarks
25.	ponds in Brzezina Polska near Nysa, Silesia, S.W. Poland 50°26'N, 17°14'E (1)	<i>S. turricula</i>	JACKIEWICZ 1988b, 1998a, 2000
26.	Siedzina, near Nysa, S.W. Poland 50°34'N, 17°27'E (1)	<i>S. turricula</i>	BARGUES et al. 2003
27.	stream, Niwnica, around Nysa, S.W. Poland 50°26'50"N, 17°23'43"E (2)	<i>S. turricula</i> (*)	new locality of <i>S. turricula</i>
28.	anthropogenic reservoirs, near Pniowiec River, Tarnowskie Góry, S.W. Poland 50°29'06"N, 18°48'40"E (2)	<i>S. turricula</i> (*)	SKOWROŃSKA-OCHMAN et al. 2012 locality confirmed in our studies
29.	San near Lesko, S.E. Poland 49°28.5'N, 22°18.9'E (1)	<i>S. turricula</i>	JACKIEWICZ 1988b, 1992b
30.	stream Solinka, near Cisna, S.E. Poland 49°12.5'N, 22°19.0'E (1)	<i>S. turricula</i>	JACKIEWICZ 1988b, 1992b
31.	private pond, near Krościenko-Ustrzyki Dolne road, S.E. Poland 49°28'15"N, 22°38'53"E (2)	<i>S. turricula</i> (*)	JACKIEWICZ 1988b, 1992b locality confirmed in our studies
32.	ditch near Ustrzyki Dolne, S.E. Poland 49°27'N, 22°38'E (1)	<i>S. turricula</i>	JACKIEWICZ 1988b, 1992b
33.	Zegrzyński Reservoir (Zegrze), Central Poland 52°27'N, 21°01'E (1)	<i>S. turricula</i>	JURKIEWICZ-KARNKOWSKA 2004
34.	Bug River (90–107 km) near Brok, E. Poland 52°41.3'N, 21°50.0'E (1)	<i>S. turricula</i>	JURKIEWICZ-KARNKOWSKA 2006
35.	Bug River (53–152 km) near Budy Nowe, E. Poland 52°37'N, 21°40'E (1)	<i>S. turricula</i>	JURKIEWICZ-KARNKOWSKA 2004
36.	Bug River, Drohiczyn, E. Poland 52°23.3'N, 22°38.7'E (1)	<i>S. turricula</i>	JURKIEWICZ-KARNKOWSKA 2004

(1) approximate geographic coordinates are given when the locality is based on reference data only;

(2) precise geographic coordinates are given when the locality was newly found or confirmed in our studies;

Specimens were identified based on reproductive system (JACKIEWICZ 1998a, 2000, GLÖER & MEIER-BROOK 2003, KRUGLOV 2005)

(\*) identification additionally confirmed in molecular studies.