Resolving riddles and presenting new puzzles in Chonorinidae Phylogenetics

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Abida secale (Mollusca, Gastropoda, Pulmonata) discrepancies between morphology and molecules

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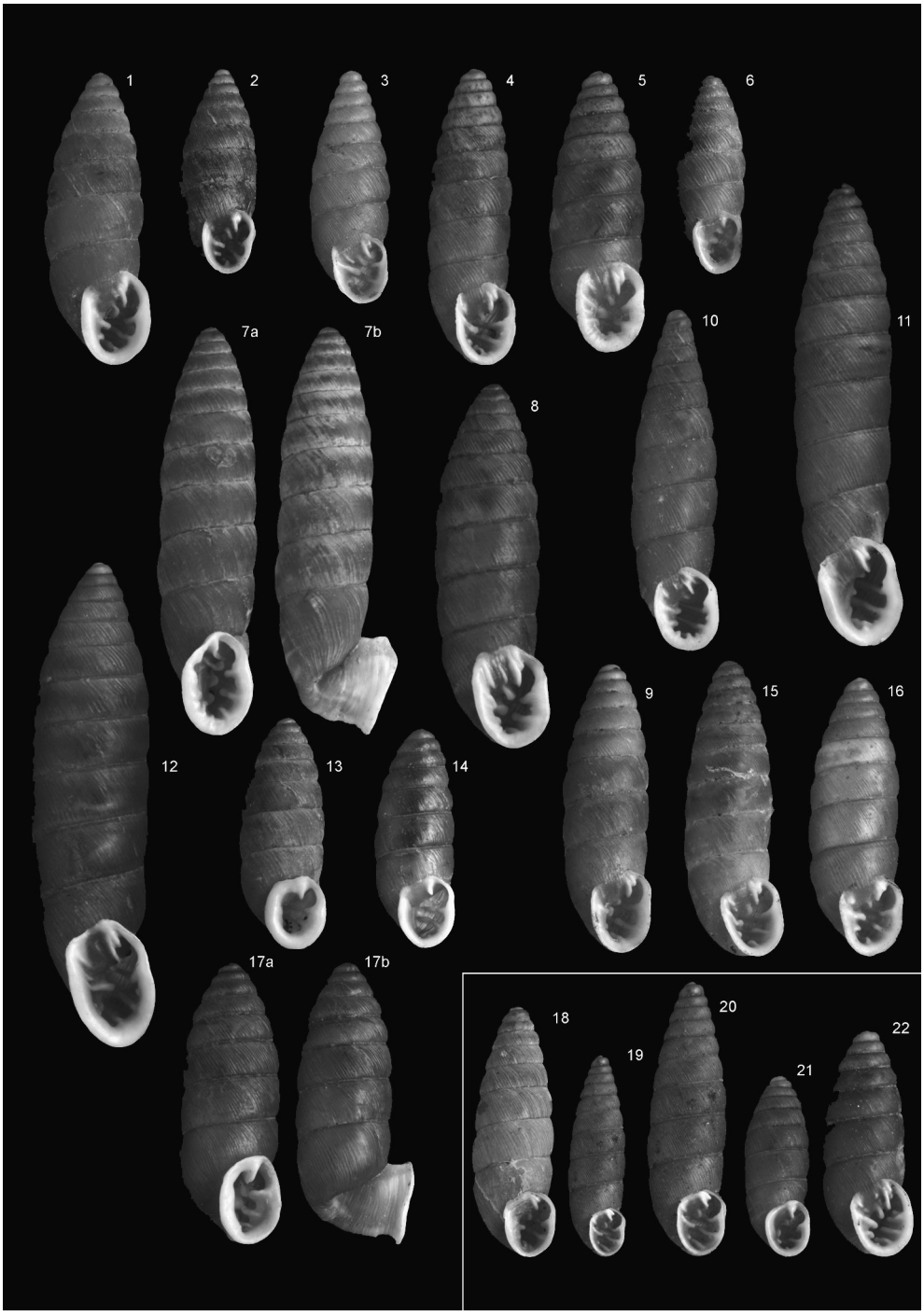
INTRODUCTION

The genus *Abida* Turton, 1831, consists of 10 currently known species, i.e. *A. vasconica* (Kobelt, 1882), *A. bigerrensis* (Moquin-Tandon, 1856), *A. pyrenaearia* (Michaud, 1831) [with two subspecies; *A. p. pyrenaearia* (Michaud, 1831) and *A. p. vergniesiana* (Küster, 1850)], *A. partioti* (De Saint-Simon, 1848), *A. cylindrica* (Michaud, 1829), *A. gittenbergeri* Bössneck, 2000, *A. attenuata* (Fagot, 1886), *A. occidentalis* (Fagot, 1888), *A. polyodon* (Draparnaud, 1801) and *A. secale* (Draparnaud, 1801). All representatives of the genus are obligate limestone dwellers, and therefore their distribution is restricted to calcareous habitats. Their distributional ranges all lie within the Cantabrian mountains in NW. Spain and the Pyrenees, with the exception of *A. polyodon* and *A. secale*, which extend beyond this area. *Abida polyodon* also occurs north of the Pyrenees in the Mediterranean zone as far as the Rhône valley in southeastern France. *Abida secale* occurs throughout Europe from England in the west, Belgium and southern Germany in the north and Slovakia and former Yugoslavia in the east. The southern part of its distribution includes the extreme northwest. of Italy, France and northeastern Spain (Gittenberger, 1973; Kerney, 1963: 235).

The morphological variation exhibited in *A. secale* is small over the largest part of its range, where it shows only minor variation in the shape and size of the shell. A striking exception to this morphological stability is found in the extreme southwestern part of the species' range, where an impressive, geographically determined morphological differentiation occurs in southern France and northeastern Spain (Gittenberger, 1973). This has resulted in the description of three subspecies for the French part of the Pyrenees, one for Andorra and a surprising eleven for the province of Catalonia, Spain (Gittenberger, 1973; Bech, 1993; Martínez-Ortí et al., 2004) (fig. 1). In France, *Abida secale secale* (Draparnaud, 1801) occurs throughout most of the country. *Abida s. boileausiana* (Küster, 1845) is restricted to the departments of Aude and Pyrenees Orientales, *A. s. saxicola* (Fagot, 1888) is known from only a single hill at Villefranche-de-Conflent in the Pyrenees Orientales, and *A. s. ateni* Gittenberger, 1973, has been reported from only a small part of the Aspe valley, SW. of Lourdes in the department of Pyrenees Atlantiques. From the Spanish province of Huesca in the central part of the Pyrenees and some localities in adjoining France, isolated from the main range of the nominate subspecies, *A. s. secale* is reported, with populations that are considered somewhat untypical (Gittenberger, 1973: 87). When their distinctness is confirmed, the name *A. s. cylindroides* (Moquin-Tandon, 1856) could be used here (maybe *Pupa secale elongata* De Saulcy, 1853, was introduced for the same form, but that is a junior homonym of both *P. elongata* Bouillet, 1836, and *P. elongata* Melleville, 1843). *Abida s. andorrensis* (Bourguignat, 1863) is endemic to three limestone areas in Andorra and the northern part of the province of Lerida in adjoining Spain. *Abida s. meridionalis* Martínez-Ortí, Gómez & Faci, 2004, occurs at scattered localities in the Spanish provinces of Castellon, Tarragona and Teruel in Spain, whereas north of that area, in the province of Barcelona near Montserrat and Cardona, *A. s. bofilli* (Fagot, 1884) is found. *Abida s.*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affinis (Rossmässler, 1839) occurs from Gerona in the east to the river Ter valley at Ripoll in the west. *Abida s. elegantissima* Gittenberger, 1973, has been described from a single locality in the mountains approximately halfway between Banyoles and Olot, but that subspecies has never been found again. We looked for it in vain twice, in different years (2004 and 2005). The remaining taxa in *A. secale*, viz. *A. s. brongersmai* Gittenberger, 1973, *A. s. margaridae* Bech, 1993, *A. s. cadiensis* Gittenberger, 1973, *A. s. cadica* (Westerlund, 1902), *A. s. tuxensis* (Westerlund, 1902), *A. s. brauniopsis* (Altimira, 1963) and *A. s. lilietensis* (Bofill, 1886), all occur in and/or around the Sierra de Cadí - Sierra Moixero mountain range, south of Andorra. The extreme morphological differentiation here is surprising since the sierra and its immediate surroundings constitute a more or less continuous stretch of suitable habitat. The separation of these forms into distinct taxa is based on shell characters only, but all subspecies can be identified quite easily, also because most populations are relatively uniform. Their distribution conforms well with the geology and geomorphology of the southern pre-Pyrenees. Subspecies are restricted to a few valleys and/or limestone 'islands'. Intermediate specimens can be found in the contact zones between some subspecies, which are generally neither very broad nor complex.

Here we present the results of an intraspecific phylogenetic study, involving both mitochondrial and nuclear DNA markers, to contribute to a better understanding of the species' extreme morphological diversification.

Material and Methods

Samples and DNA-extraction

The snails were collected during three periods of fieldwork in 2004-2006. Although many (sub)species have relatively small distributions, their density at any suitable habitat may be very high. Therefore sampling of 10-20 individuals (as was done) will not have caused a serious threat to the (sub)species. Up to 5 individuals were preserved in 96% ethanol for DNA extraction. The remaining snails were

Figure **1**. Pictures of typical representatives of *Abida secale* subspecies. **1**, *A. s. secale*, Slovakia; **2**, *A. s. secale*, England; **3**, *A. s. boileausiana*, France; **4**, *A. s. saxicola* from its type locality (Villefranche de Conflent, France); **5**, *A. s. andorrensis*, Andorra; **6**, *A. s.* cf. *cadiensis*, Andorra; **7**, *A. s. brongersmai*; **8**, *A. s. margaridae*, **9**, *A. s. lilietensis* from its type locality (La Pobla de Lillet, Spain); **10**, *A. s. elegantissima* paratype from its type locality (Sta. Maria de Finestras Mntn, Spain); **11**, *A. s. affinis*; **12**, *A. s. tuxensis*; **13**, *A. s. cadica* from its type locality (Comabona mountain, Sierra de Cadí, Spain); **14**, *A. s. cadiensis* paratype; **15**, *A. s. meridionalis*; **16**, *A. s. bofilli* from its type locality (Montserrat Mountain, Spain); **17**; *A. s. brauniopsis* from its type locality (Gosol, Spain); **18**; *A. attenuata*, Spain; **19**, *A. attenuata*, Spain; **20**, *A. attenuata*, France; **21**, *A. vergniesiana*, Andorra; **22**, *A. ateni* paratype, France. The pictures have been made with an Olympus motorized stereomicroscope SZX12 with AnalySIS Extended Focal Imaging Software.

either relaxed and stored in 70% ethanol for anatomical study or cleaned and dried for morphological analysis of the shell.

Specimens could readily be identified based on shell morphology. All identifications were checked by both authors.

COI was sequenced for a total of 97 individuals representing all the known subspecies of *Abida secale* except for *A. s. elegantissima*. All other known *Abida* species are represented by at least one individual, with the exception of *A. occidentalis*, which was not available for study. That species has always been rare and no specimens have been found alive since the 1960s. *Chondrina bigorriensis* (Des Moulins, 1835) was used as outgroup taxon in the COI dataset and *C. avenacea avenacea* (Bruguière, 1792) with the ITS-1 data (table 1).

Snail shells were broken into two parts to extract the body tissue. Shell remains, including the undamaged aperture which carries the key features for identification of these taxa, were stored as vouchers. The entire snail tissue was than sliced into pieces and genomic DNA was extracted using Qiagen DNEasy tissue kit following the manufacturers protocol. Final elution of the DNA was done in 200 ìl of provided buffer. With dry specimens, the E.Z.N.A. Mollusc DNA kit (Omega Bio-tek) was used. Elution was done here with 100 ìl.

A 600 bp fragment of the Cytochrome Oxidase I (COI) gene was amplified using primers H2198-alb (5′-ACT CAA CGA ATC ATA AAG ATA TTG G-3′) and L1490-alb (5′-TAT ACT TCA GGA TGA CCA AAA AAT CA-3′) (Uit de Weerd et al., 2004) in the polymerase chain reaction (PCR). The PCR started with a 5′ denaturing step at 94°and consisted of 40 cycles with 30″ at 94°, 30″ at 49° and 1′ at 72°, with an additional extension step at the end of all cycles of 5′. The PCR products were cleaned using the Nucleospin Extract II kit from Clontech. Sequences were subsequently obtained by either cycle sequencing using Big Dye Terminator v1.1 from Applied Biosystems and an ABI 377 gel sequencer, or by sequencing on a Megabace 1000 capillary sequencer. Some products were sequenced by Macrogen, Seoul, Korea.

The primers 18d-ALB (5′-CAC ACC GCC CGT CGC TAC TAC C-3′) and 5.8s-ALB (5′-ATG CGT TCA AGA TGT CGA TGT TCA A-3′) (Uit de Weerd & Gittenberger, 2004) were used to sequence the Internal Transcribed Spacer 1 (ITS1). The sequence product included a stretch of 180 bp of the 18s- and 50 bp of the 5.8s ribosomal genes. Direct sequencing yielded 'unreadable' and therefore unusable chromatograms due to polymorphic signals. The PCR products were therefore cloned using pGEM-T easy vectors from PROMEGA. This was done using a quarter of the prescribed reaction volume. 50ìl of the final mixture was plated on agar plates. Depending on the sample, 1 to 40 colonies were than sequenced.

Phylogenetic analyses

For both COI and ITS1 PCR products both strands of the DNA were sequenced. Forward and reverse chromatograms were aligned using the Chromas Pro package (Technelysium, Australia). In most cases ambiguous positions could easily be solved

and where this was not possible IUPAC ambiguity codes were used. Consensus sequences were exported as Fasta file and individual sequences were aligned using Clustal X (Thompson et al., 1997) with default parameters, as incorporated in BioEdit (Hall, 1999). The alignment was saved in fasta format and subsequently imported in Macclade 4.04 (Maddison & Maddison, 2002). All variable base positions were manually checked and compared with the chromatograms to rule out editing errors. Primer sites were identified and omitted from the dataset. With COI, another 20 base positions on both sides of the sequence alignment were left out of the analyses. This was done to increase the reliability of the dataset, since the terminal parts of the chromatograms were of low quality in many cases. Sequences were deposited with GENBANK (accession numbers: COI, EU395301- EU395427; ITS-1, EU394966-EU395300).

Codon positions were calculated by minimizing the number of stop codons, using the *Drosophila* (invertebrate) mitochondrial code. The final alignment was than translated and checked for stop codons. A test for saturation was performed for the entire COI dataset and for the individual codon positions. Nei's test for saturation was performed using the program DAMBE v.4.5.33 (Xia & Xie, 2001). Uncorrected pairwise distances for transition and transversion substitutions were also plotted to visualize saturation and eventually identify the taxa responsible. Additionally, base frequencies were checked for the individual codon positions and for the entire dataset using chi-square statistics implemented in Paup* (Swofford, 2002). A G1-skewness test (Hillis & Huelsenbeck, 1992) based on 1,000 random trees was used to test for phylogenetic signal. The best fitting model for substitution was calculated using MrModeltest v2.2 (Nylander, 2004). Phenetic and parsimony phylogenetic analyses were performed using PAUP* v4.0b10. A neighbor joining tree was created and subsequently bootstrapped with 1,000 replicates, using the model suggested by MrModeltest. A heuristic search was performed and followed by a parsimony bootstrap using 100 replicates with ACCTRAN optimization, 10 random sequence addition replicates and full heuristic search. The model suggested by MrModeltest was applied in MrBayes v3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) for Bayesian inference. Here 5 incrementally heated chains were used next to a cold one. The program was run until the average standard deviation between the two simultaneous runs was below 0.01 for a minimum of 1,000,000 generations. An additional 1 million generations were than run with a sample frequency of 1000. The initial trees were discarded as 'burnin' and a 50% majority rule consensus of the 2,000 remaining trees (from both runs) was created using the 'sumt' option in MrBayes.

The molecular clock hypothesis was tested a priori. An UPGMA tree based on uncorrected p-distances was constructed and subsequently used for the test as implemented in DAMBE.

Table **1** (pages 80-84). Samples used in this study.
Samples collected by the authors, unless stated otherwise.

Genbank Accession	RMNH	(Sub)species	LOCATION	ALT (m)	UTM	Collector
EU395301	102487	*Chondrina bigorriensis*	France, Aude, Axat	1100	DH3637	
EU395302	104142	*Abida vasconica*	Spain, Cantabria, Ramales de la Victoria	233	VN6387	
EU395303	104136	*Abida partioti*	France, Pyrénées Atlantiques, Cirque du Troumouse	1561	BH6137	
EU395304	100659	*Abida pyrenaearia*	France, Pyrénées Atlantiques, Coll de Pourtalet	1670	YN1344	
EU395305	99059	*Abida polyodon*	Spain, Cataluña, Pedraforca mountain	1500	CG5377	
EU395306	99196	*Abida polyodon*	France, Aude, Axat	x	x	
EU395307	100625	*Abida biggerensis*	Spain, Cataluña, Salardú	1400	CH2931	
EU395308	103374	*Abida biggerensis*	Spain, Arágon, Candanchu	1634	YN0239	
EU395309	104144	*Abida biggerensis*	Spain, Cantabria, Ramales de la Victoria	233	VN6387	
EU395310	104166	*Abida biggerensis*	France, Arrìège, Salau	1232	CH5233	
EU395311	99070	*Abida gittenbergeri*	Spain, Cataluña, Beuda	200	DG7574	
EU395312	99071	*Abida gittenbergeri*	Spain, Cataluña, Albanya	600	DG7385	
EU395313	99071	*Abida gittenbergeri*	Spain, Cataluña, Albanya	600	DG7385	
EU395314	100638	*Abida gittenbergeri*	France, Pyrénées Orientales, Coustouges	850	DG7290	
EU395315	100638	*Abida gittenbergeri*	France, Pyrénées Orientales, Coustouges	850	DG7290	
EU395316	99051	*Abida cylindrica*	Spain, Cataluña, N of Coll del Port	1300	CG8075	
EU395317	100633	*Abida cylindrica*	Spain, Cataluña, Beget			
EU395318	100439	*Abida cylindrica*	Spain, Cataluña, Sadernes	x	DG6781	
EU395319	100439	*Abida cylindrica*	Spain, Cataluña, Puig de Bassegoda			
EU395320	100453	*Abida cylindrica*	Spain, Cataluña, Pujarnol			
EU395321	100628	*Abida cylindrica*	Spain, Cataluña, Montserrat			
EU395322	100621	*Abida attenuata*	France, Aude, Axat	1100	DH3637	
EU395323	100624	*Abida attenuata*	France, Aude			A.J.W. de Winter
EU395324	104151	*Abida attenuata*	Spain, Pais Vasco, Durango	680	WN2577	
EU395325	102414	*Abida ateni*	France, Pyrénées Atlantiques, Escot	346	XN9471	
EU395326	104139	*Abida ateni*	France, Pyrénées Atlantiques, Escot	346	XN9471	
EU395327	99025	*Abida vergniesiana*	Andorra, Pal	x	CH7512	

Genbank Accession	RMNH	(Sub)species	LOCATION	ALT (m)	UTM	Collector
EU395328	99029	*Abida vergniesiana*	Andorra, Ordino	2000	CH8313	
EU395329	100519	*Abida secale affinis*	Spain, Cataluña, Sadernes	x	DG6681	
EU395330	100519	*Abida secale affinis*	Spain, Cataluña, Sadernes	x	DG6681	
EU395331	100520	*Abida secale affinis*	Spain, Cataluña, N of Ripoll	x	DG3178	
EU395332	100526	*Abida secale affinis*	Spain, Cataluña, Gombrén	x	DG2577	
EU395333	100528	*Abida secale affinis*	Spain, Cataluña, Sta. Maria de Montgrony monastery	1350	DG2479	
EU395334	100531	*Abida secale affinis*	Spain, Cataluña, Castellar de Nuch	1250	DG2179	
EU395335	100532	*Abida secale affinis*	Spain, Cataluña, Baga	950	DG0580	
EU395336	100532	*Abida secale affinis*	Spain, Cataluña, Baga	950	DG0580	
EU395337	100537	*Abida secale affinis*	Spain, Cataluña, Coll de Pal	2000	DG0983	
EU395338	100537	*Abida secale affinis*	Spain, Cataluña, Coll de Pal	2000	DG0983	
EU395339	99026	*Abida secale andorrensis*	Andorra, Pal	x	CH7512	
EU395340	99027	*Abida secale andorrensis*	Andorra, Arans	1600	CH7815	
EU395341	99027	*Abida secale andorrensis*	Andorra, Arans	1600	CH7816	
EU395342	99076	*Abida secale andorrensis*	Andorra, Pal	x	CH7512	
EU395343	104077	*Abida secale bofilli*	Spain, Cataluña, Montserrat	1000	DG0204	
EU395344	106710	*Abida secale boileausiana*	France, Ariège, Foix	x	CH85	D. Aten
EU395345	99055	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, W-flank Pedraforca mountain	2000	CG5577	
EU395346	99056	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, W-flank Pedraforca mountain	2200	CG5477	
EU395347	99062	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, N of Josa de Cadí	1600	CG5680	
EU395348	100538	*Abida secale brongersmai*	Spain, Cataluña, road to refugi Prat d'Aguilo	1625	CG9486	
EU395349	100538	*Abida secale brauniopsis*	Spain, Cataluña, S-flank Comabona mountain	1625	CG9486	
EU395350	100542	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, Gosol	1450	CG8976	
EU395351	100542	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, Gosol	1450	CG8976	
EU395352	100543	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, N of Saldes	1180	CG9479	
EU395353	100547	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, N of Saldes	1430	CG9579	
EU395354	100551	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, S of Tuxen	1430	CG8476	

Genbank Accession	RMNH	(Sub)species	LOCATION	ALT (m)	UTM	Collector
EU395355	100551	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, S of Tuxen	1431	CG8477	
EU395356	104084	*Abida secale brauniopsis*	Spain, Cataluña, Sierra del Cadí, S-flank Comabona mountain	1820	CG9480	
EU395357	100560	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Pedra	1000	DG0090	
EU395358	100561	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Bor	1000	DG0188	
EU395359	100566	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Pedra	1120	DG0188	
EU395360	100569	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, S-flank Comabona mountain	1700	CG9580	
EU395361	104108	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Orteró	1200	CG7988	
EU395362	104110	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Arseguel	1000	CG8390	
EU395363	104114	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Ansovell	1247	CG8387	
EU395364	104115	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Ansovell	1392	CG8386	
EU395365	104117	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, El Quer Foradat	1400	CG8786	
EU395366	104119	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Toloríu	1235	CG8790	
EU395367	104130	*Abida secale brongersmai*	Spain, Cataluña, Sierra del Cadí, Torres d'Alàs	850	CG7890	
EU395368	104125	*Abida secale cadica*	Spain, Cataluña, Sierra del Cadí, S-flank Comabona mountain	2429	CG9481	
EU395369	99057	*Abida secale peteri*	Spain, Cataluña, Sierra del Cadí, E-flank Pedraforca mountain	2250	CG5477	
EU395370	99057	*Abida secale peteri*	Spain, Cataluña, Sierra del Cadí, E-flank Pedraforca mountain	2251	CG5477	
EU395371	99076	*Abida secale ionicae*	Andorra, Pal	x	CH7512	
EU395372	99080	*Abida secale bechi*	Spain, Cataluña, Sierra del Cadí, Torre de Cadí mountain	2400	CG7981	
EU395373	99085	*Abida secale peteri*	Spain, Cataluña, Sierra del Cadí, Pedraforca mountain	2300	CG5477	
EU395374	100575	*Abida secale cadiensis*	Spain, Cataluña, Sierra del Cadí, N-flank Comabona mountain	2000	CG9483	
EU395375	100578	*Abida secale cadiensis*	Spain, Cataluña, Sierra del Cadí, N-flank Comabona mountain	2125	CG9483	
EU395376	100579	*Abida secale cadiensis*	Spain, Cataluña, Sierra del Cadí, N-flank Comabona mountain	2150	CG9483	
EU395377	100583	*Abida secale cadiensis*	Spain, Cataluña, Sierra del Cadí, N-flank Comabona mountain	2250	CG9482	
EU395378	100584	*Abida secale peteri*	Spain, Cataluña, Sierra del Cadí, N-flank Pedraforca mountain	1670	CG9377	
EU395379	104080	*Abida secale merijni*	Spain, Cataluña, Sierra del Cadí, Cap del Serrat Gran mountain	2163	DG1083	
EU395380	104100	*Abida secale merijni*	Spain, Cataluña, Sierra del Cadí, Cap del Serrat Gran mountain	2400	DG1084	
EU395381	104102	*Abida secale merijni*	Spain, Cataluña, Sierra del Cadí, Pedro dels Quatre Batlles mountain	2530	DG0885	

Genbank Accession	RMNH	(Sub)species	LOCATION	ALT (m)	UTM	Collector
EU395382	104104	*Abida secale merijni*	Spain, Cataluña, Sierra del Cadí, N-flank Pedro dels Quatre Batlles mountain	2326	DG0986	
EU395383	104121	*Abida secale cadiensis*	Spain, Cataluña, Sierra del Cadí, N-flank Comabona mountain	2430	CG9382	
EU395384	104123	*Abida secale cadiensis*	Spain, Cataluña, Sierra del Cadí, Comabona mountain	2511	CG9482	
EU395385	100589	*Abida secale lilietensis*	Spain, Cataluña, Sierra del Cadí, N of Baga	1325	DG0178	
EU395386	100592	*Abida secale lilietensis*	Spain, Cataluña, Sierra del Cadí, Vallcebre	1025	DG0373	
EU395387	100595	*Abida secale lilietensis*	Spain, Cataluña, Sierra del Cadí, Vallcebre	1500	CG9573	
EU395388	100598	*Abida secale lilietensis*	Spain, Cataluña, Sierra del Cadí, N of Saldes	1015	CG9677	
EU395389	100603	*Abida secale lilietensis*	Spain, Cataluña, Malanyeu	800	DG0772	
EU395390	104090	*Abida secale lilietensis*	Spain, Cataluña, La Pobla de Lillet	1201	DG1480	
EU395391	104092	*Abida secale lilietensis*	Spain, Cataluña, La Pobla de Lillet	934	DG1577	
EU395392	104096	*Abida secale lilietensis*	Spain, Cataluña, Fumanya	1681	CG9868	
EU395393	104098	*Abida secale lilietensis*	Spain, Cataluña, Fumanya	1630	DG0170	
EU395394	100606	*Abida secale margaridae*	Spain, Cataluña, N of Bellver de Cerdanya	1350	CG9993	
EU395395	100609	*Abida secale margaridae*	Spain, Cataluña, N of Bellver de Cerdanya	1200	DG0193	
EU395396	100614	*Abida secale margaridae*	Spain, Cataluña, Das	1325	DG0790	
EU395397	104165	*Abida secale meridionalis*	Spain, Valencia, Morella	1024	YL4500	
EU395398	100677	*Abida secale saxicola*	France, Pyrénées Orientales, Villefranche-de-Conflent	600	DH4815	
EU395399	100660	*Abida secale secale*	France, Pyrénées Atlantiques, Coll de Pourtalet	1970	YN1143	
EU395400	100678	*Abida secale secale*	France, Aude, Axat	1100	DH3637	
EU395401	102472	*Abida secale secale*	Belgium, Limburg, St. Pieter mountain	x	x	
EU395402	104419	*Abida secale secale*	England, West Morland, Scout scar	227	x	B. Colville
EU395403	104419	*Abida secale secale*	England, West Morland, Scout scar	227	x	B. Colville
EU395404	105219	*Abida secale secale*	France, Jura, Poligny	450	GM0690	
EU395405	105223	*Abida secale secale*	France, Ain, Gorges d'Ain	290	FM9426	
EU395406	105224	*Abida secale secale*	France, Drôme, Saou	300	FK6059	
EU395407	105233	*Abida secale secale*	France, Drôme, between Rosans and Serres	700	GK1421	
EU395408	105237	*Abida secale secale*	France, Isère, Sassenage	530	GL0709	

Genbank Accession	RMNH	(Sub)species	LOCATION	ALT (m)	UTM	Collector
EU395409	106691	*Abida secale secale*	Austria, Nieder-Oesterreich, Weissenbach a/d Triesting	x	WP71	P. Reischütz
EU395410	106697	*Abida secale secale*	Germany, Baden-Würtemberg, Grenzach	x	x	
EU395411	106698	*Abida secale secale*	Switserland, Glarus, Tierfehd	800-1050	x	Leiden students
EU395412	106700	*Abida secale secale*	Switserland, Glarus, Sernftal	1100	x	Leiden students
EU395413	106701	*Abida secale secale*	Switserland, St. Gallen, Walenstadt	400-800	x	Leiden students
EU395414	106703	*Abida secale secale*	Spain, Parque Nacional Aigûes Tortes, Lago Labreta	1500	x	C. Fransen
EU395415	106704	*Abida secale secale*	Germany, Baden-Würtemberg, Kleinkems	x	x	
EU395416	99045	*Abida secale tuxensis*	Spain, Cataluña, Sierra del Cadí, Torre de Cadí mountain	2200	CG7982	
EU395417	99046	*Abida secale tuxensis*	Spain, Cataluña, Sierra del Cadí, Torre de Cadí mountain	1700	CG7781	
EU395418	99046	*Abida secale tuxensis*	Spain, Cataluña, Sierra del Cadí, Torre de Cadí mountain	1701	CG7782	
EU395419	99047	*Abida secale tuxensis*	Spain, Cataluña, Sierra del Cadí, Torre de Cadí mountain	1600	CG7681	
EU395420	99049	*Abida secale tuxensis*	Spain, Cataluña, Alinya	1000	CG7070	
EU395421	99052	*Abida secale tuxensis*	Spain, Cataluña, N of Coll del Port	1300	CG8075	
EU395422	99052	*Abida secale tuxensis*	Spain, Cataluña, N of Coll del Port	1301	CG8076	
EU395423	100620	*Abida secale tuxensis*	Spain, Cataluña, Sierra del Cadí, Tuxen	1175	CG8176	
EU395424	104127	*Abida secale tuxensis*	Spain, Cataluña, Sierra del Cadí, Tuxen	1150	CG7878	
EU395425	104128	*Abida secale tuxensis*	Spain, Cataluña, Sierra del Cadí, Osera	1212	CG7475	
EU395426	104129	*Abida secale tuxensis*	Spain, Cataluña, Els Castells	1300	CG5687	
EU395427	106691	*Abida secale secale*	Austria, Nieder-Oesterreich, Weissenbach a/d Triesting	x	WP71	P. Reischütz

Results and Discussion

COI

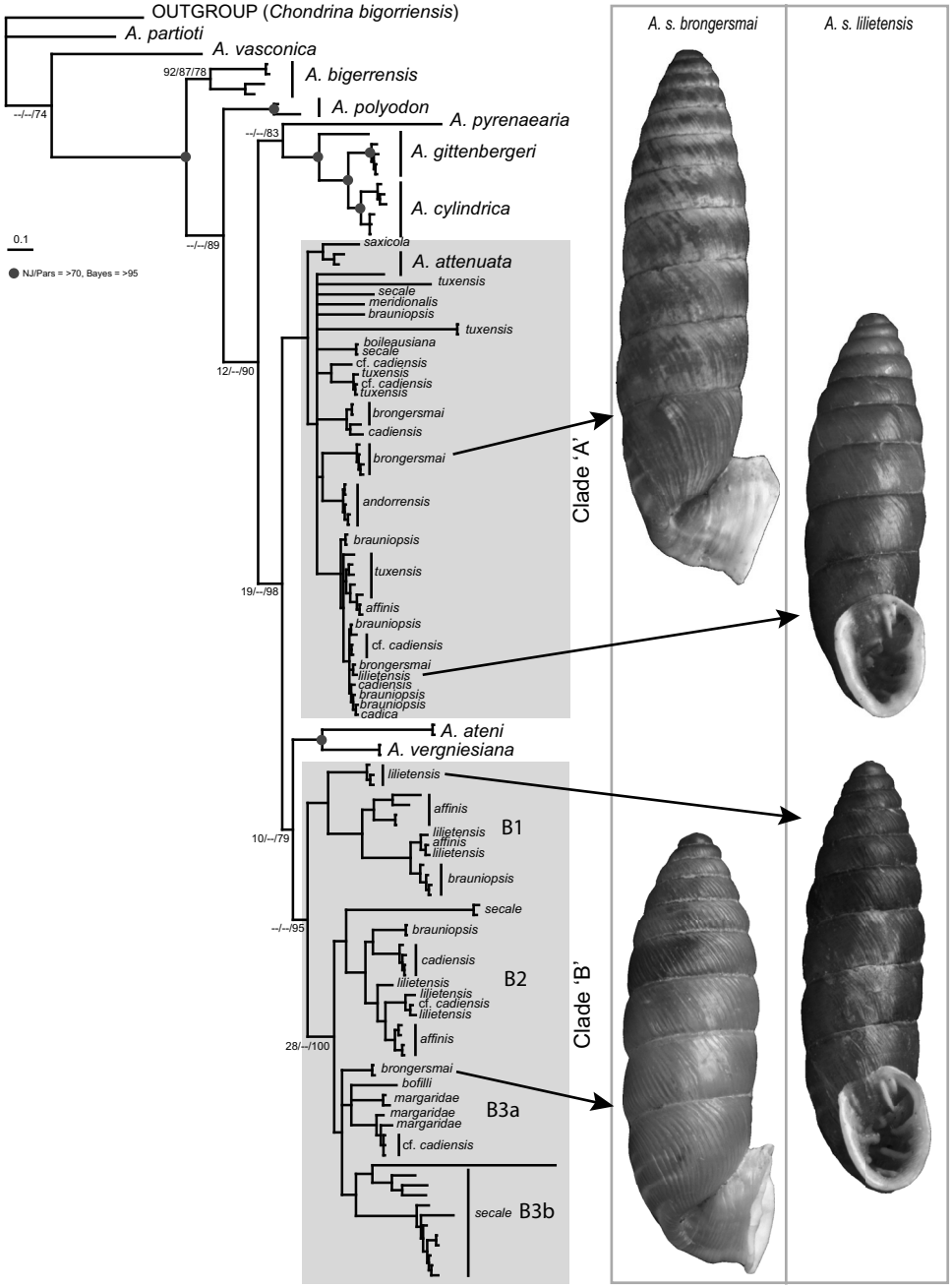
Stop codons were absent in all COI sequences. Therefore it was assumed that no pseudogenes were incidentally sequenced. The estimated proportion of invariant sites that was used in the saturation test, was obtained from the MrModeltest output. Saturation is present, but only in the third codon position. It was decided not to exclude the third codon position however, since saturation is mostly due to outgroup versus ingroup comparison. The third codon position registered 199 parsimony informative characters against 50 for the first and 11 for the second.

The chi-square test for homogeneity of base frequencies across taxa showed no significant deviation from the expected distribution (pos. 1, p=1.00; pos. 2, p=1.00; pos. 3, p=0.97; entire dataset, p=1.00). The G1 skewness statistic was highly significant (g1=-0.51, p<0.01), indicating the presence of nonrandomized signal in the dataset.

MrModeltest suggested the use of the General Time Reversible (GTR) model with an estimated proportion of invariable sites (I; 0.4722) and Gamma shape parameter (G; 0.8348). This was suggested both by the hierarchical ratio likelihood tests and the Akaike Information Criterion (AIC). The molecular clock hypothesis was rejected (p=0.00). Since the scarce fossil record does not allow for sensible calibration, it was decided to refrain from molecular clock analyses in this study.

The three different phylogenetic reconstruction methods that were used showed broadly the same topology for the COI data (fig. 2). In contrast with the Bayesian analysis, both the Neighbor Joining and Parsimony bootstrap analyses gave very low support values for most clades. However, since they all gave largely the same topology, we consider this a robust phylogenetic reconstruction. *Abida vasconica* and *A. partioti* make up the most basal clade(s) in the trees. The species' distributions are strongly vicariant, with *A. vasconica* in the Cantabrian mountains and *A. partioti* in the central Pyrenees. The next two clades are formed by *A. polyodon* and *A. bigerrensis*. These two species are mostly vicariant, but at a few locations in Navarra (Spain). The third clade shows another couple, i.e. *A. cylindrica* and *A. gittenbergeri*. The latter is known from a restricted area just west of Figueres, on the eastern edge of the distribution of the other species. Extensive field studies in that area have shown that the two species never occur in sympatry (Tarruela Ruestes, 2006; unpub. data) and that intermediate forms are not found. Nevertheless, *A. gittenbergeri* appears to be paraphyletic with *A. cylindrica* as a derived clade.

The remaining taxa show an intriguing pattern. At the base of a clade we see *A. attenuata*, which clusters with a large number of OTU's that allegedly belong to *A. secale*. Next to it we see *A. s. ateni* and *A. p. vergniesiana* branching off. The former is known from a few localities in the valley of the River Aspe in the western French Pyrenees and the latter from a slightly larger range in and north of Andorra. These


OUTGROUP (Chondrina bigorriensis)
A. partioti
A. vasconica
A. bigerrensis
A. polyodon
A. pyrenaearia
A. gittenbergeri
A. cylindrica
saxicola
A. attenuata
tuxensis
secale
meridionalis
brauniopsis
boileausiana
cf. cadiensis
brongersmai
cadiensis
andorrensis
affinis
lilietensis
A. ateni
A. vergniesiana
B1
B2
B3a
B3b
Clade 'A'
Clade 'B'
92/87/78
--/--/74
--/--/83
--/--/89
12/--/90
19/--/98
10/--/79
--/--/95
28/--/100
0.1
NJ/Pars = >70, Bayes = >95
A. s. brongersmai
A. s. lilietensis

are widely separated distributional ranges. It must be mentioned once more however, that *A. occidentalis* is missing from our analyses. Its known range fits right in between that of the former two taxa and in shell morphology it is similar to *A. p. vergniesiana*. Therefore, it is possible that *A. occidentalis* belongs to this clade. As a sistergroup to the latter clade, we see the remaining *A. secale* individuals, including those from the range of A. s. secale in northwestern Europe.

According to the mtDNA, *A. secale* is subdivided into two distinct clades, here refered to as clades 'A' and 'B', each with different sister taxa. Quite surprisingly, the geographical distribution of both clades is not congruent with the morphologically determined subspecies' ranges (fig. 3). Individuals of morphologically indistinguishable populations of *A. s. affinis*, *A. s. cadiensis*, *A. s. brauniopsis* and *A. s. lilietensis* end up in both clades 'A' and 'B'. However, despite their incongruence, both the morphology-based and the mtDNA-based geographical pattern are coherent.

The seemingly conflicting patterns might be explained by hybridization and subsequent unilateral introgression of the '*attenuata*' mitochondrion. Here we hypothesize that there has been a hybridization event between *A. attenuata* and *A. secale* in the Aude region in France, where *A. attenuata* and *A. s. boileausiana* occur sympatrically. From that region the '*attenuata*' mitochondrion could have spread into the nearby populations of *A. secale*, irrespective of the subspecies and passing intraspecific hybrid zones (fig. 4). If so, we may assume that *A. s. margaridae* and *A. s. bofilli*, and most populations of *Abida s. secale* have not or not yet been reached.

Two individuals of *A. s. affinis* from Gombrén and Sta. Maria de Montgrony Monastery, respectively, also appear in clade 'A'. This fits the introgression scenario, since *A. s. affinis* occurs also north of the Pyrenees in France. The southernmost part of the species' range in Spain is occupied by *A. s. meridionalis*, which is in clade 'A'. Just north of its range *A. s. bofilli* occurs, which shows up in clade 'B'. Introgression into *A. s. meridionalis* but not in *A. s. bofilli* could be explained by the occurrences in different river-basins. In and south of the valley of the Ebro and its tributary the Segre, *A. s. meridionalis, A. s. brongersmai* and *A. s. tuxensis* occur, all of which having the '*A. attenuata*' COI. In the Llobregat basin, where *Abida s. bofilli* occurs and populations of *A. s. lilietensis* the invasive mitochondrion has not been recorded.

Abida attenuata has a strictly disjunct distribution, with two small ranges in Orduña (Basque Country, Spain) and in the French department of Aude, respectively (fig. 4**)**, whereas its phylogeography is obscure. The species has never been found in between. Maybe, the hybridization between *A. attenuata* and *A. secale* has

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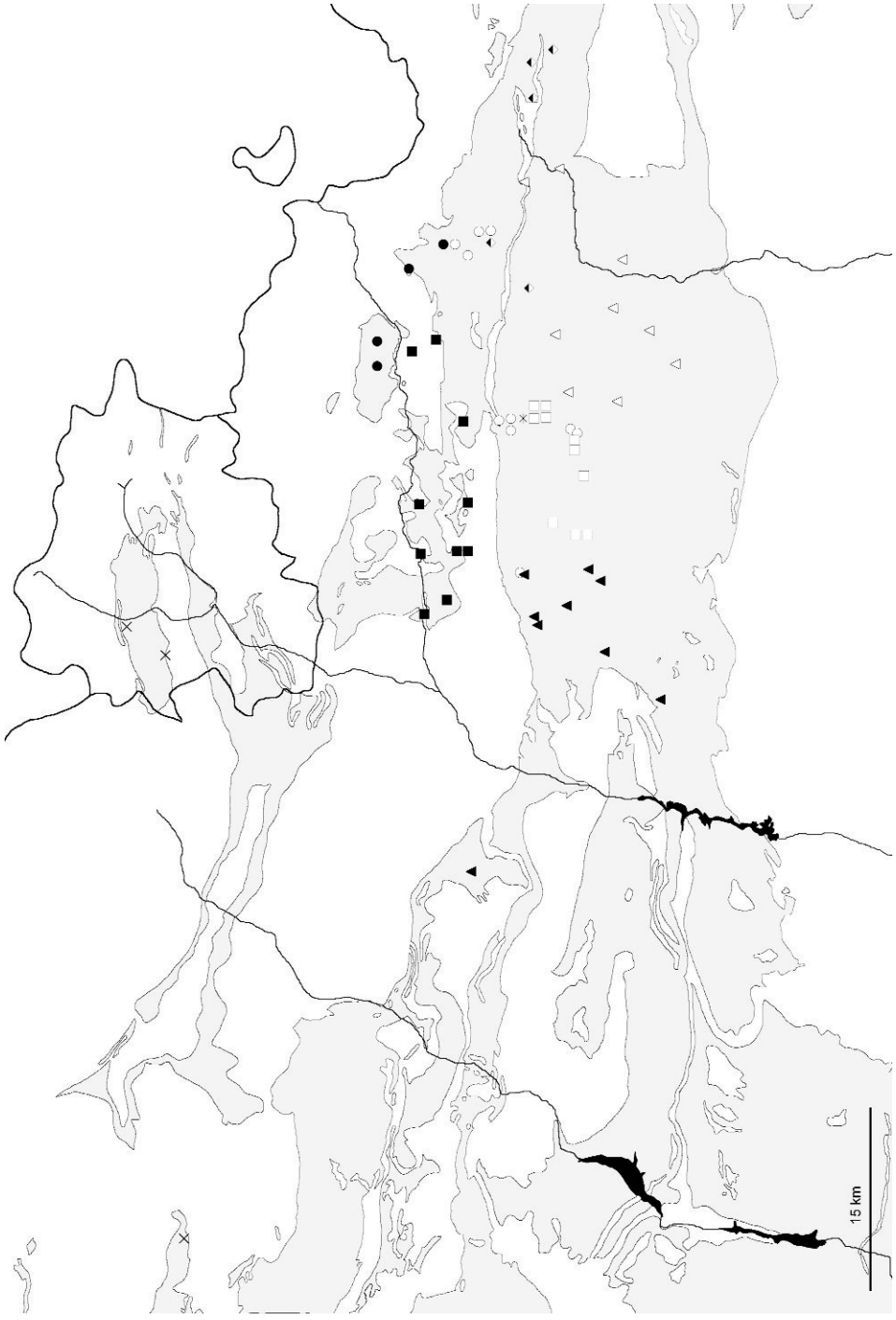
Figure **2**. Bayesian likelihood, 50% majority rule consensus tree. Two examples are shown of specimens of morphologically well defined subspecies that end up in both clade A and clade B. Values at the nodes represent Bayesian posterior probabilities, Neighbor Joining and Parsimony bootstrap support respectively. For explanation of clade names see text.


15 km

been caused by an introduction of the former species into France. Obviously, *A. s. secale* has colonized by far the largest part of its range during the Holocene. This view is supported by the monophyly and the low genetic differentiation between even the most distant populations (England versus Austria/Switzerland), as well as by the fossil record. The species appears in fossil assemblages after 13,000 yr bp in England (Kerney, 1963; Preece, 1994; Preece & Bridgland, 1999), Germany (Meyrick, 2001) and France (Lautridou & Puisségur, 1977). There is evidence however, that *A. secale* was already present in central Europe during the Lower Pleniglacial, i.e. around 60,000 years ago (Moine et al., 2005). The subsequent glacial maximum would then have forced the species into more southerly refugia.

The source of the Holocene range expansion may be situated in the Segre valley, as is suggested by the phylogeny reconstruction. Nearly all populations in that area (fig. 3), and especially those just north of the Pyrenees, now keep the '*attenuata*' mitochondrion. Therefore, this introgression should have occurred only after the northwards dispersal wave of *A. secale*. This conclusion is supported by the minimal extent of mtDNA genetic variation (short branchlengths) in clade 'A' as compared to clade 'B'.

Three distinct subclades can be identified within clade 'B'. These do not correspond with the subspecies delimitations based on morphology. *Abida s. lilietensis,* for instance, is found in two distinct clades. There is substantial genetic variation between *A. s. lilietensis* individuals in the two clades. The average, uncorrected, pairwise distance is 0.14 (n=9 pairwise comparisons), which is equal to the average genetic distance between all *A. secale* individuals (n=136) or even between two clearly differentiated *Abida* species, like *A. bigerrensis* and *A. polyodon* (n=8 pairwise comparisons). Such a contrast between morphology and mitochondrial phylogeny reconstruction has previously been observed in other organisms as well (i.e. Babik et al., 2005; Van Riel et al., 2005; Spooner & Ritchie, 2006; Hagemann & Pröhl, 2007) and several explanations for this phenomenon have been proposed. In this particular case the different mitochondrial lineages may have evolved during temporary isolation in different river valleys. The first two subclades are distributed south of the Cadí mountain range (figs 2 and 3). One (B1) is found from Tuxen in the west to Gerona in the east. The other subclade (B2) ranges from Vall de Gresolet (east of the Pedraforca mountain) in the west to La Pobla de Lillet in the east, including the crest of the mountain range. Its origin is probably linked to the northern part of the

Figure **3**. Map of the primary research area south of Andorra showing limestone areas (shaded grey). Localities from which material was sequenced for the COI dataset are shown. Symbols correspond to subspecies identifications based on shell morphology. Cross, *A. s. andorrensis*; black square, *A. s. brongersmai*; black circles, *A. s. margaridae*; white circles, *A .s. cadiensis*; black/white diamonds, *A. s. affinis*; black triangles, *A. s. tuxensis*; white squares, *A. s. brauniopsis*; star, *A. s. cadica*; white triangles, *A. s. lilietensis*. The dashed line represents the approximate border between the distribution of clade A and clade B (fig. 2). The map is in UTM projection.

Figure **4**. Map of Europe showing the locations from which material was sequenced for the COI dataset as well as the individuals of *A. s. secale* that were used for ITS-1 analysis. Black diamonds, *A. s. secale*; black/white square, *A. attenuata*; black/white circle, *A. s. ateni*. The dashed line represents the southern most edge of the distributional range of *A. secale*. The dark grey area indicates the distribution of clade A (fig. 6). The open square indicates the primary research area as shown in fig 2-3. The map has no projection.

Llobregat valley. The third subclade (B3) represents the individuals found east of Martinet and north of the mountain range (B3a), including all populations of *A. s. secale* north of the Pyrenees (B3b). Its origin might be associated with the eastern

part of the Segre valley.

Pairwise genetic distances between for instance *A. polyodon* and *A. bigerrensis* range from 0.14 to 0.16. Such a degree of variation is comparable to that between *A. s. secale* populations from the French departments of Jura and Isère (0.16). Founder effects and population bottlenecks are probable causes for the relatively large genetic variation within *A. s. secale*.

ITS-1

The ITS-1 data show a strong AT-bias of 69%, which is a common observation in functionally unconstrained spacers. *Abida vasconica, A. partioti, A. bigerrensis* and *A. polydon* were shown as monophyletic. The clones sequenced for these individuals formed monophyletic groups in the phylogeny reconstruction. These taxa had the same topology here as in the COI-based phylogeny. *Abida cylindrica* and *A. gittenbergeri* cluster together but the individual sequences are mixed. Also a single cloned sequence of *A. s. lilietensis* is found in this clade. Towards the crown of the tree the individuals representing *A. attenuata, A. p. vergniesiana, A. s. ateni* and *A. s. saxicola* cluster in monophyletic groups. These are, however, interspersed with diverse clones of the remaining *A. secale* individuals. The magnitude of intra-individual variation results in a mix of gene-trees that is phylogenetically uninformative.

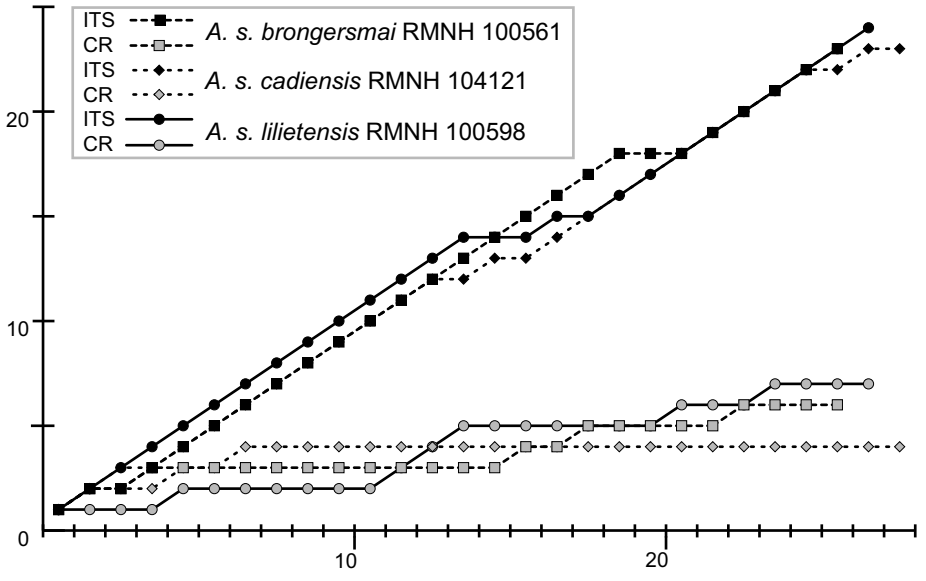


Figure **5**. Graph showing increase of unique clones for the whole ITS-1 region (ITS) and for the conserved regions (CR) only.

Excluding those characters that showed intra-individual variation (Gittenberger et al., 2006) resulted in the loss of practically all phylogenetic information. Reduction of the intra-individual variation in the ITS-1 data by combining them into a single sequence cannot be an option, since they are heterologous genes. In other words, genes or alleles with a different evolutionary history would be combined.

Eventually it was decided to reduce the amount of variation by analysing only the six conserved regions that had been identified by Armbruster et al. (2000). This resulted in a dataset of 118 base pairs and 60 'haplotypes' for all *Abida* species (55) and *Chondrina avenacea* (5), with a substantial reduction in intra-individual variation (fig. 5). The conserved regions (CRs) showed no AT-bias (50%), which is consistent with their alleged function in gene splicing. Gaps in these CRs were considered informative and therefore taken into account. One haplotype was considered part of a pseudogene since here CR 1 was completely absent. Although the animal-specific motive at the end of the 18s gene ('GATCATTA') was intact, it was decided to exclude it from subsequent analyses. This haplotype was represented by six sequences, all from *A. s. brongersmai* (RMNH 104117).

The remaining haplotypes contained practically no phylogenetic information. Fourteen out of 118 characters (11%) were parsimony informative, but all suffered from homoplasy. This resulted in a non-informative basal polytomy. The haplotypes were therefore plotted in a median joining network (fig. 6). The high number of possible connections ('loops') suggest a high number of recombination events. The haplotypes also suffer from incomplete lineage sorting. Haplotype 7 is shared, for instance, between *A. vasconica* and *A. partioti*, as well as with *A. s. ateni*. Haplotype 12 is shared between *A. polyodon* and *A. bigerrensis* and again *A. s. ateni*. Extensive sampling within *A. secale* revealed a large amount of variation, in that 41 haplotypes were found to be unique to this taxon. There are two main haplotypes, 1 (103 clones) and 4 (86 clones). The first is shared with *A. attenuata*, *A. p. pyrenaearia* and *A. p. vergniesiana*, while the second one is unique to *A. secale*. Within *A. secale* there are six haplotypes in this dataset that occur in more than a single individual. When these haplotypes are plotted on a map (fig. 4 and 7) a pattern emerges that is geographically coherent. Haplotype 12, 44 and 51 are restricted to two individuals each. Haplotype 12 (blue) is found in *A. s. lilietensis* and *A. s. brongersmai*. Haplotype 44 (yellow) is found in *A. s. brongersmai* and a geographically close individual of *A. s. cadiensis*. Haplotype 51 (black) is found in two individuals of *A. s. tuxensis*.

Haplotype 1 (green) is found in subspecies north of the Cadí-Moixero mountain range, i.e. *A. s. saxicola*, *A. s. andorrensis*, *A. s. brongersmai* and *A. s. margaridae*. The exception is *A. s. brauniopsis*, which occurs south of the mountain range and also shares this haplotype. This subspecies is morphologically similar to *A. s. brongersmai* and also shares COI clade 'A' with this subspecies. It is therefore assumed that *A. s. brauniopsis* reached its present day range south of the mountain range by dispersal. The distribution pattern of haplotype 1 coincides roughly with that of the COI based clade 'A'. Haplotype 51 (black), which occurs in two individuals of *A. s. tuxensis* that also share the *A. attenuata* mitochondrion, is derived from haplotype 1 (fig. 6 and 7).

The pattern might have originated by introgression from *A. attenuata* into *A. secale*, as was assumed for the COI data. However, here we cannot distinguish between introgression and incomplete lineage sorting as possible causes of the observed pattern.

Haplotype 46 (grey) is found in two individuals of *A. s. brongersmai*, as well as in the three individuals of *A. s. secale* from England, France and Austria. This suggests, in concordance with the COI data, that the latter subspecies has originated in the Segre valley. Haplotype 1 is missing in these three individuals. This might support the introgression scenario for haplotype 1 into *A. secale*, since it has already been established from the COI data that this occurred after the initial dispersal of this subspecies. However, the absence of haplotype 1 in these three individuals (which amount to only 14 sequenced clones) is considered non-informative in this respect.

The origin of the exceptionally large morphological variation of *A. secale* in the Sierra del Cadí remains enigmatic. The habitat is mostly homogeneous, as are the climatic conditions (This thesis, chapter 5). We could not identify any factors to which the observed shell morphologies might be considered adapted. The allopatric ranges of the subspecies and the small zones with intermediates, without any obvious differentiation in habitats, suggest a case of non-adaptive radiation (Gittenberger, 1991, 2004). This, however, is not an explanation for the extreme morphological diversity in *Abida secale*.

Barcoding

The COI marker used in this study is the one chosen for species barcoding by the international Consortium for the Barcode of Life (CBOL: http://barcoding.si.edu). In this particular case, the identification of species or subspecies on the basis of only this mitochondrial DNA barcode may result in serious error. All identifications should be verified by additionally using morphological characters or other, preferentially nuclear DNA markers.

Taxonomic implications

The results of this study necessitate a reconsideration of the status of some nominal taxa. *Abida s. ateni* and *A. p. vergniesiana* belong to the same clade in the COI-based tree. We conclude that either the so-called subspecies *A. s. ateni* does in fact not belong to *A. secale*, or that *A. p. vergniesiana* also belongs as a subspecies to that species. However, *A. p. vergniesiana* and *A. s. andorrensis* are found in sympatry. Since they belong to distinct clades we can assume that they are reproductively isolated. *Abida s. ateni* and *A. p. vergniesiana* are sister taxa, that do not belong to *Abida secale*. Furthermore, as the so-called *A. pyrenaearia pyrenaearia* is more closely related to other *Abida* species (fig. 2), they also do not belong to that taxon. We therefore propose to regard both taxa as separate species, viz. *Abida ateni* and *A. vergniesiana*.

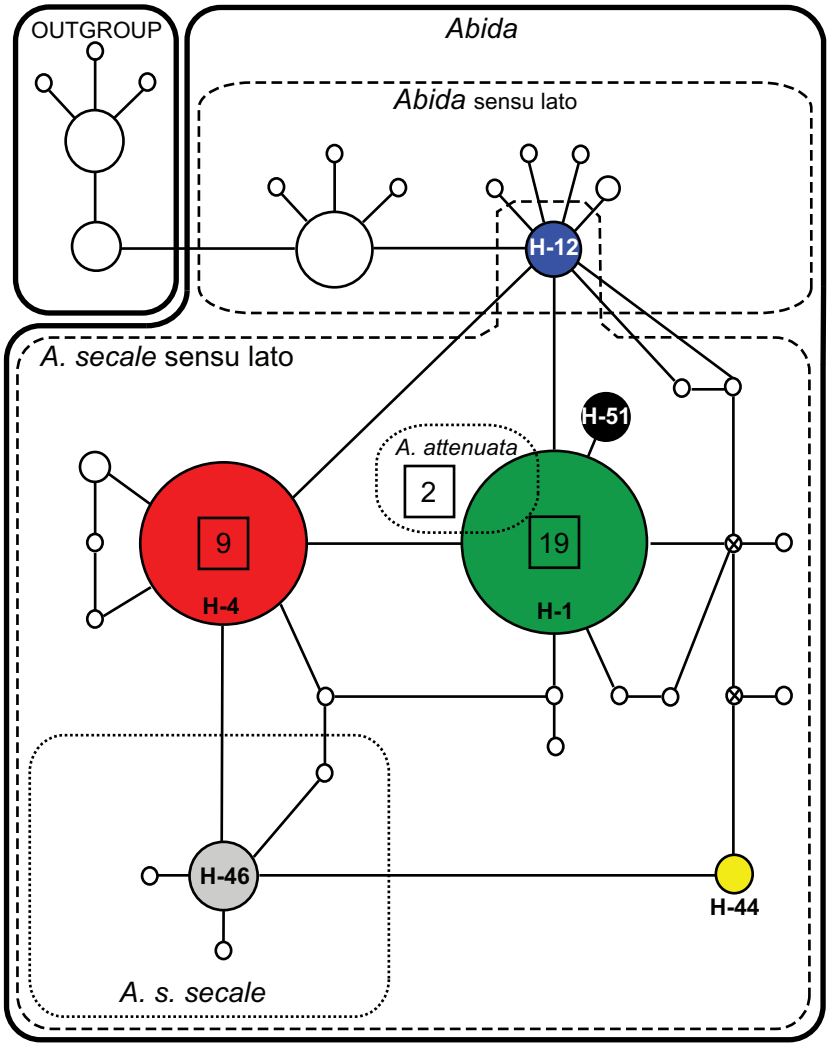


Figure 6. Median joining haplotype network of conserved region haplotypes. Green, haplotype 1; red, haplotype 4; blue, haplotype 12; yellow, haplotype 44; grey, haplotype 46; black, haplotype 51. The numbers in the open squares indicate the number of clones that differ by a single mutation from the 'parent' haplotype. Open circles with crosses indicate inferred, not observed haplotypes. The size of the nodes is related to the number of clones with that specific haplotype.

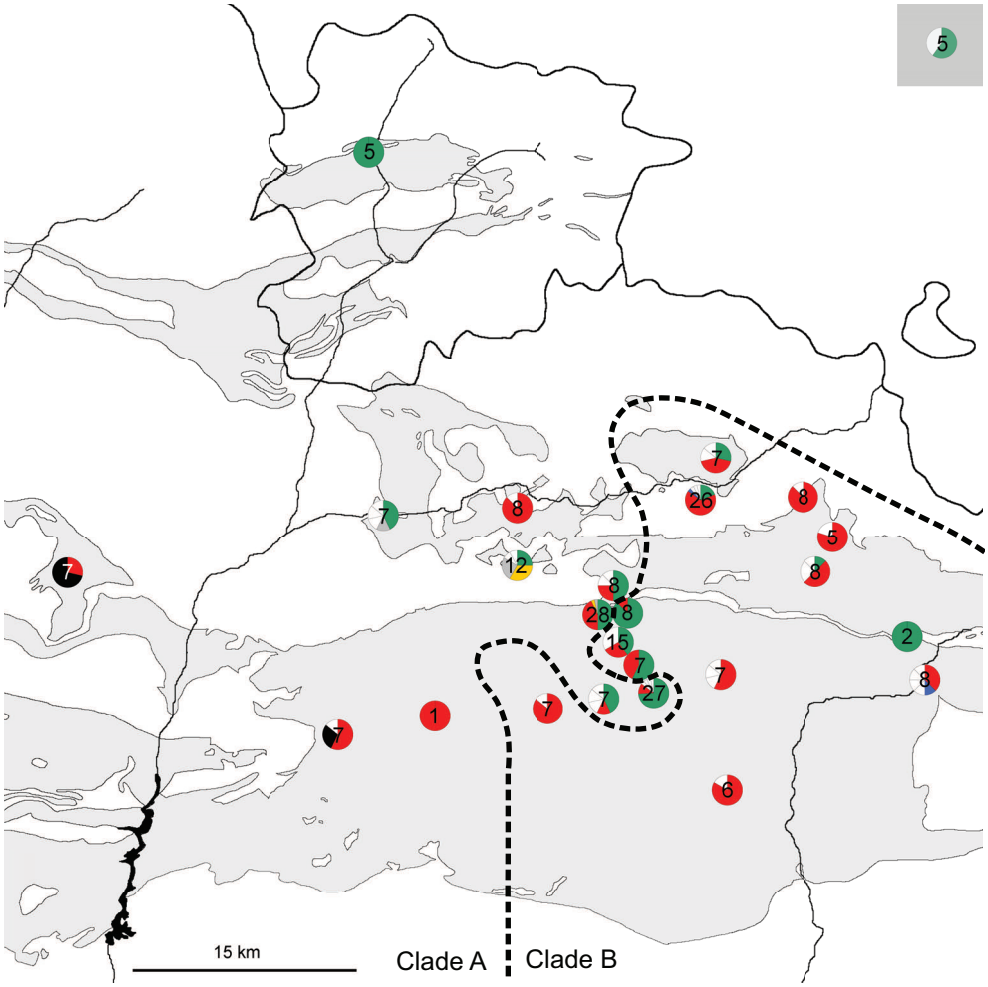


Figure 7. Map of the primary research area showing localities from which individuals were selected for ITS-1 sequences. Pie charts represent individual snails and show the percentages of the haplotypes present in the individual. The number indicates the number of clones that were sequenced. The chart in the upper right corner represents *A. s. saxicola*. Its true location lies just NE of the map area. Green, haplotype 1; red, haplotype 4; blue, haplotype 12; yellow, haplotype 44; grey, haplotype 46; black, haplotype 51. The dashed line represents the approximate border between the distribution of clade 'A' and clade 'B' as found in the COI based phylogeny reconstruction. The map is in UTM projection.

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