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aan mijn ouders

voor Wolter

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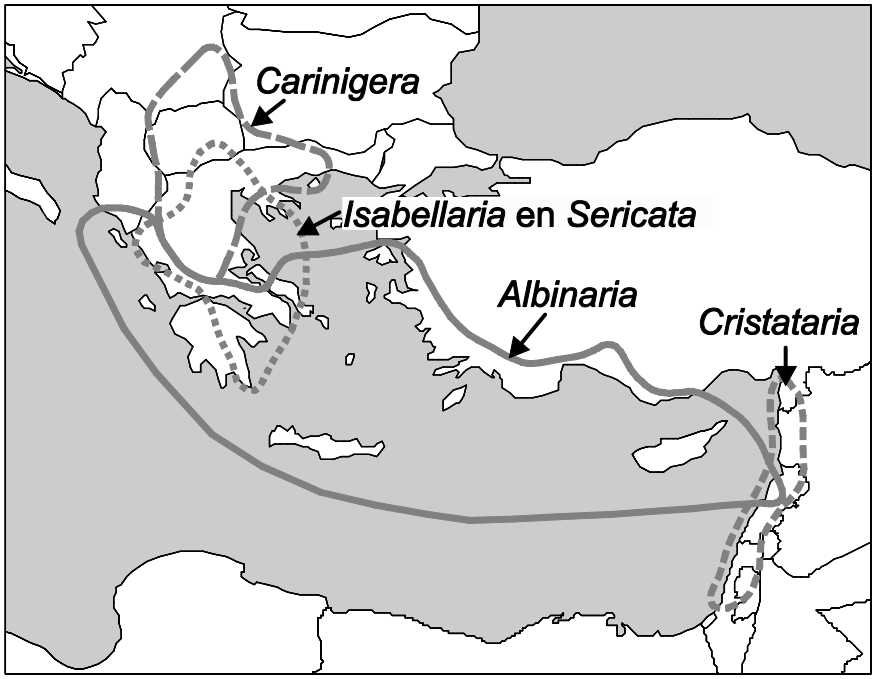
NEDERLANDSE INLEIDING EN SAMENVATTING

Albinaria en verwanten: een modelgroep in evolutie-onderzoek

Mensen zijn altijd al gefascineerd geweest door de diversiteit aan levensvormen, en hebben geprobeerd om deze diversiteit te structureren en te verklaren. In de biologie wordt aangenomen dat alle levende wezens, van bacterie tot mens, ooit een gemeenschappelijke voorouder hebben gehad, en dat hun onderlinge verschillen in de loop van vele miljoenen en zelfs miljarden jaren zijn ontstaan. Soortgelijke processen van diversificatie hebben zich ook voorgedaan tussen relatief nauwverwante soorten, die immers ook van een gemeenschappelijke — maar een meer recente — voorouder afstammen. Vanwege hun geringere omvang en kortere geschiedenis zijn zulke groepen van nauwverwante soorten beter te bestuderen. Op deze wijze hebben studies aan landslakgeslachten waardevolle kennis over evolutie opgeleverd. Landslakken staan dan ook bekend om hun diversiteit, ze zijn goed te volgen en te verzamelen in het veld, en slakkenhuizen fossiliseren relatief gemakkelijk.

Eén van de eerste groepen landslakken die de aandacht trokken van biologen, was het huidige geslacht *Albinaria*. Dit geslacht is onderdeel van de familie Clausiliidae, in het Engels ook wel ‘door snails’ (deurslakken) genoemd, naar het deur-achtige sluitmechanisme (clausiliair apparaat, CA) van hun huisje. De aandacht voor *Albinaria* is te danken aan haar enorme diversiteit, blijkend uit het grote aantal beschreven soorten. Op het moment worden er meer dan honderd soorten binnen het geslacht geplaatst, die onderling vaak zeer sterk verschillen in hun huisjes. Drie soorten uit Libanon daargelaten, komen alle *Albinaria* soorten voor binnen een relatief klein gebied, dat zich uitstrekt over de noordoostelijke kuststreek langs de Middellandse zee, van Zuid-Albanië tot Cyprus. Al in de tweede helft van de negentiende eeuw werd er gespeculeerd over de oorzaak van de enorme vormenrijkdom binnen *Albinaria*. Aangenomen wordt, dat de enorme diversiteit aan soorten binnen *Albinaria* in de loop van miljoenen jaren is ontstaan vanuit één vooroudersoort. Door verwantschappen tussen soorten te bepalen, kan mogelijk in ruwe lijnen achterhaald worden hoe dit proces is verlopen.

Albinaria lijkt in huisje sterk op de omringende oost-mediterrane geslachten *Cristataria*, *Isabellaria*, *Sericata* en *Carinigera*. Deze geslachten en *Albinaria* maken deel uit van dezelfde groep binnen de familie Clausiliidae: de onderfamilie Alopiinae. *Cristataria*, *Isabellaria* en *Sericata* worden zelfs beschouwd als de nauwste verwanten van *Albinaria*. Het verspreidings-gebied van *Albinaria* overlapt in het oosten (Libanon) met dat van *Cristataria*, en in het noordwesten (het Griekse vasteland) met dat van *Isabellaria*, *Sericata* en *Carinigera* (zie figuur). Voor zover bekend, leven de slakken uit de vijf geslachten op kalkrotsen, waar ze zich voeden met korstmossen, soms aangevuld met mossen. De slakken verplaatsen zich relatief langzaam en brengen soms maanden achtereen door op hetzelfde rotsblok. Ze zoeken vaak vochtige plekken op, en zijn meestal te vinden op verticale noordelijke wanden, in rotsspleten of onder stenen. Op zulke plaatsen kunnen tientallen, soms zelfs honderden slakken worden aangetroffen. Tijdens de hete mediterrane zomer houden de slakken een ‘zomerslaap’, om pas rond de herfst weer actief te worden. Bijzonder is dat de soorten uit de vijf geslachten onderling vaak een mozaïek-achtige verspreiding hebben, waarbij de verspreidingsgebieden als



Schematische weergave van de verspreiding (van NW naar ZO) van de geslachten *Carinigera*, *Isabellara* & *Sericata*, *Albinaria,* en *Cristataria*.

puzzelstukken in elkaar grijpen, maar slechts zelden overlappen. Aangenomen wordt daarom dat deze soorten elkaar lokaal wegconcurreren.

Het is vanwege hun onderlinge overeenkomsten belangrijk om naast *Albinaria* ook de geslachten *Cristataria*, *Isabellaria*, *Sericata* en *Carinigera* te bestuderen. Enerzijds kunnen deze overeenkomsten vergelijkingen tussen *Albinaria*, *Cristataria*, *Isabellaria*, *Sericata* en *Carinigera* mogelijk maken. Door hun grotendeels overeenkomstige levenswijze en hun geografische nabijheid valt te verwachten dat de evolutie binnen de vijf geslachten volgens dezelfde patronen verloopt. Anderzijds zijn het juist deze overeenkomsten die de onderlinge afbakening van de geslachten bemoeilijken, waardoor het lastig wordt om de eenheden in evolutionair onderzoek te definiëren. Het heeft immers weinig zin om de verwantschappen tussen bijvoorbeeld *Albinaria* soorten te bepalen, wanneer die soorten niet duidelijk als groep herkenbaar zijn. Om deze redenen richt dit proefschrift zich op de verwantschappen tussen soorten uit deze vijf oost-mediterrane geslachten.

Evolutionaire verwantschappen

Studies naar de evolutionaire geschiedenis van de geslachten *Albinaria*, *Cristataria*, *Isabellaria*, *Sericata* en *Carinigera*, zijn aangewezen op hun huidige soorten, omdat fossielen van deze geslachten, of hun voorouders, ontbreken. De evolutionaire verwantschappen tussen huidige soorten kunnen worden achterhaald door het opsporen van kenmerken die duiden op een gemeenschappelijke afstamming. Een voorbeeld van zo'n kenmerk is het huisje van de familie Clausiliidae. Het unieke en complexe clausiliair apparaat (CA), het sluitmechanisme in hun huisje, moet ooit in een gemeenschappelijke voorouder zijn ontstaan, en daarna zijn doorgegeven aan zijn afstammelingen, de huidige Clausiliidae. Men spreekt in zo'n geval van een gemeenschappelijk afgeleid kenmerk. Op soortgelijke wijze is de onderfamilie Alopiinae gedefinieerd op basis van genitaal-anatomische kenmerken.

Noch schelpkenmerken, noch genitaal-anatomische kenmerken bieden goede aanknopingspunten voor het afgrenzen van geslachten binnen de Alopiinae. Hiervoor zijn de verschillen tussen soorten vaak te klein en te weinig samenhangend. Het gemakkelijkst te benoemen waren, tot voor kort, de *Isabellaria* soorten. Al deze soorten beschikken over een clausiliair aparaat (CA) dat de opening van het slakkenhuis volledig kan afsluiten. Dit in tegenstelling tot de soorten uit *Albinaria* (destijds), *Cristataria, Sericata, Carinigera* en de meeste andere Alopiinae. Aangenomen werd daarom, dat dit type CA in de gemeenschappelijke voorouder van de *Isabellaria* soorten was ontstaan, nadat deze zich had afgesplitst van de voorouders van de andere soorten. Zo wordt ook aangenomen dat enkele (schijnbare) genitaal-anatomische verschillen tussen de *Carinigera* soorten en de soorten van de andere vier genera hun oorsprong hebben in een gemeenschappelijke voorouder van de *Carinigera* soorten. De soorten binnen ieder van de resterende geslachten, namelijk *Albinaria, Cristataria* en *Sericata*, kunnen daarentegen niet worden verenigd op basis van gemeenschappelijke afgeleide schelpkenmerken of genitaal-anatomische kenmerken. Zodoende blijft het onduidelijk of ieder van deze geslachten daadwerkelijk een afzonderlijke tak in de stamboom vormt, en daarmee een eigen evolutionaire geschiedenis heeft, die we kunnen bestuderen.

Moleculair verwantschapsonderzoek

Genitaal-anatomische kenmerken en schelpkenmerken zijn voorbeelden van zogenoemde morfologische kenmerken, genoemd naar het Griekse morphe (vorm), omdat het in beide gevallen beschrijvingen van vormen en structuren betreft. De laatste jaren is een andere categorie kenmerken, namelijk moleculaire kenmerken, steeds belangrijker geworden in verwantschapsreconstructie. Het gaat daarbij met name om DNA-sequenties, de opeenvolging van ‘letters’ in het DNA. DNA-sequenties van verschillende soorten kunnen letter voor letter met elkaar vergeleken worden, waarbij iedere letter in feite een kenmerk is, dat informatie over verwantschappen kan bevatten. Op deze manier leveren DNA-sequenties vele discrete kenmerken, die kunnen worden geanalyseerd doormiddel van modellen en statistische toetsen.

Voor zover bekend, spreken DNA-sequenties de traditionele indeling in geslachten tegen. Zo laten de DNA-gegevens zien dat de oorspronkelijke geslachten *Albinaria* en *Isabellaria* niet ieder een aparte voorouder hebben, maar dat sommige *Isabellaria* soorten in de stamboom genesteld zijn tussen *Albinaria* soorten. Dit heeft geleid tot een frustrerende situatie, waarin enerzijds getwijfeld wordt aan de waarde van morfologische kenmerken, terwijl deze kenmerken anderzijds het enige aanknopingspunt leveren voor verwantschapsbepaling zolang er niet meer soorten moleculair onderzocht zijn.

In dit proefschrift worden de evolutionaire verwantschappen tussen een aantal soorten van de vijf geslachten bepaald op basis van diverse DNA-sequenties. Aan de hand van de verkregen evolutionaire stamboom (fylogenie), worden uitspraken gedaan over verspreidingsgeschiedenis, kenmerk-evolutie en het ontstaan van verschillen tussen soorten.

Opzet van het proefschrift

Voordat de verwantschappen tussen *Albinaria*, *Cristataria*, *Isabellaria, Sericata* en *Carinigera* soorten kunnen worden onderzocht, moet eerst nagegaan worden of de soorten van al deze geslachten samen daadwerkelijk één groep, dat wil zeggen één tak in de stamboom, vormen. In het bijzonder de positie van *Carinigera* is daarbij cruciaal. Om deze te bepalen zijn twee stukken DNA uit de celkern, de zogenaamde 'internal transcribed spacers', afgekort ITS, onderzocht. DNA-sequenties van *Carinigera* soorten zijn vergeleken met die van soorten uit een aantal nauwverwante genera, waaronder *Albinaria*, *Cristataria*, *Isabellaria* en *Sericata*. De uitkomsten van deze analyses geven aan dat de laatste gemeenschappelijke voorouder van *Albinaria*, *Cristataria*, *Isabellaria* en *Sericata* tevens de *Carinigera* soorten heeft voortgebracht, terwijl alle andere geslachten eerder zijn afgetakt. *Carinigera* blijkt zelfs nauwer verweven met *Sericata* dan aanvankelijk gedacht. Twee van de *Carinigera* soorten zijn namelijk samen het nauwst verwant aan naburige *Sericata* soorten. Deze resultaten laten zien dat de genitaal-anatomische kenmerken die worden gebruikt voor het bepalen van relatief verre verwantschappen al tussen nauwverwante soorten kunnen verschillen.

De verwantschappen binnen de groep van *Albinaria*, *Carinigera*, *Cristataria*, *Isabellaria* en *Sericata* soorten, worden verder onderzocht in hoofdstuk 3 en 4. In hoofdstuk 3 worden de meest oostelijke geslachten *Albinaria* en *Cristataria* van elkaar afgegrensd. Hiertoe wordt gebruik gemaakt van DNA uit verschillende delen van de cel, namelijk ITS uit de celkern en 12S uit het mitochondrion. Het DNA in de celkern is afkomstig van beide ouders. Het mitochondrion, daarentegen, wordt in principe uitsluitend via de eicel, dus langs vrouwelijke lijn, aan het nageslacht doorgegeven. Kern-DNA en mitochondriaal DNA erven dus apart van elkaar over, waardoor de ITS en 12S sequenties twee — tot op zekere hoogte — onafhankelijke genealogische informatiebronnen vormen, die met elkaar kunnen worden vergeleken. Centraal in de analyse staat de verwantschap van *Albinaria hedenborgi*, een sleutelsoort in de classificatie van de meest oostelijke *Albinaria* soorten. Hoewel *A. hedenborgi* uitsluitend voorkomt in Libanon, midden in het verspreidingsgebied van *Cristataria*, wordt de soort tot *Albinaria* gerekend op basis van enkele genitaal-anatomische kenmerken. Zowel de mitochondriale 12S sequenties als de ITS sequenties uit de kern plaatsen *A. hedenborgi* in de evolutionaire boom tussen de onderzochte *Cristataria* soorten. Deze positie sluit beter aan bij de verspreiding van *A. hedenborgi*. De meer westelijk voorkomende *Albinaria* soorten die zijn onderzocht, inclusief de soorten van Cyprus, vormen wél gezamenlijk één groep. De gevonden verwantschappen hebben implicaties voor de classificatie binnen beide geslachten en voor hun verspreidingsgeschiedenis.

De verwantschappen tussen soorten uit de overige, westelijke, geslachten *Carinigera*, *Isabellaria* en *Sericata* staan centraal in hoofdstuk 4. Deze verwantschappen kunnen iets vertellen over de evolutie van het clausiliair apparaat (CA). Dit sluitmechanisme kan bij *Isabellaria* de gehele schelp-opening afsluiten, terwijl er bij de overige vier geslachten een soort gootje, gevormd uit twee lamellen, openblijft. Lange tijd werd gedacht dat hun CA duidde op een gemeenschappelijke afstamming van alle oorspronkelijke *Isabellaria* soorten. Recent DNA-onderzoek plaatste echter een aantal van deze *Isabellaria* soorten binnen *Albinaria*. Nieuwe DNA-sequenties van ITS en de mitochondriale genen 12S en COI tonen aan dat ook de overige *Isabellaria* soorten niet een eigen gemeenschappelijke voorouder hebben, maar vaak nauwer

verwant zijn aan naburige *Carinigera* of *Sericata* soorten. Het gevonden verwantschapspatroon suggereert dat het compleet afsluitende CA-type van de *Isabellaria* soorten meerdere keren onafhankelijk is geëvolueerd, en dus waarschijnlijk een bepaald voordeel biedt. Mogelijke voordelen van dit CA-type zijn bescherming tegen predatie en uitdroging.

Uit de resultaten in hoofdstuk 4 blijkt dat de soorten *Carinigera buresi* en *Carinigera pharsalica* elkaars nauwste verwanten zijn. In tegenstelling tot de meeste nauw verwante soorten, die in elkaars nabijheid worden aangetroffen, zijn beide soorten geografisch ver van elkaar gescheiden: *C. pharsalica* komt voor in Thessalië in Midden-Griekenland, *C. buresi* in Noord-Oost Griekenland en aangrenzend Bulgarije. De oorzaak van deze vreemde discrepantie tussen de verwantschappen en de verspreiding van *C. buresi* en *C. pharsalica* wordt onderzocht in hoofdstuk 5. *Carinigera buresi* is in schelpkenmerken een heel diverse soort, bestaande uit een groot aantal ondersoorten, die veelal aanvankelijk werden beschouwd als volwaardige soorten. *Carinigera pharsalica*, daarentegen, is voor wat betreft het slakkenhuis een erg uniforme soort. De COI sequenties plaatsen *C. pharsalica* tussen de *C. buresi* ondersoorten uit NO Griekenland. *Carinigera pharsalica* moet dus wel afkomstig zijn uit dit gebied, bijna 200 kilometer van haar huidige verspreidingsgebied. Eigenaardiger nog is de uitkomst dat *C. pharsalica* relatief recent is afgesplitst van *C. buresi*. De relatief korte evolutionaire geschiedenis van *C. pharsalica* komt tot uitdrukking in de COI sequenties van deze soort: er hebben zich nog nauwelijks verschillen in het DNA kunnen ophopen tussen de verschillende *C. pharsalica* slakken. Een mogelijke verklaring voor beide bevindingen is passief transport van de voorouders van *C. pharsalica* vanuit NO Griekenland op blokken marmer, die daar veelvuldig zijn gedolven.

Verder blijkt uit de resultaten van hoofdstuk 4 dat de twee soorten *Isabellaria lophauchena* en *Sericata dextrorsa* veel nauwer aan elkaar verwant zijn dan tot voor kort, getuige hun indeling in verschillende geslachten, werd aangenomen. Beide soorten vallen op omdat ze — in tegenstelling tot vrijwel alle verwante soorten — grotendeels overlappende verspreidingsgebieden hebben, en zelfs vaak samen op dezelfde vindplaats worden aangetroffen. Bovendien is *S. dextrorsa* rechtsgewonden en daarmee het spiegelbeeld van alle nauwverwante soorten, inclusief *I. lophauchena*. Deze situatie doet sterk denken aan observaties aan de landslak *Partula*, waaruit blijkt dat nauwverwante soorten uitsluitend in gebieden waar ze gezamenlijk voorkomen een tegenovergestelde windingsrichting hebben. Aangezien copulatie tussen links- en rechtsgewonden *Partula* slakken sterk bemoeilijkt wordt door de omdraaiing van de positie van de genitaliën, kan de tegenovergestelde windingsrichting in deze gebieden fungeren als een mechanisme tegen het paren met niet-soortgenoten. Zulke paringen met niet-soortgenoten zullen waarschijnlijk minder (vruchtbare) nakomelingen opleveren dan paringen met soortgenoten. Als iets soortgelijks zich voordoet bij voor *I. lophauchena* en *S. dextrorsa*, dan zullen ook slakken uit deze twee soorten niet of nauwelijks met elkaar paren, en vindt er weinig of geen vermenging van hun genetisch materiaal plaats. Vreemd is dan wel, dat de huisjes van beide soorten — los van de tegenovergestelde windingsrichting — soms sterk op elkaar lijken in gebieden waar ze samen voorkomen. Juist deze gelijkenis zou veroorzaakt kunnen zijn door uitwisseling van genetisch materiaal tussen de soorten. Doormiddel van COI sequenties is onderzocht in hoeverre de beide soorten genetisch van elkaar geïsoleerd zijn. De COI sequenties van de onderzochte *I. lophauchena* individuen en de onderzochte *S. dextrorsa*

individuen vormen twee duidelijk aparte clusters, zoals verwacht wanneer de verschillen in windingsrichting kruisingen tussen de soorten bemoeilijken. Desondanks kunnen ook andere factoren verantwoordelijk zijn voor de genetische isolatie tussen *I. lophauchena* en *S. dextrorsa*.

Dit proefschrift laat zien, dat het achterhalen van verwantschappen tussen soorten nieuwe inzichten kan geven in hun evolutie. Tot nu toe is meestal (impliciet) aangenomen dat de meeste morfologische kenmerken binnen de bestudeerde groepen slechts eenmaal, of hoogstens enkele malen, onafhankelijk zijn ontstaan. De fragmentarische verspreiding van deze kenmerken moet in dat geval worden verklaard door migratie van de soorten die dit kenmerk overerfden. De resultaten van het DNA-onderzoek laten het tegenovergestelde zien, namelijk geografische clusters van afstammelingen van één voorouder. Binnen afzonderlijke clusters kunnen soortgelijke kenmerken onafhankelijk ontstaan. Dit betekent dat de spreekwoordelijke traagheid van de onderzochte slakken een veel grotere rol heeft gespeeld in hun evolutie dan tot nu toe werd aangenomen. De nieuwe inzichten in de verwantschappen tussen soorten en in de verspreidingsbeperkingen van de slakken kunnen aanzet geven tot nieuwe studies, zoals hoofdstuk 5 en 6 aantonen.

Chapter 1

GENERAL INTRODUCTION AND SUMMARY

Land snails as a model in evolutionary biology

Humans have always been fascinated by the diversity of life forms, and have tried to recognize structure and patterns within this diversity. Adding a historical dimension to these observed patterns, the concept of evolution eventually opened the way for the study of the underlying processes generating this diversity. Today systematics and evolutionary biology, the study of evolutionary patterns and processes, are two sides of the same coin. Systematics greatly relies on theories of evolutionary process, such as the fixation of new character states, while knowledge of historical relationships is central to evolutionary biology (Lewontin, 2002; Pagel, 1998).

Land snails are a popular group of study organisms in systematics and evolutionary biology, being renowned both for their diversity (Barker, 2001) and for their potential role in elucidating evolutionary processes leading to this diversity (Davison, 2002; Lewontin, 2002). Our understanding of such processes has been greatly enhanced by studies on the interrelationships between species of selected land snail genera, such as *Cerion* (Gould, 2002), *Partula* (for an overview see Johnson *et al*., 1993), *Mandarina* (for an overview see Chiba, 2002) and *Albinaria* (see below).

The clausiliid genera *Albinaria, Cristataria, Isabellaria, Sericata* and *Carinigera*

The species currently classified with the genus *Albinaria* were among the first land snails to attract the attention of evolutionary biologists. This genus is found in the eastern Mediterranean coastal regions, and is part of the Clausiliidae, a family characterized by slender spindle-shaped shells and the presence of a door-like so-called clausilial apparatus (CA) inside the ultimate whorl of the shell. The clausiliid subfamily Alopiinae, in which *Albinaria* is placed, is one of nine subfamilies recognized within the family. Already in 1883, Boettger referred to several *Albinaria* species, noting that "it is evident that, in producing the astonishing variety of species and forms (…) in the Greek islands, 'isolation' was one of the principal factors, and that the question about 'struggle for life' or 'natural selection' was but secondary to it." The last two decades have seen a renewed interest in *Albinaria*, with studies on ecological differentiation (Gittenberger, 1991), morphological evolution (Kemperman & Gittenberger, 1988; van Moorsel *et al*., 2000), molecular evolution (van Moorsel *et al*., 2001b), biogeography (Douris *et al*., 1998a; Welter-Schultes, 2000a), species barriers (Schilthuizen, 1994; Schilthuizen *et al*., 1999a; Giokas *et al*., 2000) and comparative life history (Giokas & Mylonas, 2002). Most of these studies rest on assumptions about the interrelationships between the species studied, and therefore phylogenetic reconstruction is central to this research.

The Alopiinae genera *Carinigera*, *Cristataria*, *Isabellaria* and *Sericata* that surround *Albinaria* have received far less attention. Together with *Albinaria* the four taxa represent the south-easternmost Alopiinae genera. *Albinaria*, *Isabellaria*, *Sericata*, and *Cristataria* are thought to be closely related (Nordsieck, 1977a), although they share no synapomorphic

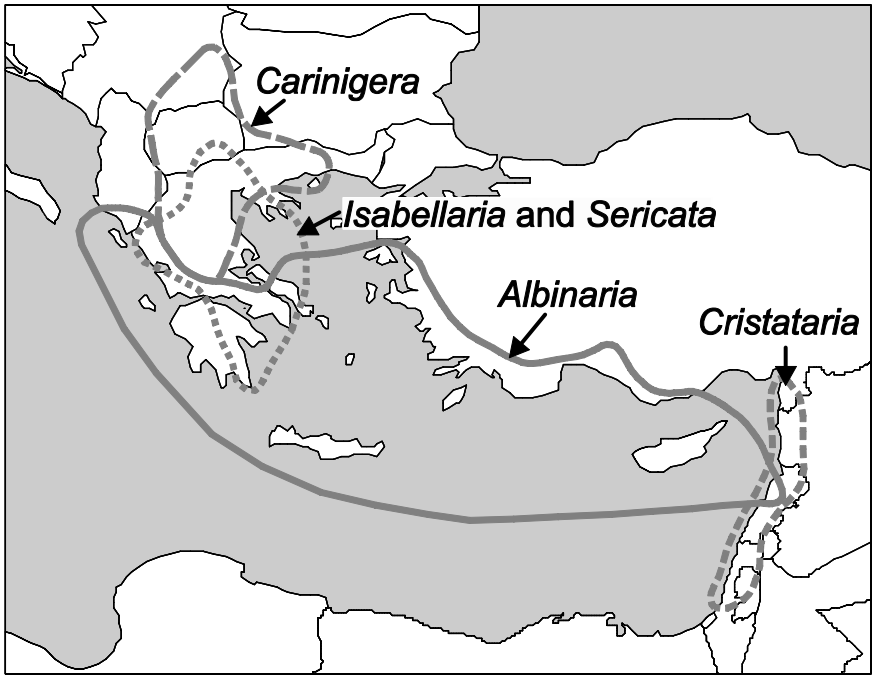


Figure 1.1. Schematic distribution (from NW to SE) of the genera *Carinigera*, *Isabellara* & *Sericata*, *Albinaria* and *Cristataria*, based on the literature and data in the National Museum of Natural History in Leiden.

morphological characters (Nordsieck, 1997). *Carinigera*, in turn, may be most closely related to *Sericata* (Nordsieck, 1972), despite some genital-anatomical features grouping *Carinigera* with a different tribe (Nordsieck, 1997).

There are several reasons to include species from *Carinigera*, *Cristataria*, *Isabellaria* and *Sericata* in phylogenetic studies along with *Albinaria* species. These genera are in many respects highly similar to *Albinaria*. As far as studied, species of all five genera live on limestone substrate, feeding predominantly on lichens, sometimes supplemented with bryophytes (Heller & Dolev, 1994; Giokas & Mylonas, 2002). *Cristataria* species, which occur in relatively arid regions, are found predominantly on vertical north-facing rocks (Bar, 1977). Species of the other genera occur on limestone outcrops and boulders, as well as under stones (Giokas & Mylonas, 2002; pers. obs.). The often mosaic distribution of both congeneric and allogeneric species does not coincide with obvious differences in habitat and niche, and has been attributed to competitive exclusion (Nordsieck, 1974; Gittenberger, 1991). Only species of the genus *Albinaria* have been found syntopical with other species, viz. *Sericata* and *Isabellaria* species, across a large area. Their co-occurrence may be facilitated through niche differentiation (Nordsieck, 1974). In addition, reciprocal displacement of their niches may allow some *Albinaria* species to coexist in areas where their ranges overlap (Gittenberger, 1991). Generally, snails from all genera are active around the winter, aestivating during the summer (Bar, 1977; Heller & Dolev, 1994; Giokas & Mylonas, 2002; pers. obs.). Under favourable weather conditions, tens (Giokas & Mylonas, 2002) or even hundreds (Heller & Dolev, 1994) of snails can be found on a square meter of limestone substrate. Individual snails may spend months or longer on a single limestone boulder, not crossing even small distances of unfavourable habitat, as shown by observations on *Cristataria elonensis* (Bar, 1977) and on *Albinaria corrugata* (Schilthuizen & Lombaerts, 1994). This low vagility and confinement to limestone rocks may have played a role in the speciation and morphological divergence within *Albinaria* (Nordsieck,

1997). If true, similar evolutionary processes may well operate in the adjacent genera. (see also Nordsieck, 1997).

Phylogenetic studies of *Albinaria*, *Carinigera*, *Cristataria*, *Isabellaria* and *Sericata* can build on extensive knowledge from previous taxonomical, biogeographical and palaeogeological studies. The reality of the intrageneric taxa, recognized on basis of a combination of conchological characters, is generally undisputed, apart from occasional disagreement about their rank as either species or subspecies. Ranges have been well documented, and several species may occur at a relatively small spatial scale, viz. within tens of kilometres from each other. This close proximity of species, the generally well-documented collection sites, the high local abundance of the snails, and their low mobility make it possible to collect large samples from a wide array of species within a relatively short time span without endangering the populations sampled. Finally, the geological history of the eastern Mediterranean area is well known. Being slow-dispersing limestone-dwelling animals, well-dated geological events have left their traces not only within the phylogeny of *Albinaria* (e.g. van Moorsel *et al*., 2001c; Douris *et al*. 1998a), but possibly also in the interrelationships among the eastern Mediterranean Alopiinae species as a whole.

Most important, however, the genera *Albinaria*, *Cristataria*, *Isabellaria*, *Sericata* and *Carinigera* are interlinked to the extent that it is difficult to separate them as distinct morphological and biogeographical units (see below). The monophyly of none of the genera is certain, and we therefore do not know whether the species included share a common evolutionary history to the exclusion of species from other alleged genera. The poor delineation and uncertain monophyly of each of the genera thus hampers studies into their evolutionary history.

The delineation of *Albinaria, Cristataria, Isabellaria, Sericata* and *Carinigera*

The morphological delineation of *Albinaria*, *Cristataria*, *Isabellaria*, *Sericata* and *Carinigera* has always been problematic. From the nineteenth century onward, researchers have struggled to recognize groups within the multitude of forms of eastern Mediterranean Alopiinae, and the current classification of the species into five taxa has its roots in this period. The genera were originally defined on basis of conchological characters, which were supplemented with genital-anatomical ones during the twentieth century. Nevertheless, the distinction between the genera became increasingly blurred, as ever more ‘intermediate’ species were described. The current classification largely dates back to the nineteen seventies. At that time an attempt was made by Nordsieck to revise all five nominal genera by detailed studies of both conchological and genital-anatomical characters using their type species as a reference (Nordsieck, 1971; 1972; 1974; 1977a; 1977b). Being the most extensive studies so far, the proposed allocation of species to each of the five genera has been largely maintained to the present day.

Supposedly derived morphological characters supporting generic monophyly have been documented from the genera *Isabellaria* and *Carinigera* only. *Isabellaria* has a CA that facilitates complete sealing of the aperture, while *Carinigera* is characterized by a set of mostly graded genital-anatomical features. These respective characters are absent in the other four nominal genera, but are encountered within other Alopiinae. Even so, species of both genera

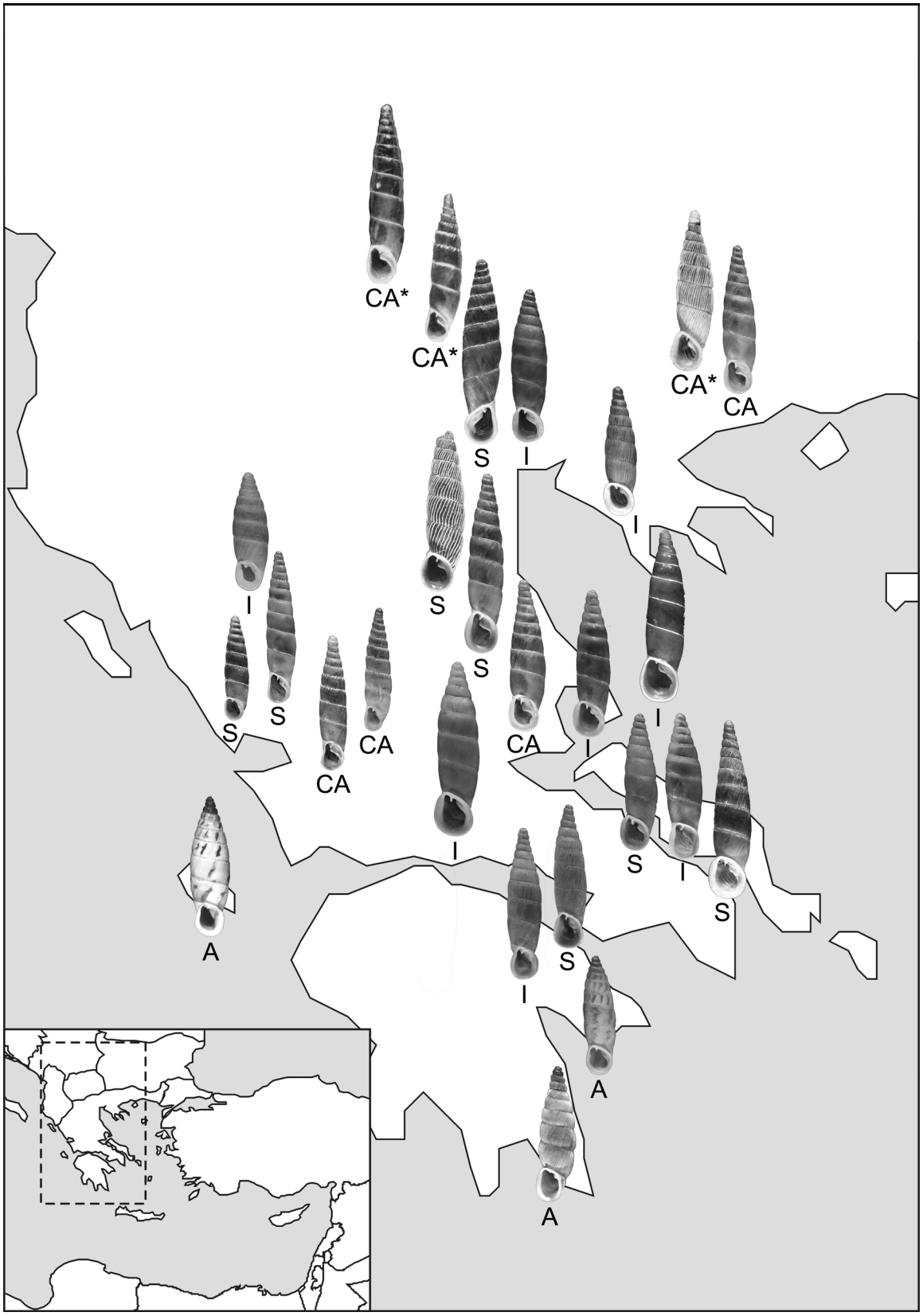


Figure 1.2. Shells and approximate area of occurrence of selected *Albinaria* (A), *Isabellaria* (I), *Sericata* (S) and *Carinigera* (CA) species. * After Brandt (1962).

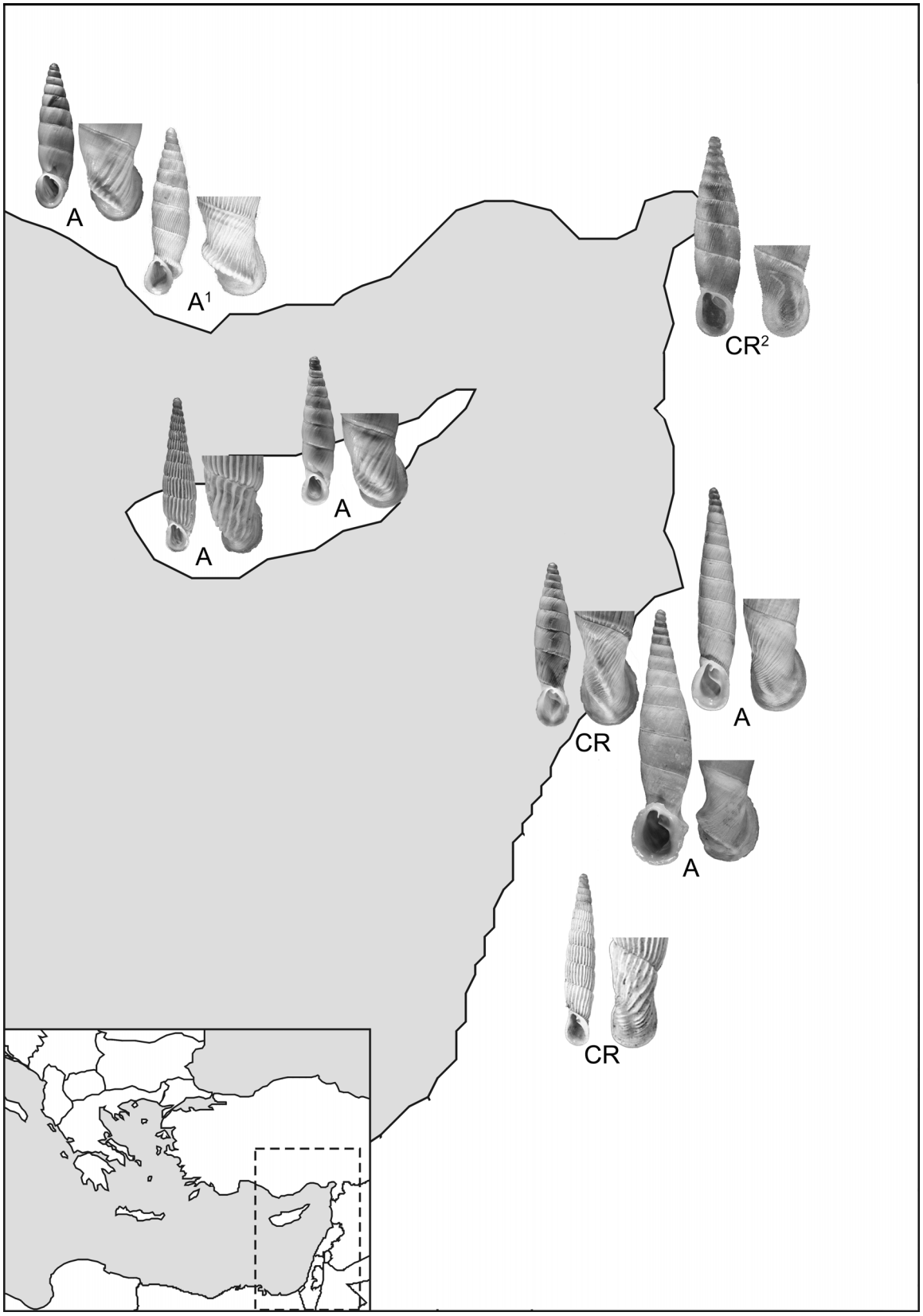


Figure 1.3. Shells and approximate area of occurrence of selected *Albinaria* (A) and *Cristataria* (CR) species. [1] After Gittenberger & Menkhorst (1992); [2] After Szekeres (1998).

exhibit striking conchological similarities with *Sericata* species. The genera *Albinaria*, *Cristataria* and *Sericata* intergrade both conchologically and genital-anatomically. In fact, Nordsieck (1997) later broadened *Albinaria* to include *Sericata* as a subgenus, although he maintained *Cristataria* as a separate genus on basis of graded genital-anatomical features. In order to avoid unnecessary confusion, this classification is not followed here.

The genera originally recognized by Nordsieck intertwine spatially like the segments of a chain, often showing a mosaic distribution in their regions of overlap. From the southern Balkans to Israel we find the successive overlapping ranges of *Carinigera*, *Isabellaria* & *Sericata*, *Albinaria*, and finally *Cristataria* (Figs. 1.1-1.3). In the regions of overlap between genera, the generic assignment of species often becomes arbitrary.

Molecular data and morphology

Molecular data are an important source of information on phylogenetic relationships and are independent of morphological characters (Collin, 2003). Moreover, molecular data show evolutionary change that can be easily modelled, usually providing an ample supply of character change even in morphologically static lineages, and typically producing data sets that can be analysed statistically. So far, molecular studies have focussed on the genus *Albinaria* and Peloponnesian *Isabellaria* species (Douris *et al.*, 1998b; van Moorsel *et al.*, 2000). These studies confirmed the doubts about the monophyly of *Albinaria*, and even refuted the monophyly of *Isabellaria*. They could not, however, completely resolve the relationships between the species placed within the genera studied, due to a combination of incomplete species sampling and poorly supported branches within the tree. Ironically, by refuting the monophyly of *Isabellaria*, the molecular studies clearly demonstrated that the apomorphic CA-type of *Isabellaria*, which was considered the only clear-cut synapomorphy, has evolved several times in parallel and may even have undergone reversals (Douris *et al*., 1998b; van Moorsel *et al*. 2000). On the basis of these results, Gittenberger (1998a) transferred the *Isabellaria* species from the south-eastern Peloponnese to *Albinaria*, retaining a yet poorly studied and ill-defined group of more northerly distributed so-called 'true' *Isabellaria* species.

At this point, the ideas about the phylogenetic relationships between the eastern Mediterranean Alopiinae, are in a state of limbo. On the one hand, morphological characters appear to be misleading, and the groups recognized on the basis of such characters may not be monophyletic. On the other hand, molecular data are missing for most species and even for entire genera such as *Carinigera* and *Cristataria*. This thesis aims to construct a molecular phylogenetic framework for eastern Mediterranean Alopiinae, focussing in particular on the yet

Table 1.1. Number of species classified with *Albinaria, Cristataria, Isabellaria, Sericata* and *Carinigera*.

Nominal (sub)genus	Following Nordsieck (1999)	Following Gittenberger (1998a)
Albinaria	100 (excluding subgenus *Sericata*)	107
Cristataria	24	24
Isabellaria	21	14
Sericata	14 (as a subgenus of *Albinaria*)	14
Carinigera	11	11
total	170	170

poorly studied 'true' *Isabellaria*, *Sericata* and *Carinigera* species, all of which occur in northern Greece and the southern Balkans. Using this framework, questions about phylogeography, character evolution and evolutionary processes within the taxa involved can then be addressed and new theories can be formulated.

Outline of this thesis

Prior to studying eastern Mediterranean Alopiinae in more detail, its monophyly, either including or excluding *Carinigera*, needs to be ascertained. This issue is addressed in the second chapter. ITS data from a wide array of Alopiinae species indicate that *Carinigera Albinaria*, *Cristataria*, *Isabellaria* and *Sericata* constitute a monophyletic group. Within this group, at least two separate *Carinigera* clades were found, more closely related to adjacent *Sericata*, and *Sericata* plus *Isabellaria* species, respectively, than to each other. These results imply that the genital-anatomical characters on which the classification of *Carinigera* was based are homoplasious even at the generic level.

The monophyly of the individual genera *Albinaria*, *Carinigera*, *Cristataria*, *Isabellaria* and *Sericata*, constituting this newly found clade, and the interrelationships between their composite species are investigated in Chapters 3 and 4. Chapter 3 sets out to delimit the easternmost genera *Albinaria* and *Cristataria*. In particular the uncertain phylogenetic position of *A. hedenborgi* from Lebanon is examined, since this is a key species in the classification of eastern Mediterranean species with *Albinaria*. Occurring within the range of *Cristataria,* this *A. hedenborgi* is, nevertheless, placed within *Albinaria* on the basis of genital-anatomical characters. Both 12S and ITS sequences of *A. hedenborgi* are nested among *Cristataria* sequences, as would be expected on the basis of distributional data, whereas *Albinaria* sequences from the more westerly parts of the range, including Cyprus, constitute a monophyletic group. The systematic and phylogeographic implications of this discovery are discussed.

The interrelationships between species from the remaining western genera *Carinigera*, *Sericata* and *Isabellaria* (sensu Gittenberger, 1998a) are examined in Chapter 4. These interrelationships are of particular interest with respect to the evolution of the CA-type. Combined ITS, 12S and COI sequences demonstrate that *Isabellaria* sensu Gittenberger (1998a) is not monophyletic, in spite of its supposedly apomorphic CA-type. Instead, species placed within this genus are nested among geographically close *Sericata* and *Carinigera* species, a topology indicating the recurrent evolution of the *Isabellaria*-type CA, which facilitates a more complete seal of the aperture. Such recurrent evolution may be promoted by improved protection against either predation or desiccation.

In contrast to the other species examined in Chapter 4, the geographically widely disjunct nominal species *C. buresi* from NE Greece and *C. pharsalica* from Thessalia were found to cluster in the phylogenetic tree. Chapter 5 further examines these two taxa. *Carinigera buresi* is considered a very heterogeneous species, consisting of many subspecies, several of which were formerly ranked as species. The analyses of COI sequences demonstrate that the *C. pharsalica* sequences are a monophyletic group, nested among *C. buresi* sequences. This result is congruent with the very small divergence between the *C. pharsalica* sequences as compared to the sequences of *C. buresi*, indicating a relatively recent long-distance dispersal event. Such a

mode of dispersal may be attributable to limestone transports in antiquity. Based on these results, the nominal species *C. pharsalica* is classified as a subspecies with *C. buresi*.

Another insight revealed by Chapter 4 is that the species *Sericata dextrorsa* and *Isabellaria lophauchena* are far more closely related than had previously been acknowledged. These species share largely overlapping ranges and are often found syntopically, as discovered during fieldwork for this thesis. Interestingly, the two species have an opposite direction of coil. Such differences in chirality have been reported from the land snail *Partula* and in that case apparently prevent or limit copulation and subsequent hybridization between similarly closely-related species in regions of range overlap. Using the marker COI, Chapter 5 examines whether transfer of mitochondrial DNA has occurred at population and species level between *S. dextrorsa* and *I. lophauchena*. No evidence for such transfer was found. This is consistent with the hypothesis that the difference in chirality acts as an isolating mechanism, although other mechanisms cannot be ruled out.

Apart from establishing a phylogenetic framework for future studies, this thesis provides new insights into the evolution of eastern Mediterranean Alopiinae. So far, it has been implicitly assumed that within eastern Mediterranean Alopiinae most new traits evolved singularly or a few times at most, and that the scattered spatial distribution of these traits resulted from their subsequent spread. Conversely, the general picture that emerges from this thesis is one of geographically confined clades, in which morphological traits evolved in parallel. These results demonstrate that their vagility has constrained the spread of the snails studied far more and far deeper in time than previously thought. The insights into the interspecific interrelationships, in particular between *Carinigera*, *Isabellaria* and *Sericata* species, and into the long-term vagility of the snails, offer new prospects for further evolutionary studies, as demonstrated by Chapters 5 and 6.

Chapter 2

RE-EVALUATING *CARINIGERA*: MOLECULAR DATA OVERTURN THE CURRENT CLASSIFICATION WITHIN THE CLAUSILIID SUBFAMILY ALOPIINAE (GASTROPODA, PULMONATA)

ABSTRACT

The current subdivision of the clausiliid subfamily Alopiinae relies for a large part on genital-anatomical characters. Based on a few such characters *Carinigera* is placed within the tribe Montenegrinini, whereas *Isabellaria* and *Sericata* are included within the tribe Medorini. This classification might not be expected on the basis of two observations: (1) *Carinigera* is conchologically indistinguishable from *Sericata* and highly similar to *Isabellaria* and (2) *Carinigera*, *Isabellaria* and *Sericata* have mosaic distributional patterns in central and northern Greece**,** which are difficult to explain given the low vagility of snails of these genera.

The complete ITS1&ITS2 and partial 18S rRNA, 5.8S rRNA and 28S rRNA sequences used in this study reveal that all *Carinigera* sensu auct. species are nested among Medorini, and should therefore be placed within that tribe. Apart from this, the results largely support the current higher classification of the Clausiliidae. *Carinigera* sensu auct. consists of at least two clades, which are not sister groups. Both are related to geographically close species hitherto classified with *Sericata* or *Isabellaria*. The two groups of *Carinigera* do not correspond to the alleged subgenera *Angiticosta* and *Carinigera* s.s. This study shows that, like conchological characters, the traditional diagnostic genital-anatomical characters used at tribe level suffer more often from homoplasy than previously thought. Therefore, classifications based on only a few of such characters can be erroneous and should be mistrusted, especially when they conflict with both conchological and distributional patterns, as in *Carinigera*.

INTRODUCTION

Classifications have traditionally been based on morphological data. Unfortunately, many morphological structures are prone to parallelism or convergence, especially those structures that are in direct contact with the external environment. Gastropod shells, providing the external protection of snails, exemplify how similar environments may cause similar structural adaptations (Goodfriend, 1986). Not surprisingly then, in gastropod systematics, shell morphological characters are often considered inferior to anatomical ones (e.g. Schmidt, 1855; Kool, 1993). However, recent studies (Schander & Sundberg, 2001; Collin, 2003) revealing an equal amount of homoplasy in both character types, have questioned this practice.

Various authors emphasize that within the stylommatophoran land snail family Clausiliidae, parallelism and convergence in shell morphological characters have often been mistaken for homology (Nordsieck, 1978a: 69; Douris *et al.*, 1998b; van Moorsel *et al.*, 2000). Due to the supposed paucity of informative conchological characters, the higher classification within the clausiliid subfamily Alopiinae is based largely on genital anatomy. On the basis of

Dennis R. Uit de Weerd and Edmund Gittenberger. Re-evaluating *Carinigera*: molecular data overturn the current classification within the clausiliid subfamily Alopiinae (Gastropoda, Pulmonata).

Table 2.1. Division of alopiinid genera into tribes, according to Nordsieck (1979, 1997, 2002). Genera marked with an asterisk are represented in the analyses. On the basis of the molecular data, *Carinigera* has to be transferred from the Montenegrinini to the Medorini.

tribe	genera
Montenegrinini H. Nordsieck, 1972	*Carinigera* Moellendorf , 1873 *
	Montenegrina O. Boettger, 1877 *
	Protoherilla A. J. Wagner, 1921
Medorini Brandt, 1961	*Agathylla* H. & A. Adams, 1855 *
	Albinaria Vest, 1867 *
	Cristataria Vest, 1867 *
	Isabellaria Vest, 1867 *
	Lampedusa O. Boettger, 1877
	Leucostigma A. J. Wagner, 1919
	Medora H. & A. Adams, 1855 *
	Muticaria Lindholm, 1925 *
	Sericata O. Boettger, 1878 *
	Strigilodelima A. J. Wagner, 1924 *
Alopiini A.J. Wagner, 1913	*Alopia* H. & A. Adams, 1855
	Herilla H. & A. Adams, 1855 *
	Triloba Vest, 1867
Cochlodinini Lindholm, 1925	*Cochlodina* Férussac, 1821 *
	Macedonica O. Boettger, 1877 *
Delimini Brandt, 1956	*Barcania* Brandt, 1956
	Charpentieria Stabile, 1864
	Delima Hartmann, 1842
	Dilataria Vest, 1867
	Papillifera Hartmann, 1842 *

these characters, Nordsieck (1997: 54) divided the subfamily into five tribes (Table 2.1): Medorini, Alopiini, Cochlodinini, Montenegrinini and Delimini.

This current division is problematic for *Carinigera* Moellendorf, 1873, a so-called genus from the southern Balkan Peninsula. *Carinigera* is intermediate in genital anatomy between genera currently placed in either the Montenegrinini or the Medorini (see Nordsieck, 1963: 92; 1969: 251, 252, 259, 263), and closely resembles some genera of the Medorini conchologically. Nordsieck (1972) placed *Carinigera* in the Montenegrinini, together with the genera *Montenegrina* O. Boettger, 1877 and *Protoherilla* A. J. Wagner, 1921, which occur in the south-western part of the Balkan Peninsula.

The correct systematic position of *Carinigera* is important for at least two reasons. First, it permits an evaluation of the systematic value of genital-anatomical characters used to characterize and classify *Carinigera*. These characters are considered important tools for the classification within the subfamily as a whole. Second, it may clarify whether the tribe Medorini sensu Nordsieck (1997) is monophyletic. The tribe Medorini is the best studied tribe of the Clausiliidae, since it includes the genus *Albinaria* Vest, 1867, which is used as a model in numerous phylogeographical, evolutionary and ecological studies. Phylogenetic analyses of *Albinaria* and supposedly related genera are hampered by the uncertain monophyly of the Medorini, which complicates the selection of ingroup and outgroup species.

According to Nordsieck (1972: 9, 26), the distinction between the genera currently placed in the tribe Montenegrinini and those grouped in the Medorini is based on two characters (Fig.

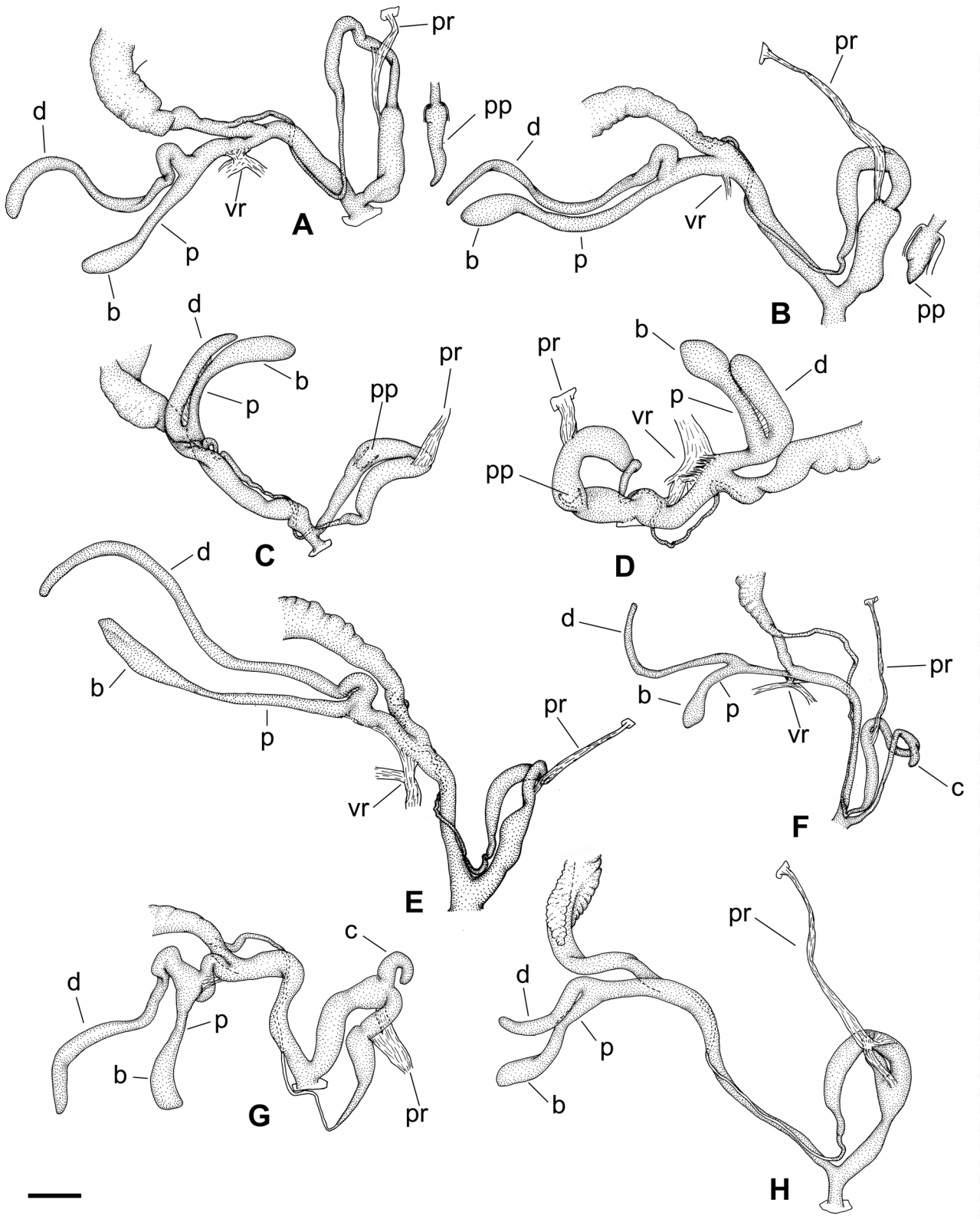


Figure 2.1. Genital anatomy of Montenegrinini (**A-D**) and Medorini (**E-H**). **A** *Carinigera (Carinigera) hausknechti hiltrudae*; **B** *Carinigera. (Carinigera) megdova tavropodensis;* **C** *Carinigera (Angiticosta) superba*; **D** *Montenegrina janensis maasii*; **E** *Sericata albicosta*; **F** *S. inchoata inchoata*; **G** *S. sericata sericata;* **H** *Isabellaria vallata errata*. The scale bar (left below) represents 1.0 mm. Abbreviations: c: caecum; b: bursa; d: diverticulum; p: pedunculus; pp: penial papilla; pr: penial retractor; vr: vaginal retractor. The insertion of the vaginal retractor could not be clearly located in *C. superba*, *S. sericata* and *I. vallata.*

2.1): (1) a perforated penial papilla, i.e. an inward bulge at or near the opening of the epiphallus into the penis, which is present in the Montenegrinini but absent in the Medorini; (2) a vaginal retractor, which is muscular in the Montenegrinini and connective-tissue-like in the Medorini. The presence of a penial papilla and the muscular vaginal retractor are considered apomorphic character states (see Nordsieck, 1969: 252, 255; 1978a: Anmerkung 14) and have been used to place *Carinigera* within Montenegrinini (Nordsieck, 1972). According to Nordsieck (1969: 255), the penial papilla in the Montenegrinini is probably derived from a caecum, a vermiform extension of the penis, which is often found in the Medorini. However, the homology of both structures is disputed by Kemperman (1992: 77), who showed that both a papilla and a caecum may be present in a single individual. In contrast to the Montenegrinini, the Medorini have only symplesiomorphic character states (Nordsieck, 1997: 54), which makes the monophyly of this tribe questionable.

On closer examination, the definition of the Montenegrinini is rather poor. Neither of the character states considered diagnostic for the Montenegrinini is found in all three genera of this alleged tribe, and some occur outside the tribe as well. The penial papilla is obsolete in *Protoherilla*, where the opening of the epiphallus is protruding slightly into the penis (Nordsieck, 1972: 36; 1979: Anmerkung 3). Moreover, a penial papilla is also found in some *Albinaria* species (Kemperman, 1992: 50-62, 72-74), a genus considered to belong to the Medorini, and it may even have evolved independently in the ancestors of the *Carinigera* subgenera, *Carinigera* s.s. and *Angiticosta* (Nordsieck, 1977b: 83). The vaginal retractor is rather muscular in *Montenegrina* (Nordsieck, 1969: 259; 1972: 27) and *Protoherilla* (Nordsieck, 1972: 36), but only weakly so in *Carinigera* (Nordsieck 1969: 251, 259; 1972: 9; 1974: 146), which in this respect approaches the connective-tissue-like vaginal retractor in the Medorini.

A third genital-anatomical character, viz. the penial retractor muscle, is supposed to point to a ‘common ancestor’ (‘gemeinsame Stammform’, Nordsieck, 1972: 9) of *Carinigera* and *Sericata* (definition Nordsieck, 1972, 1974), a genus placed in the Medorini. However, the penial retractor muscle is polymorphic in both genera: both a single and a bifurcate state occur (Nordsieck, 1972, 1974, 1977b). All other Montenegrinini have a single retractor, whereas the penial retractor can be single, bifurcate or polymorphic in the other genera of the tribe Medorini (see Nordsieck, 1969, 1972).

While the genital-anatomical characters remain rather inconclusive about the systematic position of *Carinigera* in either the Montenegrinini or the Medorini, conchological characters seem to favour its classification with the Medorini. *Carinigera* shows striking conchological similarities with some so-called genera of the Medorini, especially with *Sericata* and *Isabellaria* (definition Gittenberger, 1998a), but also with *Albinaria* (definition Gittenberger, 1998a) and *Cristataria* (definition Nordsieck, 1971). Before their genital anatomy was studied, species now placed in *Carinigera* were frequently grouped on a conchological basis with species now included in one of those genera (see von Möllendorf, 1873: 141; Boettger, 1877: 49; Westerlund, 1894: 174, 175; Wagner, 1927: 327 (65)-333 (71); Brandt, 1962: 132-141; Nordsieck, 1972: 16).

Of all Medorini, the genera *Isabellaria* and *Sericata* are most similar to *Carinigera* in both conchology and in their ranges (Fig. 2.2), which extend from the north-eastern Peloponnese in

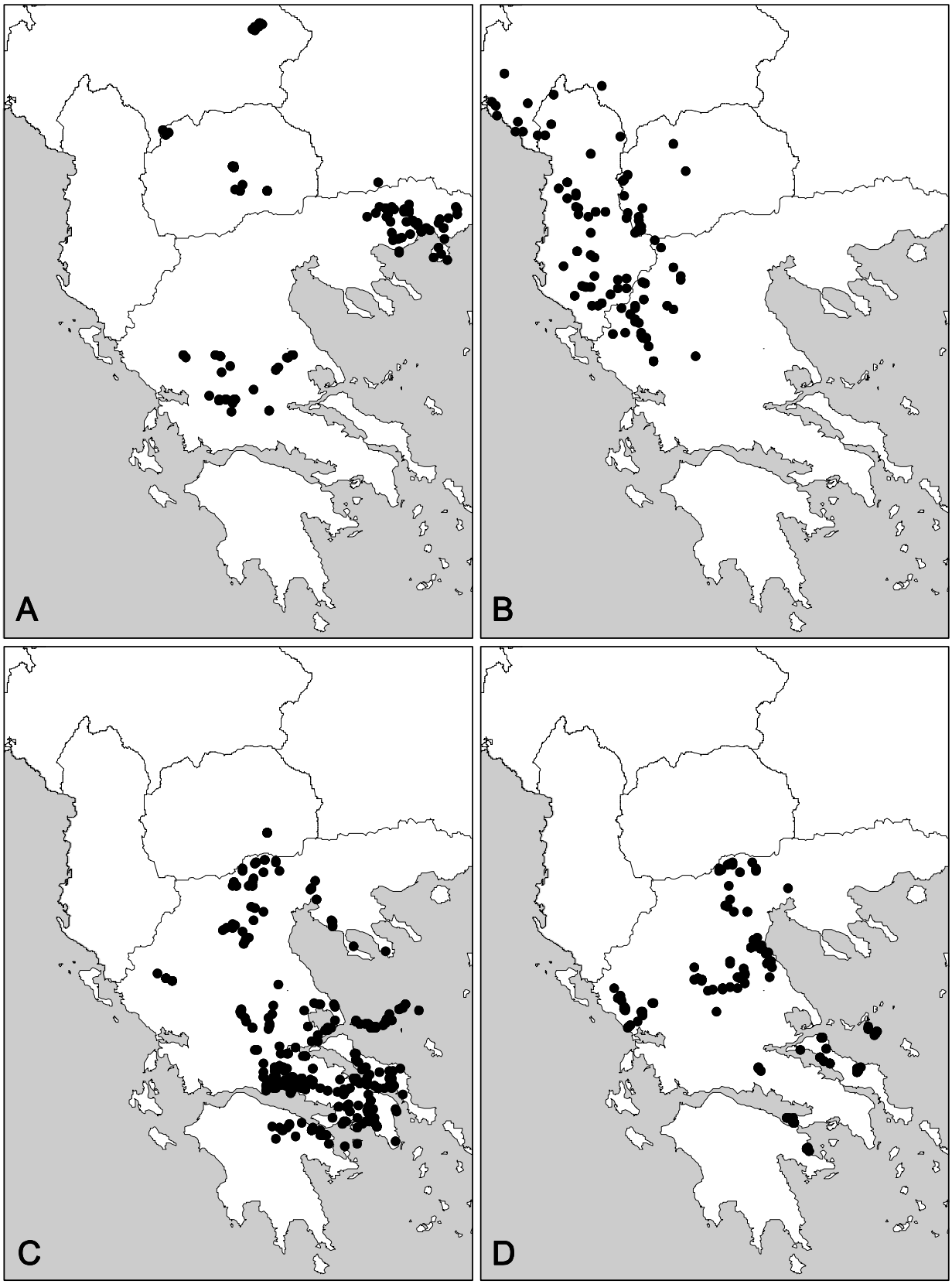


Figure 2.2. Distribution of: *Carinigera* (**A**); *Montenegrina* (**B**); *Isabellaria* (**C**) and *Sericata* (**D**). Distributional data, based on the literature and data in the National Museum of Natural History in Leiden, the Netherlands, are available on request.

Greece into Macedonia. *Carinigera* and *Sericata* cannot be distinguished conchologically. The distinction between these genera is based on the presence of a relatively muscular vaginal retractor and a penial papilla in *Carinigera* only (see Nordsieck, 1972, 1974). *Carinigera* and *Sericata* differ from *Isabellaria* in certain characters of the clausilial apparatus, which are known to be homoplasious within the subfamily (Nordsieck, 1963: 92; 1979: 251, 252; Douris *et al*., 1998b; van Moorsel *et al*., 2000). In the region from central Greece to southern Macedonia, where the range of *Carinigera* overlaps with that of *Isabellaria* and *Sericata*, the three genera have a mosaic (Nordsieck, 1974: 127, 131, 146) pattern of distribution (Fig. 2.2), maybe due to competitive exclusion (Nordsieck, 1974: 132). Here, we find pairs of neighbouring species showing an overall similarity in shell morphology, but placed in different genera on the basis of allegedly diagnostic characters. Two such pairs containing *Carinigera* species have been described (Nordsieck, 1974: 132): *Carinigera hausknechti* (O. Boettger, 1886) forms a species pair with *Sericata inchoata* (O. Boettger, 1889) (see Fig. 2.3), and *Carinigera pharsalica* Nordsieck, 1974, with *Isabellaria clandestina* (Rossmässler, 1857) (see Fig. 2.4). The conchological resemblance between *Carinigera hausknechti* and *Sericata inchoata* initially led Nordsieck (1972: 16) to include *C. hausknechti* in *Sericata*. Both *C. hausknechti* and *S. inchoata* possess shells with a white sutural line and papillae at least on the upper whorls. Such white sutural lines and papillae, however, are also found in other species of both genera, among which the reputed closest relatives of *C. hausknechti* and *S. inchoata*, viz.

Figure 2.3. The species pair *Carinigera hausknechti* (**B**) and *Sericata inchoata* (**C**) together with their respective putative closest relatives *Carinigera megdova* (**A**) and *Sericata regina* (**D**). Scale line 1 mm. Photographs by A 't Hooft (IBL, Leiden).

C. megdova (see Nordsieck, 1974: 147, 148) and *S. regina* (see Nordsieck, 1974: 127), respectively (see Fig. 2.3). Of all *C. hausknechti* subspecies, *C. hausknechti alticola*, *C. hausknechti hausknechti* and *C. hausknechti semilaevis* most closely resemble *S. inchoata*, with which they share the parietal interruption of the peristome. Nevertheless, this character state is also found in other *Carinigera* species. The species of the second species pair, viz. *Carinigera pharsalica* and *Isabellaria clandestina*, have no distinct characters that unite them, but they are similar in habitus (Nordsieck, 1974: 147). Similar pairs of species, occurring in the eastern Peloponnese and originally classified as *Albinaria* and *Isabellaria*, respectively, were found to be sister species, or congeneric at least, in previous studies (Douris *et al*., 1998b, Gittenberger, 1998a; van Moorsel *et al.*, 2000).

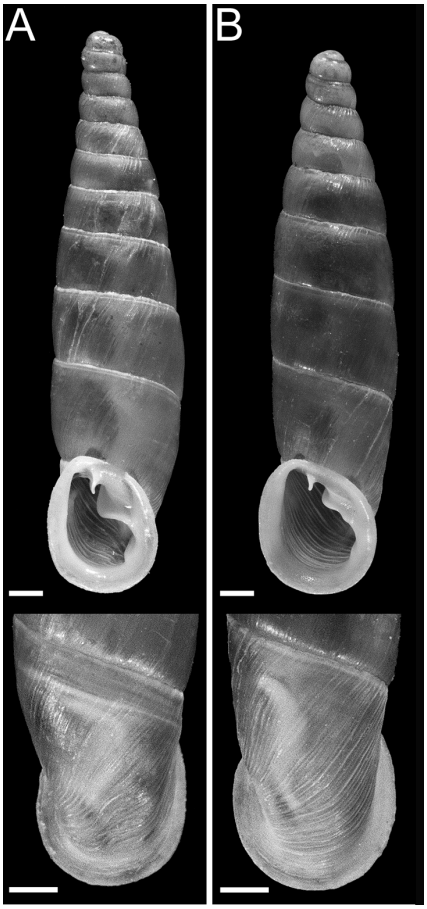


Figure 2.4. The species pair *Carinigera pharsalica* (**A**) and *Isabellaria clandestina clandestina* (**B**). Scale line 1 mm. Photographs by A 't Hooft (IBL, Leiden).

The co-occurrence of similarities in both conchological and distributional patterns between *Carinigera* and the genera *Isabellaria* and *Sericata* can be explained in two ways. Nordsieck (1972, 1974) prefers to give most weight to their genital-anatomical differences. He concludes that *Carinigera* is not closely related to *Isabellaria* and *Sericata*, and that local conchological similarities are the result of convergent evolution (Nordsieck, 1974: 127, 132). This convergence may have resulted from similar habitat conditions owing to the proximity between *Carinigera* and the adjacent ranges of *Isabellaria* and *Sericata* species, in combination with their shared ecological niches (Nordsieck, 1974: 127, 132). Alternatively however, the mosaic distributional patterns, the similar niches, and the conchological similarities of *Carinigera* species and species of *Isabellaria* and *Sericata* could result from recent common descent.

To test these opposing hypotheses, we used an independent data set, viz. the complete nucleotide sequences of the nuclear internal transcribed spacers 1 (ITS1) and 2 (ITS2) and partial sequences of the nuclear 18S rRNA, 5.8S rRNA and 28S rRNA genes of species of *Albinaria*, *Isabellaria*, *Sericata*, *Carinigera*, *Montenegrina* and eight additional genera from the subfamily Alopiinae. We extended this study to the subfamily level, since the Alopiinae is the hierarchically lowest taxon that uncontroversially includes *Carinigera*, *Montenegrina*, *Sericata* and *Isabellaria*. In gastropods, ITS1 is divided into conserved and variable regions (Armbruster *et al.,* 2000) that can resolve relationships at different taxonomic levels. ITS2 is used successfully from the genus level up to the family level in gastropods (Wade & Mordan, 2000; Coleman & Vacquier, 2002). Combined ITS1 and ITS2 data are informative about phylogenetic relationships within the Alopiinae (van Moorsel *et al.*, 2001d).

MATERIALS AND METHODS

Species selection

Samples of the 37 species of Alopiinae investigated in this study were collected in south-eastern Europe and nearby Asia (Fig. 2.5). Shells were deposited in the National Museum of Natural History, Leiden, the Netherlands. The total set of included alopiinid samples is given in Table 2.2. For 25 of these, complete ITS1 and ITS2, and partial 18S rRNA, 5.8S rRNA and 28S rRNA sequences were determined in this study. The corresponding sequences of the other species had already been determined previously (see Table 2.2). Additional partial 18S rRNA, ITS1 and partial 5.8S rRNA sequences were obtained for *Sericata sericata*, *Isabellaria isabellina*, and *Isabellaria perplana*, of which ITS1 sequences had been only incompletely determined previously (van Moorsel *et al.*, 2000). Individuals from the same localities as the original samples were used. The sequences obtained in this study were submitted to GenBank (Accession numbers AY382098 to AY382150).

Our aim was to have all systematic levels addressed in this study represented, and we sampled species accordingly: (1) all tribes within the subfamily Alopiinae, (2) nearly all genera within the tribe Medorini, and (3) all known species of *Carinigera* (except the most recently described *C. pellucida*) and the most divergent species, both anatomically and geographically, within *Isabellaria* and *Sericata*. Thus the so-called systematically isolated *I. praecipua* (see Nordsieck 1972: 17, 18; 1999: note 27), *I. vallata* (see Nordsieck, 1974) and *S. albicosta* (see Nordsieck, 1972: 17; but see Nordsieck, 1999: note 2) are represented, as well as the type species and other species from both genera. The species *S. albicosta* and *I. praecipua* were set apart because of genital anatomy, whereas *I. vallata* is considered systematically isolated (Nordsieck, 1974) for unspecified reasons. Finally, *I. clandestina* and *S. inchoata*, both of which are part of so-called species pairs with *Carinigera* species, are represented, as well as *S. regina*, which is thought to be closely related to *S. inchoata* (see Nordsieck, 1974).

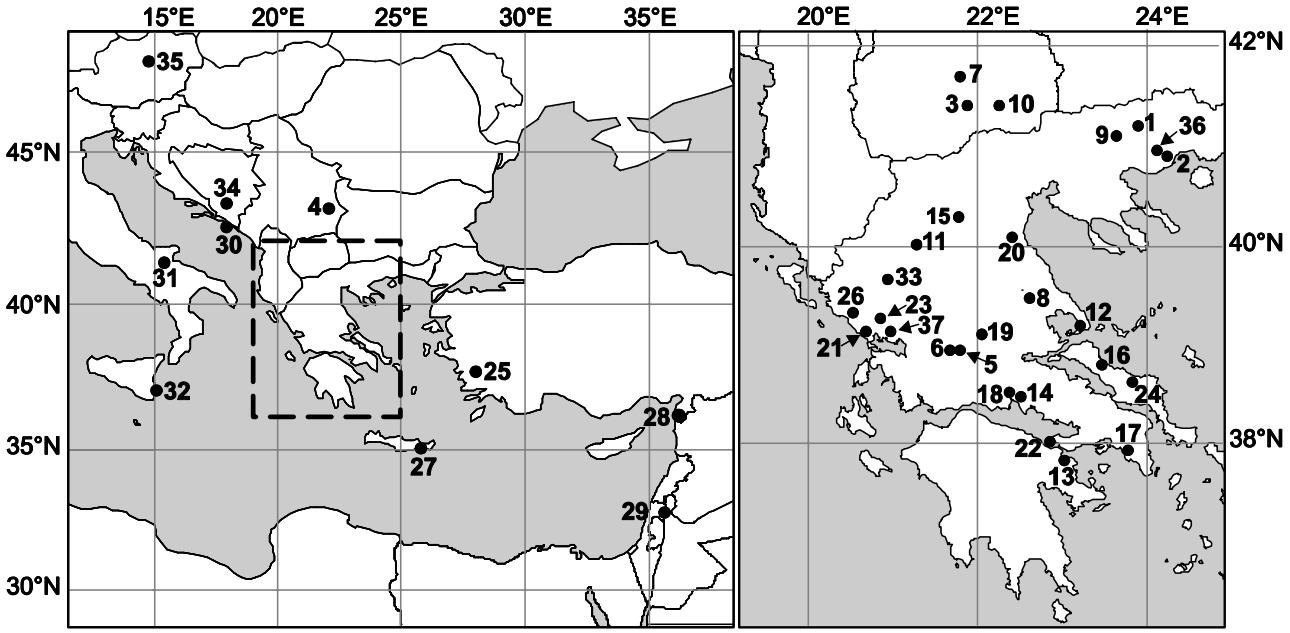


Figure 2.5. Collection sites of the species used in this study. The numbers refer to Table 2.2.

Table 2.2. Sample and sequence information. Numbers refer to the collection sites shown in Figure 2.5. Accession numbers of sequences retrieved from GenBank are given in parentheses.

tribe	genus (subgenus)	species	No.	UTM code	source (GenBank accession number)
Montenegrinini	*Carinigera (Angiticosta)*	*C. superba* Nordsieck, 1977	1	GL4266	this study
	Carinigera (Carinigera)	*C. buresi nordsiecki* Gittenberger, 2002	2	KF6731	this study
		C. drenovoensis (Brandt, 1961)	3	EL78	this study
		C. eximia (Moellendorf, 1873)	4	EN99	this study
		C. hausknechti alticola Nordsieck, 1974	5	EJ6910	this study
		C. megdova tavropodensis Fauer, 1993	6	EJ5910	this study
		C. octava Brandt, 1962	7	EM61	this study
		C. pharsalica Nordsieck, 1974	8	FJ3871	this study
		C. schuetti Brandt, 1962	9	GL2554	this study
		C. septima Brandt, 1962	10	FL08	this study
	Montenegrina	*M. dennisi dennisi* Gittenberger, 2002	11	EK2429	this study
Medorini	*Isabellaria*	*I. clandestina clandestina* (Rossmässler, 1857)	12	FJ9042	this study
		I. isabellina isabellina (L. Pfeiffer, 1842)	13	FG8190	van Moorsel *et al*., 2000 (AF254618); this study
		I. perplana perplana (O. Boettger, 1877)	14	FH3261	van Moorsel *et al.*, 2000 (AF254614); this study
		I. praecipua serviana Nordsieck, 1972	15	EK84	van Moorsel *et al.*, 2000 (AF254602)
		I. riedeli Brandt, 1961	16	GH1498	van Moorsel *et al.*, 2000 (AF254619)
		I. saxicola aperta (Küster, 1861)	17	GH4505	van Moorsel *et al.*, 2000 (AF254613)
		I. thermopylarum faueri Nordsieck, 1974	18	FH2066	van Moorsel *et al.*, 2000 (AF254620)
		I. vallata errata Fauer, 1985	19	EJ93	this study
	Sericata	*S. albicosta* (O. Boettger, 1877)	20	FK2038	this study
		S. inchoata inchoata (O. Boettger, 1889)	21	DJ7233	this study
		S. lutracana Nordsieck, 1977	22	FH6311	van Moorsel *et al.*, 2000 (AF254609)
		S. regina Nordsieck, 1972	23	DJ8647	this study
		S. sericata sericata (L. Pfeiffer, 1850)	24	GH4876	van Moorsel *et al.*, 2000 (AF254612); this study

(Table 2.2. continued)

tribe	genus (subgenus)	species	No.	UTM code	source (GenBank accession number)
Medorini (continued)	*Albinaria*	*A. puella puella* (L. Peiffer, 1850)	25	NB29	van Moorsel *et al.*, 2001
		A. senilis senilis (Rossmässler, 1836)	26	DJ66	van Moorsel *et al.*, 2000 (AF254585)
		A. wiesei Gittenberger, 1988	27	LV6706	van Moorsel *et al.*, 2001
	Cristataria	*C. colbeauiana* (L. Pfeiffer, 1861)	28	BA40	this study
		C. genezerethana (Tristam, 1865)	29	YB35	this study
	Agathylla	*Agathylla lamellosa* (Schubert & Wagner, 1829)	30	BN62	this study
	Medora	*Medora italiana garganensis* (A.J. Wagner, 1918)	31	WG71	this study
	Muticaria	*Muticaria syracusana* (Philippi, 1836)	32	WB20	this study
	Strigilodelima	*Strigilodelima conspersa* (L. Peiffer, 1848)	33	DJ9591	this study
Alopiini	*Herilla*	*Herilla bosniensis rex* Nordsieck, 1971	34	BP61	this study
Cochlodinini	*Cochlodina*	*Cochlodina laminata* (Montagu, 1803)	35	Rotgraben, Austria	van Moorsel *et al.*, 2001
	Macedonica	*Macedonica pangaionica pang*. (Brandt, 1961)	36	KF5532	this study
Delimini	*Papillifera*	*Papillifera papillaris* (Müller, 1774)	37	DJ93	this study; Wade *et al*., 2001 (AY014049)
Outgroup family	**subfamily**	**species**			**reference**
Clausiliidae	Baleinae	*Balea biplicata* (Montagu, 1803)			Winnepenninckx *et al*., 1998 (X94278); van Moorsel *et al.*, 2001
	Clausiliinae	*Macrogastra ventricosa* (Draparnaud, 1801)			van Moorsel *et al.*, 2001
	Mentissoideinae	*Idyla bicristata* (Rossmässler, 1839)			van Moorsel *et al.*, 2000 (AF254616)
	Phaedusinae	*Stereophaedusa japonica* (Crosse, 1871)			Wade *et al*., 2001 (AY014053)
Helicidae		*Arianta arbustorum* (L., 1758)			Armbruster *et al*., 2000 (AF124052)
Cochlicopidae		*Cochlicopa lubricella* (Porro, 1838)			Armbruster & Bernard, 2000 (Y16760); Wade *et al*., 2001 (AY014020)

For rooting this ingroup of Alopiinae (see Table 2.2), we used as outgroup taxa not only species of Clausiliidae, but also two pulmonate stylommatophoran species that are far less closely related, viz. *Arianta arbustorum* (L., 1758) [Sigmurethra, Helicoidea] and *Cochlicopa lubricella* (Porro, 1838) [Orthurethra, Pupilloidea]. The sequence of spacers and ribosomal sequences of *Arianta arbustorum* were determined by Armbruster *et al.* (2000). The sequence of *Cochlicopa lubricella* included a partial 18S rRNA, complete ITS1, and partial 5.8S rRNA sequence from Armbruster & Bernhard (2000), and a partial 5.8S rRNA, complete ITS2, and partial 28S rRNA sequence from Wade *et al.* (2001). The outgroup additionally consisted of representatives of four clausiliid subfamilies: (1) *Stereophaedusa japonica* (Crosse, 1871) [Phaedusinae], (2) *Balea biplicata* (Montagu, 1803) [Baleinae], (3) *Macrogastra ventricosa* (Draparnaud, 1801) [Clausiliinae] and (4) *Idyla bicristata* (Rossmässler, 1839) [Mentissoideinae]. The subfamilies (2), (3) and (4), viz. Baleinae, Clausiliinae and Mentissoideinae, together are thought to form a monophyletic group (see Nordsieck, 1979). Of *Stereophaedusa japonica,* only partial 5.8S rRNA, complete ITS2 and partial 28S rRNA sequences were available (Wade *et al*., 2001). For *Balea biplicata* the terminal (3') 149 base-pair sequence of 18S rRNA, determined by Winnepenninckx *et al.* (1998), was combined with the ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA sequence obtained by van Moorsel *et al*. (2001d). Sequences of *Macrogastra ventricosa* and *Idyla bicristata* were determined by van Moorsel *et al*. (2001d).

DNA sequencing

The specimens, of which sequences were obtained in this study, were collected in the field and stored at -80°C, with exception of *Carinigera drenovoensis*, *Carinigera eximia, Carinigera octava*, *Carinigera septima*, *Cristataria colbeauiana* and *Cristataria genezerethana*. Tissue of *Cristataria genezerethana* was briefly stored in 96% ethanol between collecting and processing, whereas that of the other species had been stored in ethanol for 7 to 34 years. Total genomic DNA was extracted from foot tissue. According to the condition of the tissue, two protocols were used. For tissue that had been frozen since collection and tissue of *C. genezerethana*, the protocol described by Schilthuizen *et al.* (1995) was followed. For older ethanol-preserved tissue, we used a slightly modified protocol to increase DNA yield. This tissue was dissolved in a CTAB buffer with 20 mg/ml proteinase K and 0.2% (v/v) 2-mercapto-ethanol by incubation at 60°C for 10 to 12 hours. The samples were then extracted with chloroform - isoamyl alcohol (24:1), and DNA was precipitated with isopropanol after cooling to 4°C for 10 to 12 hours. The supernatant was removed and the pellet was washed by soaking it in 0.5 ml ethanol/ammonium-acetate for 30 minutes at room temperature. Subsequently, the supernatant was removed and after it had dried, the DNA pellet was dissolved in 300 µl distilled water.

Partial 18S rRNA, complete ITS1 and partial 5.8S rRNA were amplified using modified versions of the universal 18d and 5.8c primers (Hillis & Dixon, 1991): the forward primer 18d-ALB (CACACCGCCCGTCGCTACTACC) and the reverse primer 5.8c-ALB (ATGCGTTCAAGATGTCGATGTTCAA). Our reverse primer (5.8c-ALB) matches the 5.8S rRNA sequences determined by van Moorsel *et al.* (2000), whereas the forward primer (18d) was shortened by 5 bases on the 3' side, to be more compatible with the reverse one.

For amplification of partial 5.8S rRNA, ITS2 and partial 28S rRNA, newly designed primers were used. The forward primer ITS2U-ALB (GGCGGCCTCGGGTCCATCC) was

designed on basis of 5.8S rRNA sequences obtained by van Moorsel *et al.* (2000), while the reverse primer ITS2L-ALB (TTCCCGCTTCACTCGCCGTTACTG) was based on 28S rRNA sequences by Wade *et al.* (2001).

All PCR reactions consisted of 35 cycles (1 min. 94°C, 1 min. 61°C, and 1 min. and 15 sec. 72°C). Total PCR product was isolated by gel purification using spin columns (Qiaquick® Gel Extraction Kit by Qiagen®). Sequencing reactions were performed directly on purified PCR products using a Big Dye Kit (PE Biosystems®). Electrophoresis was performed on an ABI 377 automated sequencer (PE Biosystems®). Forward and reverse sequences were assembled and edited using Sequencher (Gene Codes Corp.®).

Sequence alignment

Sequences were aligned in MegAlign 4.03© (DNASTAR Inc., 1999), using the Clustal V align option with the default parameter settings, and were further aligned manually in MacClade 4.0 (Maddison & Maddison, 2000). Spacer boundaries were determined by comparison with other pulmonate ITS sequences in GenBank. Since the calculation of the secondary structure of ITS is problematic for the group studied (Armbruster, 2001), stem and loop regions could not be identified this way. Therefore, we checked the alignment for presence of previously identified conserved regions. These were identified in ITS1 throughout Stylommatophora (Armbruster *et al.*, 2000) and in ITS1 and ITS2 throughout Clausiliidae (van Moorsel *et al*., 2001d). Positions that were aligned ambiguously within the ingroup (Alopiinae) were excluded from the analysis. Included positions that contained ambiguously aligned characters in the outgroup taxa were coded as unknown for these taxa. The data matrix was submitted to TreeBASE (http://www.treebase.org).

Tests for data quality

The quality of the data set was tested in PAUP* 4.0b10 (Swofford, 2002). To obtain an indication of the quality of the alignment and the selection of unambiguous sites, we compared the GC content of unambiguously and ambiguously aligned positions within ITS1 and ITS2 using a Wilcoxon Signed Ranks Test as implemented in SPSS 10.0. To test for homogeneity of base frequencies across taxa, we performed separate chi-square tests in PAUP* for unambiguously aligned sites of ITS1 and ITS2. Outgroup sequences were excluded from these analyses, since positions aligned unambiguously within the ingroup often could not be aligned between in- and outgroup, and thus had to be coded as unknown in the outgroup. We tested for the presence of phylogenetic signal in the total data set and separately in the ITS1 and ITS2 data sets. This was done by using the skewness statistic of 10,000 random trees (Hillis & Huelsenbeck, 1992) and by performing a permutation test (PTP) (Archie, 1989; Faith & Cranston, 1991) with 200 replicates, using the heuristic search option with TBR and one random addition per replicate. Because most of the variation was expected to occur within the two separated and functionally independent (Musters *et al.*, 1990) spacer regions, we checked for a contradicting phylogenetic signal between ITS1 and ITS2 with a partition homogeneity test. This test was based on 100 replicates, using heuristic search with TBR and one random addition in each replicate.

Phylogenetic analyses

Heuristic maximum parsimony searches were performed in PAUP* 4.0b10 (Swofford, 2002), using unambiguously aligned sites only and weighting transitions and transversions equally. We did not code gaps, neither as a fifth character state nor as a separate character, since most of these supposed gaps were found in relatively variable positions that were difficult to homologize throughout the ingroup. To increase the chance of finding the optimal tree, we used TBR, steepest descent and 1000 random addition replicates. Non-parametric parsimony bootstrap analyses were performed with 1000 bootstrap replicates, excluding uninformative characters, using TBR and one random addition per bootstrap replicate. To study the effect of transition/transversion weighing schemes on bootstrap support values, we repeated this analysis, using 250 bootstrap replicates, this time weighting transversions four times over transitions. This weighing factor is slightly higher than that used by van Moorsel *et al.* (2000), which was based on an estimate of the transition/transversion bias within an overlapping taxon set. To examine the effect of rooting on the ingroup topology, we repeated the parsimony analysis with three different outgroups: (1) the combined outgroup consisting of *Balea*, *Idyla* and *Macrogastra*, (2) *Stereophaedusa*, and (3) *Arianta* and *Cochlicopa*.

RESULTS AND DISCUSSION

Sequences

For *Papillifera papillaris* 306 bases out of 570 positions of ITS1 could not be determined. We used 53 bases of the 5.8S rRNA sequence of this species determined by Wade *et al.* (2001) to bridge the non-sequenced part of 5.8S rRNA for *Papillifera papillaris*. This 53 base sequence was identical to the majority of ingroup sequences previously determined by van Moorsel *et al.* (2000) and van Moorsel *et al*. (2001d). For *Sericata sericata, Isabellaria isabellina* and *Isabellaria perplana* newly obtained partial 18S rRNA, ITS1 and partial 5' 5.8S rRNA sequences were used, to which the partial 3' 5.8S rRNA, complete ITS2 and partial 28S rRNA sequences determined by van Moorsel *et al.* (2000) were concatenated. Fifteen bases from ITS2 of *Sericata regina* could not be identified; all of these were located in a variable region that was excluded from the phylogenetic analyses.

Table 2.3. Total number of bases, and number of alignable, variable and informative positions within each of the rRNA genes and spacers. The number of variable and informative positions is given for the total set of taxa and for the ingroup only. The regions of the 18S rRNA, 5.8S rRNA and 28S rRNA genes that were missing for some taxa were assumed to contain no additional gaps. Total sequence lengths could not be determined, since no such complete sequences were available.

	18S	**ITS1**	**5.8S**	**ITS2**	**28S**	**total**
number of bases	149	436-585	156-160	345-502	80	?
unambiguously aligned positions						
total unambiguously aligned	149	248	158	185	80	820
variable	3	70	11	79	1	164
parsimony informative	1	39	3	43	0	86
variable within ingroup (Alopiinae)	3	50	6	58	0	117
parsimony informative within ingroup	1	23	0	30	0	54

Sequence comparisons

Most of the variable and parsimony informative sites in the sequences were located in ITS1 and ITS2, whereas the 18S rRNA, 5.8S rRNA and 28S rRNA genes, proved to be highly conserved (see Table 2.3). Since complete ITS1 and ITS2 sequences were available for nearly all species sampled, hardly any potential phylogenetic information was lost from our analyses, despite the otherwise incomplete overlap of the sequences obtained from different studies. Summed over 18S rRNA, 5.8S rRNA and 28S rRNA, only one position was informative within the ingroup. Assuming there were no gaps in the missing parts of the sequences of the three genes, only 5.8S rRNA showed variation in length, corresponding to three gaps. One of these gaps comprised two nucleotides, the other two only one.

In contrast, ITS1 and ITS2 were highly variable in length and in nucleotide composition. The number of alignable, variable and parsimony informative positions in both spacers is given in Table 2.3. ITS1 ranged in length from 436 nucleotides in *Montenegrina* to 585 in *Macrogastra ventricosa*. Of these, 248 positions, holding 210 to 239 nucleotides (41.9-48.8%), could be aligned unambiguously in the ingroup. These positions include nearly all conserved sites described in Armbruster *et al.* (2000), with the exception of five positions at the 5' side of region 2 and two positions at the 5' side of region 5. The unambiguously aligned regions were rather conserved; only 23 positions were parsimony informative in ITS1 within the ingroup.

ITS2 was shorter on average than ITS1, varying from 345 nucleotides in *Montenegrina* to 502 in *Idyla bicristata*. In ITS2, 185 positions, corresponding to 167-178 nucleotides (37.2-51.0%), could be aligned unambiguously between the ingroup sequences, with the exception of the *Medora* sequence. This sequence contained a region of five nucleotides (294-298 of ITS2) that could not be aligned unambiguously with other ingroup sequences. Since this region could easily be aligned between the other ingroup and outgroup sequences, we coded these five positions as unknown in *Medora*, but used them in the analyses for the other taxa. Again most of the unambiguously aligned positions were invariable and only 30 of these were parsimony informative within the ingroup.

ITS1 indel

Previous studies emphasized the taxonomic value of an indel in ITS1 in the group studied here (van Moorsel *et al.,* 2000; Schilthuizen *et al.*, 1995). Schilthuizen *et al.* (1995) found that an insertion of 22 nucleotides in ITS1 of *Isabellaria praecipua* corresponds to an extra hairpin in its predicted secondary structure. Instead, van Moorsel *et al*. (2000) found an insertion of 20 to 26 nucleotides in the ITS1 sequences of the *Isabellaria* and *Sericata* species sampled, shifted in position nine bases to the 5' side of ITS1 relative to the insertion inferred by Schilthuizen *et al*. (1995). Again, this insertion was absent in *Albinaria*. Although the polarity of this character could at that time not be deduced, van Moorsel *et al.* (2000) concluded that this indel might be used as a diagnostic character for placement of species either with *Albinaria* or with *Isabellaria* and *Sericata*.

We located the so-called insertion in ITS1 (van Moorsel *et al.*, 2000) of all *Isabellaria*, and *Sericata* species, and also in ITS1 of *Carinigera*, *Cristataria*, *Strigilodelima*, *Macedonica* and *Cochlodina*. It varied in length from 20 to 32 bases. Nearly all of this variation in length was situated at the 3' side of the insertion, whereas the 5' side was highly conserved. Nevertheless,

Table 2.4. Mean values for number of nucleotides and base composition of the complete sequence, the unambiguously aligned regions, and ambiguously aligned regions of ITS1 and ITS2. Standard deviations of the means are placed in parenthesis. Calculations for ITS1 are based on all ingroup sequences minus that of *Papillifera papillaris*, calculations for ITS2 on the complete set of ingroup sequences.

ITS1 (36 species)	Length	A(%)	C(%)	G(%)	T(%)	AT(%)	CG(%)
Complete spacer	525.03	17.07	29.98	31.43	21.51	38.58	61.42
	(26.48)	(0.81)	(0.78)	(0.84)	(1.00)	(1.21)	(1.21)
Unambiguously aligned	236.42	15.64	30.49	37.16	16.71	32.35	67.65
	(6.74)	(0.54)	(0.52)	(0.79)	(0.49)	(0.76)	(0.76)
Ambiguously aligned	288.61	18.25	29.56	26.73	25.47	43.72	56.28
	(21.57)	(1.34)	(1.42)	(1.16)	(1.72)	(2.00)	(2.00)
ITS2 (37 species)	Length	A(%)	C(%)	G(%)	T(%)	AT(%)	CG(%)
Complete spacer	391.46	19.09	28.85	30.36	21.70	40.79	59.21
	(26.67)	(1.02)	(1.82)	(1.27)	(2.20)	(2.75)	(2.75)
Unambiguously aligned	175.97	18.01	30.71	33.38	17.90	35.91	64.09
	(1.83)	(0.81)	(1.00)	(0.97)	(1.17)	(1.18)	(1.18)
Ambiguously aligned	215.49	19.96	27.34	27.85	24.85	44.80	55.20
	(26.72)	(1.75)	(2.77)	(2.40)	(3.60)	(4.54)	(4.54)

the nucleotides could not be unambiguously aligned with the outgroup sequences, and the insertion was ignored in subsequent phylogenetic analyses. A gap was found in *Albinaria*, *Agathylla*, *Medora*, *Montenegrina*, *Muticaria* and *Herilla*. These gaps overlapped but varied in length between genera; only in *Medora*, *Montenegrina* and *Muticaria* was an identical gap found. Because of the (mostly) different lengths of the gaps and the position of the taxa in the tree (see Fig. 2.6), the gaps in the sequences of *Albinaria*, *Agathylla*, *Herilla*, *Medora*, *Montenegrina* and *Muticaria* are best considered independent deletions in the same hyper-variable region of ITS1. A similar hot spot has been described in ITS2 (Denduangboripant & Cronk, 2001). The opposite explanation, an insertion in the sequences of *Carinigera*, *Cristataria*, *Isabellaria* and *Sericata*, fails to account for the occurrence of a similar insertion in *Strigilodelima, Macedonica* and *Cochlodina*, given the phylogenetic tree.

Data quality

Base composition differed between the ambiguously and unambiguously aligned regions of each spacer (Table 2.4). In both ITS1 and ITS2, the GC content in the unambiguously aligned regions of the ingroup taxa was significantly higher than that in the ambiguously aligned regions (one-tailed Wilcoxon Signed Ranks Test: $P<0.001$). The unambiguously aligned regions of ITS1 and ITS2 had a similar base composition compared over all ingroup sequences (ITS1 X^2: P=1.00; ITS2 X^2: P=1.00); so had each of the rRNA genes compared over all sequences (X^2: P=1.00). G1 statistics and a permutation test both revealed a significant phylogenetic signal in the data set ($P<<0.01$) as a whole, and in ITS1 and ITS2 separately. This phylogenetic signal in ITS1 and ITS2 was not significantly contradicting (partition homogeneity test, P=0.50).

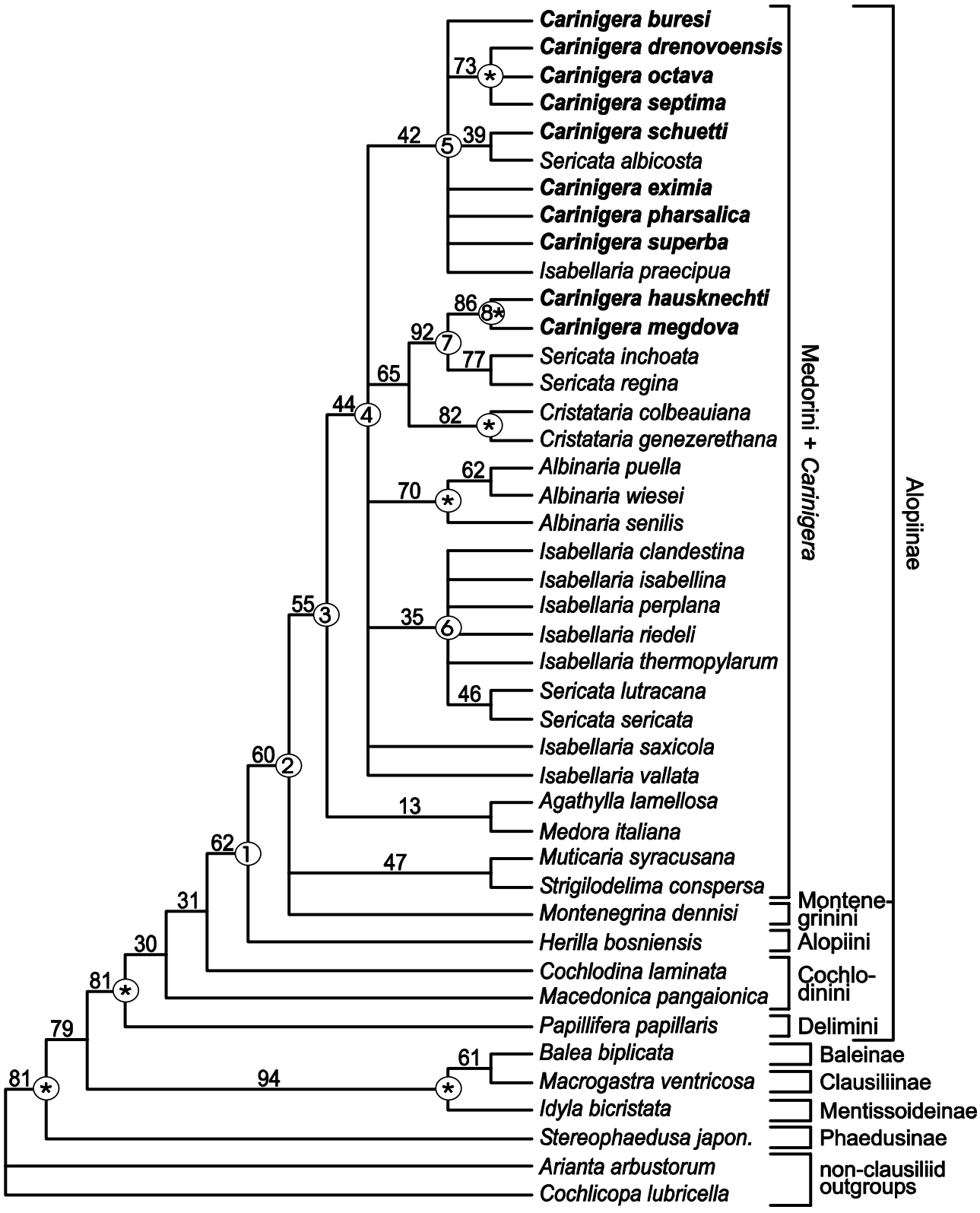


Figure 2.6. Strict consensus cladogram of the 349 most parsimonious trees, requiring 350 transformations. Numbers above the branches indicate bootstrap support. Bootstrap values below 5% are not shown. Encircled numbers on nodes refer to clades mentioned in the text. Encircled asterisks indicate groups previously recognized as monophyletic on morphological grounds.

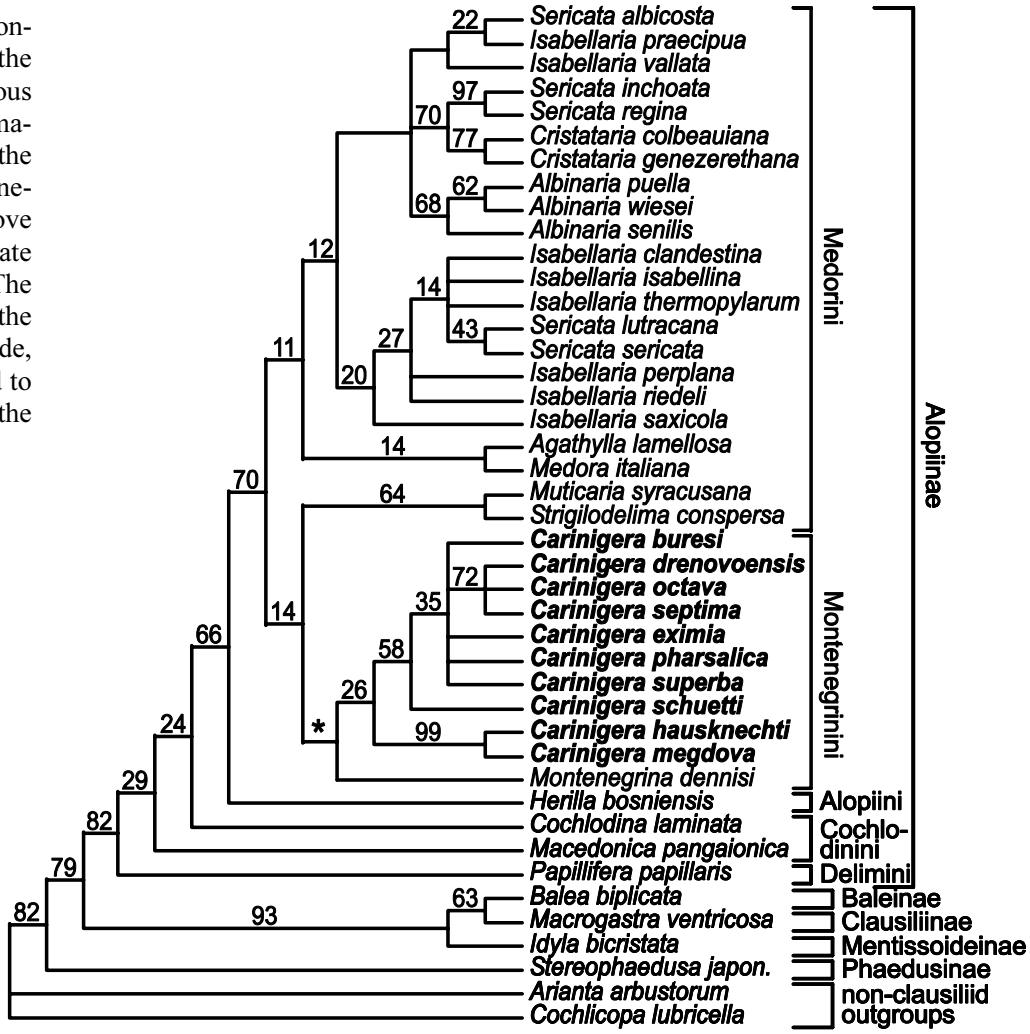


Figure 2.7. Strict consensus cladogram of the 144 most parsimonious trees (367 transformations) supporting the monophyly of Montenegrinini. Numbers above the branches indicate bootstrap support. The asterisk indicates the Montenegrinini clade, which was constrained to be monophyletic in the analyses.

Suprageneric phylogenetic relationships

The maximum parsimony analyses excluding gaps yielded 349 most parsimonious trees with a score of 350 and a CI of 0.477 (uninformative characters excluded), which is higher than the expected CI of 0.348 for phylogenetic analyses with 43 taxa (see Sanderson and Donoghue, 1989). The strict consensus of the most parsimonious trees is depicted in Figure 2.6. Most of the variation between the trees is found within clade 4 (*Albinaria*, *Carinigera*, *Cristataria*, *Isabellaria* and *Sericata*).

Rooting with different outgroups, viz. (1) *Balea*, *Idyla* and *Macrogastra*, (2) *Stereophaedusa* or (3) *Arianta* and *Cochlicopa*, had only a slight effect on ingroup topology. These different roots resulted in 1740, 418 and 348 different maximum parsimony topologies, respectively, the majority rule consensus of which differed only in the relationship between the 'peripheral' ingroup taxa *Cochlodina*, *Macedonica* and *Papillifera*.

With respect to the taxonomic ranks above the disputed tribe level, the MP consensus tree broadly corresponds to the current classification. Thus maximum parsimony bootstrap analyses (Fig. 2.6) support the monophyly of both Clausiliidae (81%) and its subfamily Alopiinae (81%) by more than 70%, which is considered a threshold value generally corresponding to a probability of >95% that a clade has been correctly inferred (Hillis & Bull, 1993).

Representatives of the tribes Montenegrinini, Medorini and Alopiini (represented by *Herilla*) (see Tables 2.1 and 2.2) together constitute a monophyletic group (clade 1), corroborating the earlier conclusion (Nordsieck, 1972: 26) that *Montenegrina* and *Carinigera* are closely related to genera now placed in the Alopiini and the Medorini. Within this group, the Montenegrinini and Medorini form a subclade (clade 2). Clade 1 and clade 2 have moderate MP bootstrap support (62% and 60%, respectively).

The ITS data favour placement of *Carinigera* with the Medorini, rather than with *Montenegrina* in the Montenegrinini. Most of the 349 MP trees (81%) supported the monophyly of the Medorini with the nested *Carinigera* species. In contrast, the 144 most parsimonious trees supporting the monophyly of the Montenegrinini (see Fig. 2.7 for strict consensus) required 367 instead of 350 transformations and were significantly less parsimonious (one-tailed Templeton test, $P<0.01$). At least two clades (clade 3 and clade 4) found in all MP trees include all *Carinigera*, *Isabellaria* and *Sericata* species but exclude *Montenegrina.* Clade 4 consists of *Albinaria*, *Carinigera Cristaratia*, *Isabellaria* and *Sericata*. Clade 3 groups these five genera with *Agathylla* and *Medora*. Neither clade is significantly supported though (clade 3: bootstrap value 55%; clade 4: bootstrap value 44%). Downweighting transitions four times relative to transversions increased the bootstrap support of clade 3 to 57%, while *Medora* and clade 4 together constituted a 49% bootstrap supported clade.

Apart from the addition of *Carinigera*, the phylogenetic relationships found between the Medorini largely confirm morphology-based ideas about their interrelationships. Nevertheless, the addition of *Carinigera* to subgroups previously recognized within the Medorini does not introduce conflicts with any alleged morphological synapomorphies of these subgroups. Excepting *Carinigera*, the genera in clade 3 and those in clade 4 (Fig. 2.6) were considered closely related on the basis of overall similarities (see Nordsieck, 1972: 8 and 1977a: 285, respectively), not synapomorphies (see Nordsieck, 1997: 55). A supposed genital-anatomical synapomorphy (Nordsieck, 1997: 55; but see Uit de Weerd & Gittenberger, Chapter 3, this thesis) uniting the genera *Albinaria*, *Isabellaria* and *Sericata*, viz. a relatively long diverticulum (longer than the bursa with pedunculus), is also present in *Carinigera* (see Nordsieck, 1974: 146). In this respect, the nested position of *Cristataria* among these genera in clade 4 is unexpected, since *Cristataria* does not have this long diverticulum (Nordsieck, 1971: 238). Neither does any morphological evidence support the sister group relationship between *Cristataria* and clade 8 (consisting of *Sericata inchoata*, *S. regina*, *Carinigera hausknechti* and *C. megdova*), as far as we know. Moreover, both sister groups are widely separated geographically. In the most recent classification (Nordsieck, 1997) *Cristataria* is grouped with *Medora* and *Agathylla*, but this grouping is not based on any synapomorphies.

Non-monophyly of *Carinigera*

The MP analyses divide the genus *Carinigera* into two separate clades: (1) *C. hausknechti* and *C. megdova* (clade 8, 86% MP bootstrap support), placed as a sister group to *Sericata inchoata* plus *Sericata regina* in the highly supported clade 7 (92% MP bootstrap support), and (2) a relatively unresolved and poorly supported clade (clade 5, 42% MP bootstrap support) containing the remaining *Carinigera* species as well as *Isabellaria praecipua* and *Sericata albicosta*. The well-supported sister group position of *C. hausknechti* and *C. megdova* confirms

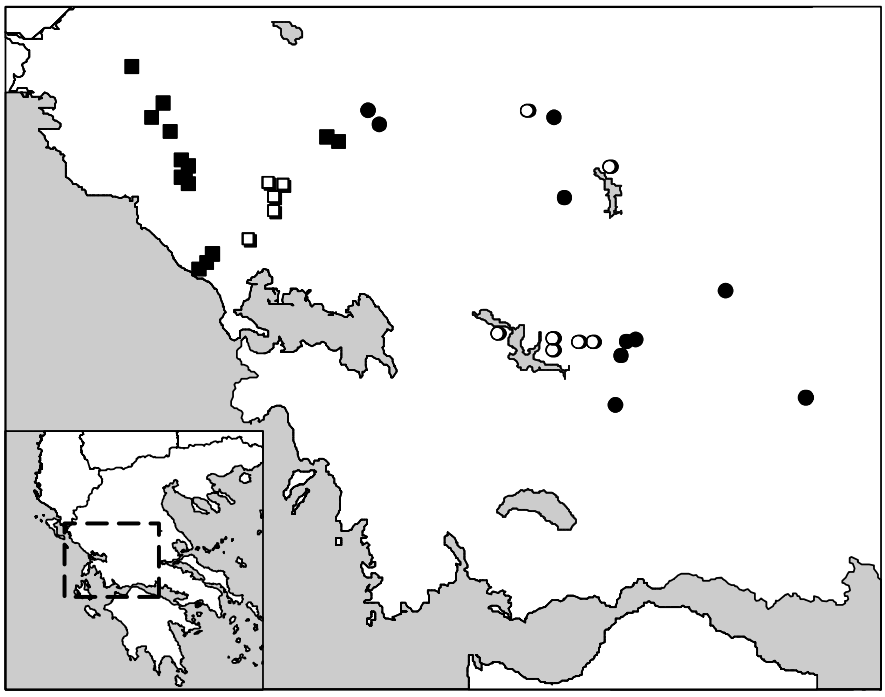


Figure 2.8. Distribution of the species within clade 7. Circles indicate species in subclade 8: *Carinigera hausknechti* (dots) and *C. megdova* (open circles); squares indicate species in its sister clade: *Sericata inchoata* (filled squares) and *S. regina* (open squares).

Nordsieck's view (Nordsieck, 1974), which was based on unspecified conchological characters. A second well-supported *Carinigera* clade is the subclade of clade 5 consisting of *Carinigera drenovoensis*, *Carinigera octava* and *Carinigera septima* (73% MP bootstrap support). This subclade also confirms earlier conchological inferences (see Brandt, 1962: 138-139). Even when ignoring the other clades, which are supported by MP bootstrap values below 70%, *Carinigera* is still paraphyletic with respect to *Sericata inchoata* and *Sericata regina*.

Our results are discordant with the division of *Carinigera* into two subgenera, viz., *Carinigera* s.s. and *Angiticosta*, based on differences in the papilla. Neither do our results reflect the distribution of a single versus a bifurcate penial retractor. In clade 8, consisting of *C. hausknechti* and *C. megdova*, only the bifurcate retractor is found, whereas the *Carinigera* species in clade 5 represent both forms.

The two clades of *Carinigera* species and their position in the tree, as found in this study, are concordant with distributional patterns, and correspond to more or less separate geographic clusters. *Carinigera hausknechti* and *C. megdova* are geographically isolated from the other *Carinigera* species, while their combined range is largely parapatric with that of their sister group *S. inchoata* plus *S. regina* (Nordsieck, 1974) (see Fig. 2.8). Although the phylogenetic position of the *Carinigera* species in clade 5 is poorly supported, their clustering with *Isabellaria praecipua* and *Sericata albicosta* is also congruent with distributional data. The ranges of these remaining *Carinigera* species almost completely surround the ranges of *Isabellaria praecipua* and *Sericata albicosta*, but not that of the other species of *Isabellaria* and *Sericata* included in this study.

The genus *Carinigera* has never been defined in terms of synapomorphies. As such, its monophyly has not been firmly established. *Carinigera* can readily be distinguished conchologically from the supposedly nearest genera *Montenegrina* and *Protoherilla*. These genera also differ from *Carinigera* by the presence of a strongly muscular vaginal retractor.

With its classification as Medorini, however, only two anatomical character states remain as possible synapomorphies, viz. a relatively muscular vaginal retractor and a penial papilla. Our results suggest that these character states evolved in parallel in the ancestors to the genus *Montenegrina* and the two *Carinigera* clades.

The penial papilla and the vaginal retractor differ between the two *Carinigera* clades. Clade 8, with *C. hausknechti* and *C. megdova*, is characterized by a penial papilla that is relatively long compared to that of the other *Carinigera* species. This has been recognized as a possible synapomorphy uniting *C. hausknechti* and *C. megdova* (Hausdorf, 1987: 174). Our results suggest that rather than being a transformation of a short papilla, the characteristic papilla in *C. hausknechti* and *C. megdova* reflects an independent origin, since *C. hausknechti* and *C. megdova* are nested among taxa lacking a penial papilla. A somewhat muscular vaginal retractor is mentioned as the second diagnostic character state for *Carinigera*. However, in *C. megdova* the so-called retractor is composed of hardly more than connective tissue (Hausdorf, 1987: 174). To our knowledge, the vaginal retractor of *C. hausknechti* has never been described.

Species pairs

Although the tree (Fig. 2.6) indicates some geographic congruence with the relatedness of *Carinigera*, *Isabellaria* and *Sericata* species in general, none of the previously recognized neighbouring species pairs was found monophyletic. Nevertheless, *Carinigera hausknechti* and *Sericata inchoata* are closely related and some of their conchological similarities, notably the white sutural line and the presence of papillae, may have some phylogenetic significance. These character states may be synapomorphies for clade 7, since they are present in all species constituting this clade, although not restricted to these species.

The systematic position of *C. pharsalica* and *I. clandestina*, together constituting the second species pair, is less clear from our results. These species are depicted as part of different clades within the polytomous clade 4. *Carinigera pharsalica* is grouped with *C. buresi*, *C. drenovoensis*, *C. octava*, *C. septima*, *C. schuetti*, *S. albicosta*, *C. eximia*, *C. superba* and *I. praecipua* in clade 5, while *Isabellaria clandestina* is part of clade 6 together with *Isabellaria isabellina*, *Isabellaria perplana*, *Isabellaria riedeli*, *Isabellaria thermopylarum*, *Sericata sericata* and *Sericata lutracana.* Both clades have low bootstrap values (clade 5: 42% MP bootstrap support; clade 6, 35% MP bootstrap support). (see Fig. 2.6). Additional molecular studies, grouping *C. pharsalica* and *C. buresi* (Chapters 4 and 5, this thesis), confirm the position of *C. pharsalica* within clade 5. Therefore, we consider the geographic proximity and the shell morphological similarity of *C. pharsalica* and *I. clandestina* misleading.

CONCLUSIONS

Carinigera as part of the Medorini

Although many of the clades grouping *Carinigera* with Medorini are poorly supported by MP bootstrap values, we consider this position of *Carinigera* far better supported than its alternative, a grouping with Montenegrinini. First, the monophyly of Montenegrinini has never been confirmed by cladistic analyses including representatives of the other tribes within the

Alopiinae. Second, our data support a position of *Carinigera* within Medorini significantly better than the current classification, with *Carinigera* as part of the Montenegrinini. Third, the phylogeny inferred corresponds far better with distributional data than does the classification of *Carinigera* with Montenegrinini. Fourth, according to the descriptions of its genital anatomy and the homoplasy even at genus level in the presumably tribe-diagnostic genital-anatomical characters, *Carinigera* can be as easily fitted among the Medorini as among the Montenegrinini. Taken together, these observations necessitate a redefinition of the tribe Medorini, so that it includes *Carinigera*.

Given the still incomplete sampling of species placed in the apparently para- or polyphyletic nominal genera *Isabellaria* and *Sericata*, and the low support for most subclades of clade 4, a revision of the genera *Carinigera*, *Isabellaria* and *Sericata* would be premature at this point. Awaiting a more complete and resolved phylogenetic tree, we propose to temporarily maintain the generic name *Carinigera* for the species *C. hausknechti* and *C. megdova*, even though they form a clade separate from the other *Carinigera* species (including the type species *C. eximia*). For the same reason, we postpone a revision of the so-called genera *Isabellaria* and *Sericata*.

Implications for future research

This study shows that parallelism in so-called diagnostic genital-anatomical characters within the clausiliid subfamily Alopiinae may be more common than previously thought. Especially classifications based on one or a few genital-anatomical characters that conflict with both conchology and patterns of distribution should be mistrusted. In those cases, molecular sequences can provide an additional and independent source of information.

Although *Carinigera* is placed within the newly defined Medorini, the monophyly of this tribe as a whole and the interrelationships of the constituent genera are still weakly supported. Therefore, the data set used in this study should eventually be extended with more taxa and sequences from additional DNA regions. The observation that *Carinigera* sensu auct. is not monophyletic and that its constituent species are closely related to species of the genera *Albinaria*, *Cristataria*, *Isabellaria* and *Sericata* suggests that further research is required on the phylogenetic relationships among the species of these five nominal taxa, particularly because of the implications for taxonomy and character evolution. The relationships between species of *Albinaria*, *Isabellaria* and *Sericata* have been the basis of a case study into parallel evolution (van Moorsel *et al.*, 2000). Our results show that the sampling of genera in that study was incomplete. The *Carinigera* species should have been added.

ACKNOWLEDGEMENTS

We are grateful to a number of collectors of species that would otherwise have remained unsampled. J. Heller collected *Cristataria genezerethana*; T.A.C. Reydon collected *Agathylla lamellosa*. H. Nordsieck collected the samples of *Cristataria genezerethana* and *Cristataria colbeauiana*; foot tissue of these samples was provided to us by H.-J. Niederhöfer from the Staatliches Museum für Naturkunde Stuttgart. W.J. Moes assisted with collecting *Muticaria*

syracusana.

Our thanks also go to P. van Riel, for providing a protocol for DNA extraction from old tissue. The sequences of *Cristataria genezerethana*, *Strigilodelima conspersa* and *Herilla bosniensis rex* were determined by D.S.J. Groenenberg. D. Schneider isolated DNA of *Carinigera eximia* and *Carinigera octava*. C.H.M. van Moorsel kindly provided sequences of *Albinaria puella*, *Albinaria wiesei*, *Balea biplicata*, *Cochlodina laminata* and *Macrogastra ventricosa* and offered valuable advice and suggestions.

Chapter 3

TOWARDS A MONOPHYLETIC GENUS *ALBINARIA* (GASTROPODA, PULMONATA): THE FIRST MOLECULAR STUDY INTO THE PHYLOGENETIC RELATIONSHIPS OF EASTERN *ALBINARIA* SPECIES

ABSTRACT

Recent molecular phylogenetic studies of the terrestrial snail genus *Albinaria* have caused a radical re-assessment of its taxonomy. These studies, however, were limited to western species. This paper examines mitochondrial 12S sequences and nuclear ITS1&2 sequences of both eastern and western species, and demonstrates that *Albinaria,* in its most recent definition, is not monophyletic.

Both molecular data sets place '*Albinaria*' *hedenborgi* from Lebanon in a well-supported clade with species of the genus *Cristataria*, distributed south-east of the vicariant range of *Albinaria*. The remaining species from Cyprus, Turkey and Greece, constitute a well-supported monophyletic group. These two clades form geographic clusters, whereas *Albinaria* in the current definition — including '*A.*' *hedenborgi* — has a disjunct distribution. '*Albinaria*' *hedenborgi* should therefore be classified with *Cristataria*, together with the morphologically similar and geographically close '*A*'. *nadimi*. The taxonomy of some additional species, now classified as *Albinaria,* should be considered questionable because the transfer of '*A*'. *hedenborgi,* on the basis of molecular data.

INTRODUCTION

Of all genera within the pulmonate land snail family Clausiliidae, the genus *Albinaria* is the most speciose (Nordsieck, 1979) and most extensively studied. *Albinaria* serves as a model for studies on ecological differentiation (Gittenberger, 1991), morphological evolution (Kemperman & Gittenberger, 1988; van Moorsel *et al*., 2000), molecular evolution (van Moorsel *et al*., 2001b), biogeography (Douris *et al*., 1998a; Welter-Schultes, 2000a), species barriers (Schilthuizen, 1994; Schilthuizen *et al*., 1999a; Giokas *et al*., 2000) and comparative life history (Giokas & Mylonas, 2002). Essential for most of these studies is an understanding of the phylogeny of the group and a solid definition of the genus based on synapomorphic character states. Yet many aspects of *Albinaria*'s phylogeny remain uncertain, not the least of which is its monophyly.

Two types of definitions of *Albinaria* have been formulated: (1) traditional morphology-based typological definitions, and (2) definitions based on mostly molecular synapomorphies. The former offers no unequivocal synapomorphies for the monophyly of *Albinaria*. The molecular studies cover a sample of western species, and therefore fail to represent the full diversity of *Albinaria* as defined by morphology, leaving *Albinaria* as a whole still unsupported by synapomorphies.

The original definition of *Albinaria* (von Vest, 1867) as well as the subsequent revision (Boettger, 1878) were based on conchological characters. These characters were combined with

Dennis R. Uit de Weerd and Edmund Gittenberger. Towards a monophyletic genus *Albinaria* (Gastropoda, Pulmonata): the first molecular study into the phylogenetic relationships of eastern *Albinaria* species.

Figure 3.1. Ranges of *Albinaria* (**A**) and *Cristataria* (**B**).

genital-anatomical ones in later revisions (Wagner, 1923, 1924; Nordsieck, 1971, 1977a). All these morphology-based definitions, including the most recent ones (Nordsieck, 1977a, 1997, 1999), were based on a combination of non-diagnostic characters, and do not identify synapomorphic character states supporting the monophyly of *Albinaria*. According to Nordsieck (1977a), *Albinaria* is found from Albania to Lebanon, with the highest number of species in southern Greece, the Aegean islands and south-western Turkey. In Lebanon, the range of *Albinaria* is supposed to overlap with that of *Cristataria* as defined by Nordsieck (1971). The latter genus is reported from the mountain range running from southern Turkey to Israel. The north-western limit of the distribution of *Albinaria* borders and partly overlaps the range of the genera *Isabellaria*, as defined by Nordsieck (1969, 1972, 1974), and *Sericata*, as defined by Nordsieck (1972, 1974). Later on, Nordsieck (1997; 1999: note 1) added *Sericata* as a subgenus to *Albinaria*. This latest revision was based on the same set of morphological characters as used in earlier work (Nordsieck, 1977a), and the nominal genera were combined because their definitions largely overlapped, not because they shared synapomorphic character states.

Since then, studies of DNA sequences (Douris *et al*., 1998b; van Moorsel *et al.*, 2000) have led to new revisions of *Albinaria* (Douris *et al.*, 1998b; Gittenberger, 1998a). These studies have shown that some Peloponnesian species that Nordsieck (1969, 1972, 1974) had placed in *Isabellaria* should be classified with *Albinaria* instead, and they reject the hypothesis that *Sericata* is a subgenus of *Albinaria*. So far, all molecular studies have focussed on species from only the western part of the range of *Albinaria*, from the western coast of Turkey to the island of Crete and Epiros. As a consequence, only these species can be characterized by molecular

synapomorphies, whereas the eastern ones have to be classified solely on the basis of their distribution and morphology.

There are reasons to question the existence of an *Albinaria*-clade that includes all eastern *Albinaria* species sensu Nordsieck (1977a) with the exclusion of *Cristataria*. These species are disjunctly distributed, occurring in southern Turkey, Cyprus and Lebanon (Fig. 3.1). Cyprus is situated in between the undisputed ranges of *Albinaria* and *Cristataria*, and Lebanon is within the range of *Cristataria*. Furthermore, it is impossible to distinguish unequivocally the eastern *Albinaria* species from *Cristataria* species on shell characters alone. Admittedly, the genera *Albinaria* and *Cristataria* do not, on the whole, fully differ conchologically and can be distinguished at best by a few genital-anatomical characters (Nordsieck, 1971, 1977a; Fig. 3.2): in *Albinaria* species the diverticulum is generally longer; the vagina tends to be shorter; the granular zone of the penis tends to be larger; the caecum tends to be shortened; and the vaginal retractor inserts at the proximal part of the pedunculus, whereas in *Cristataria* it is attached at the transition between the pedunculus and the vagina. All of these differences are gradual, however, and tendencies are difficult to use in practice. Furthermore, the genital anatomy of only a limited number of samples and species have been studied (Nordsieck, 1977a), and this paucity may result in erroneous systematic inferences (Pfeiffer, 1955).

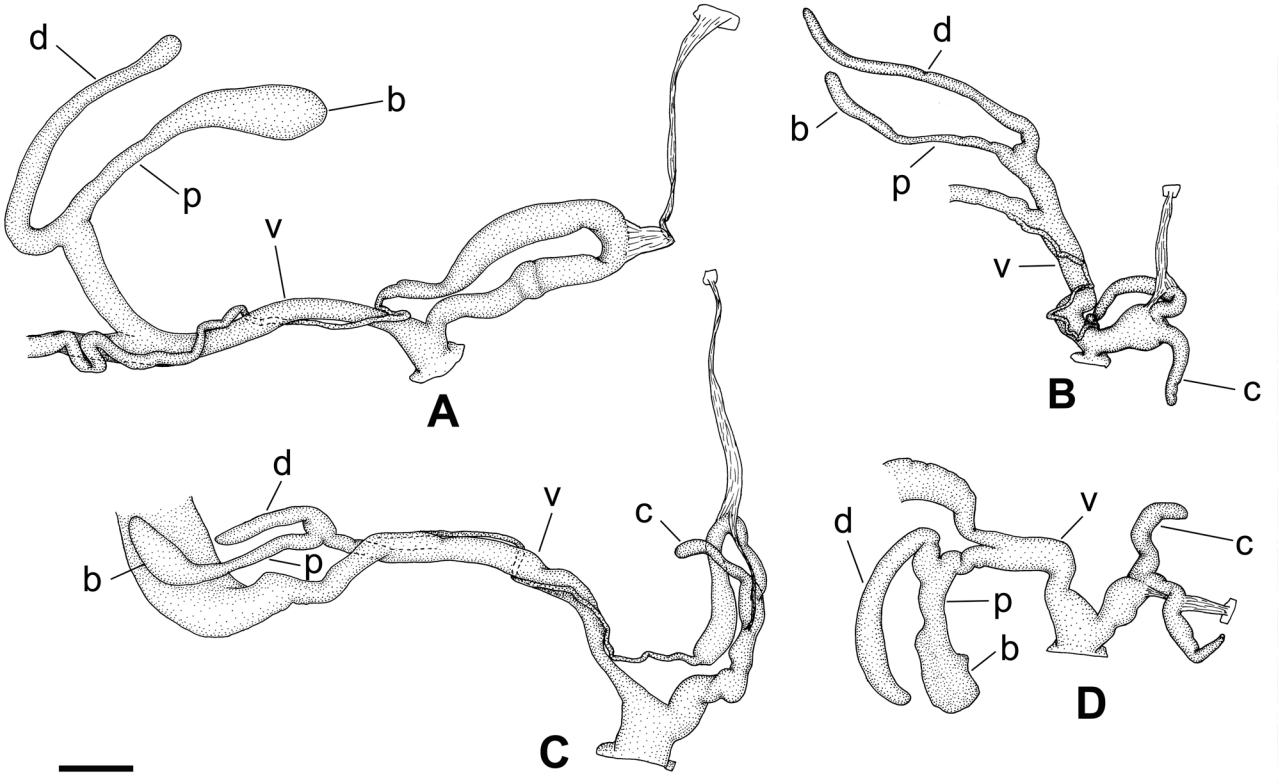


Figure 3.2. Genital anatomy of *Albinaria hippolyti holtzi* (**A**), *Albinaria hedenborgi* (**B**), *Cristataria genezerethana* (**C**) and *Cristataria elonensis* (**D**). The scale bar (left below) represents 1.0 mm. Abbreviations: c: caecum; b: bursa; d: diverticulum; p: pedunculus; v: vagina. The insertion of the vaginal retractor and the granular zone of the penis could not be clearly located.

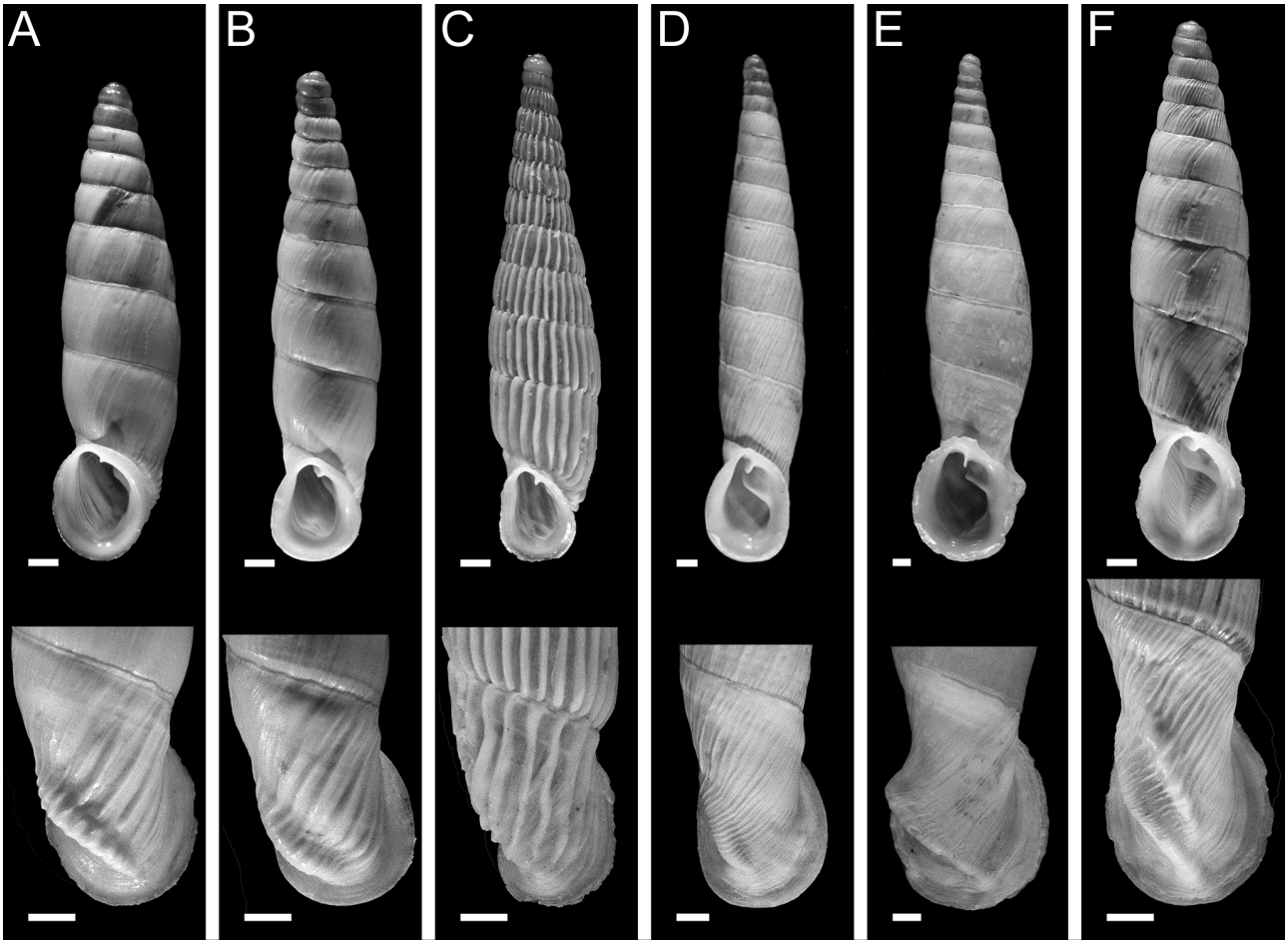


Figure 3.3. Eastern *Albinaria* species as compared to *Cristataria boissieri* from Lebanon. Species are arranged according to their distribution, from north-western to south-eastern occurrence: **A** *Albinaria schuetti* from southern Turkey; **B** *Albinaria virgo* from northern Cyprus; **C** *Albinaria saxatilis* from Cyprus; **D** *Albinaria hedenborgi* from Lebanon; **E** *Albinaria staudingeri* from Lebanon; **F** *Cristataria boissieri* from Lebanon. Scale line 1.0 mm. Photographs by A. 't Hooft (IBL, Leiden).

Of all species hitherto classified with *Albinaria*, the systematic positions of the Lebanese species *Albinaria hedenborgi* (Fig. 3.3), *A. nadimi* and *A. staudingeri* (Fig. 3.3) are most problematic. These species inhabit a relatively small range in central Lebanon. *Albinaria hedenborgi* and *A. nadimi* live west of mount Lebanon, *A. staudingeri* is found south and south-west of these species. As such, the Lebanese *Albinaria* species are widely separated geographically from other supposedly congeneric species, and they occur in the central part of the range of *Cristataria* (Fig. 3.1). This distributional pattern could be explained by postulating either (1) long-distance dispersal or (2) extinction of *Albinaria* and the establishment of *Cristataria* species in the area between south-western Turkey and Lebanon. Alternatively, these ad hoc assumptions would be moot if the Lebanese *Albinaria* species were in fact part of a *Cristataria*-clade.

The reasons for their inclusion in *Albinaria* instead of *Cristataria* differ between the three Lebanese species. Classification in *Albinaria* is supposed to be supported by genital anatomy for *A. hedenborgi* only, as such data are lacking for the other species. *Albinaria hedenborgi* was

placed in *Albinaria* on the basis of its relatively long diverticulum (Nordsieck, 1971) (see Fig. 3.2), though not unreservedly (Nordsieck, 1971: 238; 1977a: 300). A relatively long diverticulum is assumed to be an apomorphy of *Albinaria*, shared with the genera *Isabellaria* and *Sericata*, and thus would separate *A. hedenborgi* from *Cristataria* (Nordsieck, 1997). The classification of *Albinaria nadimi* and *A. staudingeri* ultimately hinges on that of *A. hedenborgi*. Both species were originally placed in *Cristataria*, and were recently moved to *Albinaria* (Nordsieck, 1993: 22, 23; 1999) on the basis of conchological characters only. *Albinaria nadimi* and *A. hedenborgi* are considered closely related (Nordsieck 1999: note 24) because of their conchological resemblance and geographic proximity. *Albinaria staudingeri* has been indirectly linked to *A. hedenborgi* on conchological grounds, through *A. schuetti* from Turkey (Nordsieck, 1984: 198; 1993; 1999: note 24). The cladistic support for the classification of *A. hedenborgi*, *A. nadimi* and *A. staudingeri* with *Albinaria* consists of the presence of a basal keel in these species, which is considered an apomorphy uniting them with species of the *munda* subgroup in *Albinaria* (Nordsieck, 1997), among which *A. schuetti*. However, a basal keel, as found in the Lebanese *Albinaria* species, is also present in most of the surrounding *Cristataria* species (cf. *Cristataria boissieri*, Fig. 3.3F), but is here accompanied by either a dorsal or a transverse keel, or a combination of both (see Nordsieck, 1971; 1999: note 24).

In this study, we have investigated whether molecular data support the monophyly of *Albinaria* including *A. hedenborgi* and other eastern species. We used the mitochondrial small ribosomal subunit 12S, and the nuclear ribosomal internal transcribed spacer regions ITS1&2. Since these DNA regions inherit differently, they can be used as independent estimators of a supposedly common phylogeny. Such an approach has already been applied in studies of the western *Albinaria* species, of which the monophyly was supported by both mitochondrial DNA (Douris *et al.*, 1998b) and nuclear DNA (van Moorsel *et al.*, 2000). Moreover, the species of *Albinaria* so far studied share a deletion in ITS1, which has to be considered synapomorphic (Chapter 2, this thesis). Should this deletion occur in the eastern species as well, then it could be used as a diagnostic character for the genus *Albinaria* as a whole.

MATERIALS AND METHODS

Selection of taxa

The species examined in this study are listed in Table 3.1, and the locations of the collection sites are given in Figure 3.4. These species were selected on basis of their availability, systematic position and distribution. The five lineages within *Albinaria*, recognized by Nordsieck (1997, 1999), are represented, including the three so-called eastern groups (Table 3.1). The Lebanese species are represented by *A. hedenborgi*. Both Cyprian groups originally recognized by Nordsieck (1977a) are represented, the *virgo* group by *Albinaria virgo*, the *saxatilis* group by *A. saxatilis*. We also studied specimens of the four species groups (not identical to the so-called lineages, see Nordsieck, 1999) occurring in Turkey. The *anatolica* group is represented by *Albinaria lycica*, the *forbesiana* group by *A. forbesiana*, the *caerulea* group by *A. caerulea* and the *munda* group by *A. puella*. Additional species from the westernmost part of the generic range are *Albinaria discolor*, *A. profuga* and *A. scopulosa*.

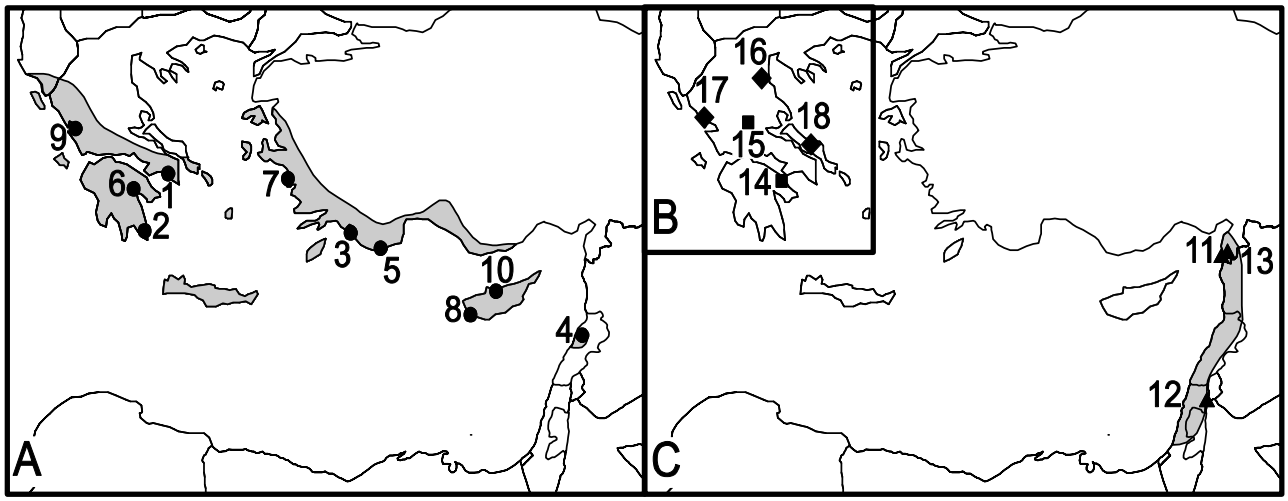


Figure 3.4. Generic ranges and collection sites, numbers refer to Table 3.1. **A** Distribution of *Albinaria* with the collection sites of the *Albinaria* species (dots); **B** Collection sites of *Isabellaria* species (squares) and *Sericata* species (diamonds); **C** Distribution of *Cristataria* and *Cristataria* collection sites (triangles).

We investigated *Cristataria* species from the regions north and south of the Lebanese *Albinaria* species. *Cristataria colbeauiana* and *C. turcica* from southern Turkey represent the northern part of the generic range, *C. genezerethana* from Israel the southern part. The genera *Isabellaria* and *Sericata*, which are considered closely related to *Albinaria* and *Cristataria* (Nordsieck, 1977a), are represented by two and three species, respectively. Since all ingroup species are placed in the tribe Medorini, we included four genera of this tribe in the outgroup, viz. *Agathylla*, *Medora*, *Muticaria* and *Strigilodelima*. The presumably closely related tribe Montenegrinini (Nordsieck, 1972) is represented by *Montenegrina*.

For nearly all species, we combined 12S and ITS1&2 sequences from the same individual. The ITS1&2 and the 12S sequences of *Albinaria puella puella* and *Albinaria forbesiana bigibbosa* are from different individuals of the same population, as is the case for the ITS1+12S and the ITS2 sequences of *Isabellaria isabellina isabellina* and of *Sericata sericata sericata.* The ITS1&2 and the 12S sequences of *Albinaria discolor discolor, Albinaria scopulosa epirotes* and *Albinaria caerulea caerulea* represent different populations (see Table 3.1). All 12S sequences were determined in this study, apart from the 12S sequence of *Albinaria caerulea caerulea*, which was obtained from Hatzoglou *et al.* (1995). The ITS data set consisted of both newly determined and existing sequences (see appendix). The ITS1&2 sequences of *Albinaria discolor* and *A. scopulosa* were obtained by Van Moorsel *et al*. (2000). Van Moorsel *et al.* (2001d) determined the ITS1&2 sequences of *A. puella*, *A. forbesiana* and *A. caerulea.* Finally, the ITS2 sequences of *Isabellaria isabellina isabellina* and *Sericata sericata sericata* were determined by van Moorsel *et al.* (2000).

DNA extraction, amplification and sequencing

DNA was extracted from foot tissue. Dependent on the condition of the tissue, different protocols were used. Fresh tissue from *Albinaria discolor*, *A. forbesiana*, *A. profuga*, *A. puella*, *A. scopulosa*, *Isabellaria isabellina*, *I. vallata*, *Sericata albicosta*, *S. inchoata*, *S. sericata* and the outgroup species, which was frozen at –80˚C after collecting, was extracted using the

Table 3.1. Species used in the analyses and their collection sites. Group numbers according to Nordsieck (1997). ∗ : ITS sample from: UTM= FF6967; ∗∗ : ITS sample from: UTM= DK7613.

No.	ingroup species	group	country	UTM	co-ordinates
1	*Albinaria caerulea caerulea* (Deshayes, 1833)	IV	Greece	GH	37º59'N 23º44'E
2	*Albinaria discolor discolor* (L. Pfeiffer, 1846)	I	Greece	FF8362*	36º41'N 23º03'E*
3	*Albinaria forbesiana bigibbosa* (Charpentier, 1847)	IV	Turkey	PA85	36º39'N 29º06'E
4	*Albinaria hedenborgi* (L. Pfeiffer, 1850)	V	Lebanon	YC69	34º17'N 35º54'E
5	*Albinaria lycica lycica* Nordsieck, 1993	III	Turkey	QA61	36º17'N 29º58'E
6	*Albinaria profuga* (Charpentier, 1852)	I	Greece	FG5367	37º38'N 22º44'E
7	*Albinaria puella puella* (L. Pfeiffer, 1850)	V	Turkey	NB29	37º52'N 27º16'E
8	*Albinaria saxatilis* (L. Pfeiffer, 1846)	II	Cyprus	VD44	34º46'N 32º25'E
9	*Albinaria scopulosa epirotes* (Nordsieck, 1974)	I	Greece	EJ31**	38º59'N 21º23'E**
10	*Albinaria virgo* (Mousson, 1854)	V	Cyprus	WE30	35º19'N 33º22'E
11	*Cristataria colbeauiana* (L. Pfeiffer, 1861)		Turkey	BA40	36º14'N 36º07'E
12	*Cristataria genezerethana* (Tristam, 1865)		Israel	YB35	32º48'N 35º31'E
13	*Cristataria turcica* Neubert, 1993		Turkey	YF60	36º07'N 35º56'E
14	*Isabellaria isabellina isabellina* (L. Pfeiffer, 1842)		Greece	FG8190	37º50'N 23º02'E
15	*Isabellaria vallata errata* Fauer, 1985		Greece	EJ93	39º07'N 22º03'E
16	*Sericata albicosta* (O. Boettger, 1877)		Greece	FK2038	40º05'N 22º25'E
17	*Sericata inchoata inchoata* (O. Boettger, 1889)		Greece	DJ7233	39º09'N 20º41'E
18	*Sericata sericata sericata* (L. Pfeiffer, 1850)		Greece	GH4774	38º35'N 23º50'E
	outgroup species	**tribe**	**country**	**UTM**	**co-ordinates**
	Agathylla lamellosa (Schubert & Wagner, 1829)	Medorini	Croatia	BN62	42º39'N 18º05'E
	Medora italliana garganensis (A.J. Wagner, 1918)	Medorini	Italy	WG71	41º40'N 15º53'E
	Montenegrina dennisi dennisi Gittenberger, 2002.	Monte-negrinini	Greece	EK2429	40º01'N 21º17'E
	Muticaria syracusana (Philippi, 1836)	Medorini	Italy	WB20	37º04'N 15º18'E
	Strigilodelima conspersa (L. Pfeiffer, 1848)	Medorini	Greece	DJ9591	39º40'N 20º57'E

Table 3.2. Amplification primers used in this study and their annealing temperatures.

region	forward primer (5' to 3')	reverse primer (5' to 3')	T
ITS1	CAC ACC GCC CGT CGC TAC TAC C	ATG CGT TCA AGA TGT CGA TGT TCA A	61°C
ITS2	GGC GGC CTC GGG TCC ATC C	TTC CCG CTT CAC TCG CCG TTA CTG	61°C
3' $tRNA^{Met}$ & 5' 12S rRNA	TAA GCT GTA GGG CTC ATA AC	GAG AGT GAC GGG CGA TTT G	47°C

protocol described by Schilthuizen *et al*. (1995), as was nearly fresh 96%-ethanol-stored tissue of *Cristataria genezerethana*. Tissue of *Albinaria lycica*, *A. hedenborgi*, *A. saxatilis*, *A. virgo*, *Cristataria colbeauiana* and *C. turcica*, which had been stored in 70% ethanol for 7-20 years was extracted using a modified protocol, with a single chloroform extraction and a prolonged digestion and precipitation period (10-12 hours) (see Chapter 2, this thesis).

The complete nuclear ITS1&2 regions and the partial mitochondrial $tRNA^{Met}$ and 12S (5' side) genes were amplified with PCR (35 cycles), using the primer sequences and annealing temperatures listed in Table 3.2. All of these primers were developed on the basis of previously published DNA sequences of *Albinaria* species. PCR product was gel-purified using spin columns (Qiaquick® Gel Extraction Kit by Qiagen®), dye-terminator cycle-sequenced (Big Dye™ by PE Biosystems®) in both directions, and run on an ABI 377 automated sequencer (PE Biosystems®). The forward and reverse sequences were assembled and edited in Sequencher 3.0 (Gene Codes Corp.®). Sequences were submitted to GenBank (Accession numbers AY382064-AY382085 for the 12S sequences, and AY382086-AY382097 for the ITS1&2 sequences).

Data analysis

Sequences were aligned using the Clustal V algorithm implemented in MegAlign 4.03© (DNASTAR Inc., 1999) with the default parameter settings. The ITS1&2 alignment was checked for the occurrence of previously identified conserved regions. Using MacClade 4.0 (Maddison & Maddison, 2000), minor adjustments were made manually in the alignment of the ITS and 12S sequences. Ambiguously aligned positions and indels were ignored in subsequent analyses. The data matrix was submitted to TreeBASE (http://www.treebase.org).

The data set was imported in PAUP* 4.0b10 (Swofford, 2002) for phylogenetic analyses. The presence of phylogenetic signal was tested for ITS1&2 and the 12S independently, using a permutation test (Archie, 1989; Faith & Cranston, 1991) of 1000 replicates with one random addition (TBR) each, and by calculating the g1 value, a measure of the skewness of tree length distribution (Hillis & Huelsenbeck, 1992). To find out if this underlying signal was similar for both data sets, we carried out a partition homogeneity test, consisting of 1000 replicates (10 random addition replicates, TBR, and steepest descent in each replicate).

Maximum parsimony analyses were performed on both data sets, independently as well as combined, weighting transitions and transversions equally. We used a heuristic search, with 1000 random addition replicates, using the TBR option with steepest descent, and saving multiple trees to find the most parsimonious ones. Parsimony bootstrap support values were calculated through 1000 bootstrap replicates. Each bootstrap replicate consisted of a heuristic search with 1 random addition, using TBR and saving multiple trees.

RESULTS

We failed to obtain a complete 12S sequence for *Albinaria virgo*. We therefore excluded this sequence from the 12S bootstrap analyses, but included it in all of the other analyses. Base frequencies were homogeneous across all taxa for both 12S and ITS1&2 (P=1.00). The total set of 12S sequences contained 157 variable and 105 parsimony informative positions. These numbers were 161 and 84, respectively, for ITS. Both the 12S and the ITS1&2 data set contained a strong phylogenetic signal. In both cases the permutation test was highly significant (P=0.001), as were the g1 values of –0.511 for 12S (P<0.01) and –0.606 for ITS1&2 (P<0.01). The partition homogeneity test showed that this signal did not significantly deviate between the

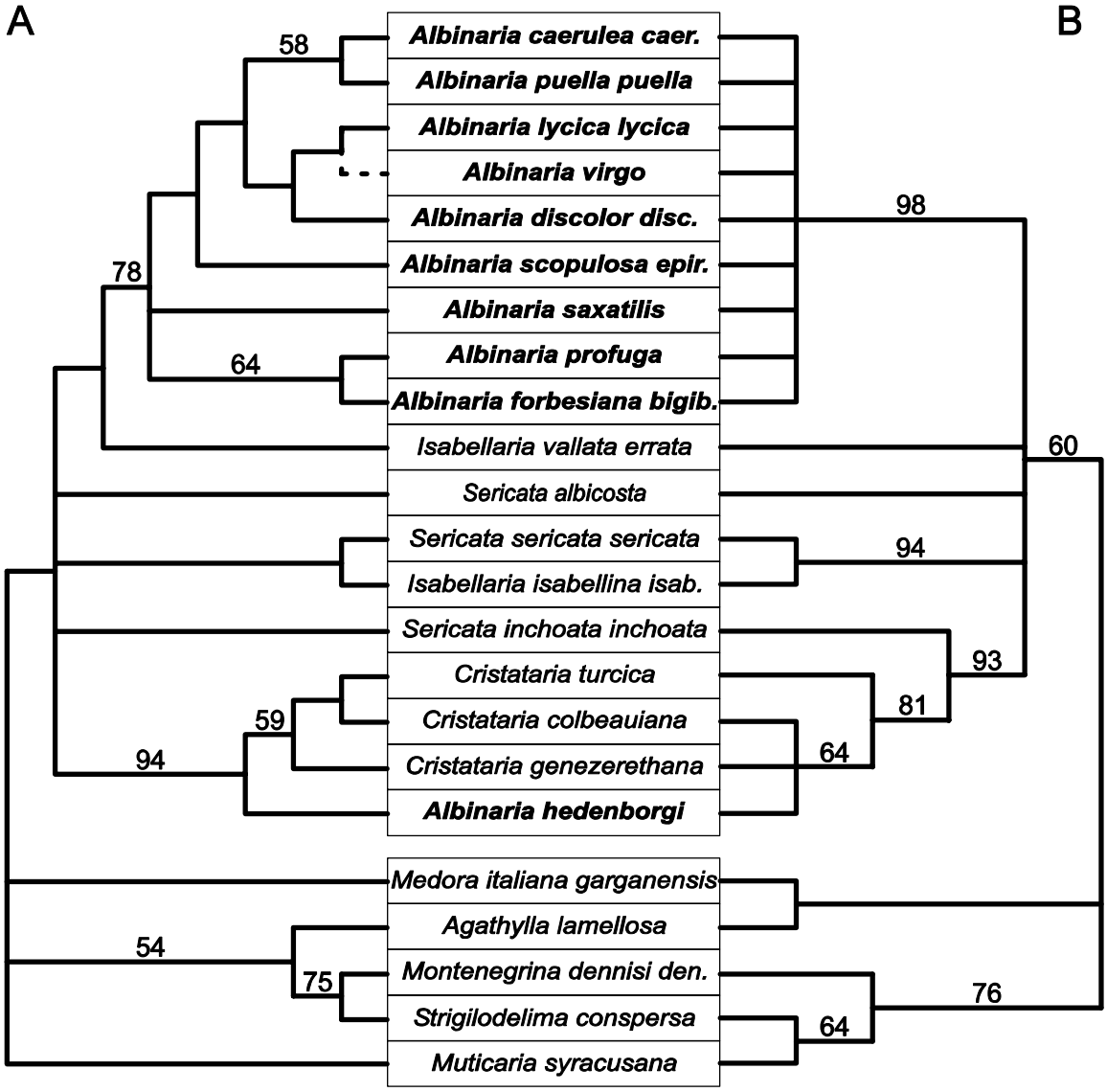


Figure 3.5. Strict consensus cladogram of (**A**) the 12S sequence data set and (**B**) the ITS sequence data set. Numbers indicate parsimony bootstrap values. The branch to *A. virgo* in the 12S strict consensus cladogram is dashed, indicating that this species was excluded from the 12S bootstrap analysis.

two data sets, neither with *A. virgo* included (P=0.489), nor without this species (P=0.304).

We found 15 most parsimonious trees for the 12S data set including *A. virgo*. Their score was 463 and their consistency index 0.440, excluding uninformative characters. The heuristic maximum parsimony search on the ITS1&2 data set produced 418 most parsimonious trees, with a score of 301 and a consistency index of 0.519, again excluding uninformative characters. The consistency index values of both data sets are near the CI of 0.507, expected in a cladistic analysis of 23 taxa (Sanderson and Donoghue, 1989). After combining both datasets, 12 MP trees, with a score of 776 and a CI of 0.459 (uninformative characters excluded), were found.

Both 12S- and ITS1&2-based trees (Fig. 3.5) strongly support a *Cristataria*-clade including *Albinaria hedenborgi*, and a separate *Albinaria*-clade consisting of the remaining *Albinaria* species sampled. The grouping of *Albinaria hedenborgi* with the *Cristataria* species is 94% bootstrap supported by 12S sequences and 81% by ITS1&2 sequences. The ITS1&2 data set supports the monophyly of the remaining *Albinaria* species, including *A. virgo*, by 98%. Apart from substitutions, this clade is also supported by a large deletion in ITS1, of 20 base pairs at a minimum. The bootstrap support in the 12S tree for the clade of all *Albinaria* species except *A. hedenborgi*, without the incomplete *A. virgo* sequence, is with 78% slightly lower. After combining ITS1&2 and 12S, these bootstrap values add up to 100% for both clades (Fig. 3.6). Most other clades that are found differ between the two trees, and are only weakly supported in each of them and in the combined analysis.

Although the topology within the *Cristataria*-clade is different for the ITS1&2 and the 12S data set, most of these differences are not highly supported. The combined analysis produced subclades that were identical to those found in the 12S tree, but with better support. In the cladogram derived from the combined data sets, *A. hedenborgi* is the sister group of the *Cristataria* species. Within this *Cristataria* subclade, the two Turkish species *C. colbeauiana* and *C. turcica* constitute the sister group of the Israeli species *C. genezerethana*.

The relationships within the '*Albinaria*-clade' remain largely unclear. Different clades are equally parsimonious in the ITS1&2 data set, resulting in a complete polytomy. In the 12S tree, only two clades receive more than 50% bootstrap support: (1) *Albinaria caerulea* and *A. puella* (58%), and (2) *A. profuga* and *A. forbesiana* (64%).

DISCUSSION AND CONCLUSIONS

Albinaria versus *Cristataria*

Both the ITS1&2 and the 12S data set provide convincing evidence that there is a monophyletic group of species, the *Cristataria*-clade, to which *A. hedenborgi* belongs. The Cyprian, Turkish and Greek *Albinaria* species together form a separate clade, which can also be defined by synapomorphic substitutions and additionally by a large deletion. The congruence between the data sets is best explained as a result of a similar phylogenetic signal. Incongruence between trees derived from mitochondrial and nuclear DNA has been observed within *Albinaria*, and was attributed to either introgression of mitochondrial DNA or ancestral polymorphisms (van Moorsel *et al.*, 2001c). These phenomena do not appear to have affected the 12S and ITS1&2 trees at the systematically higher level addressed in this study.

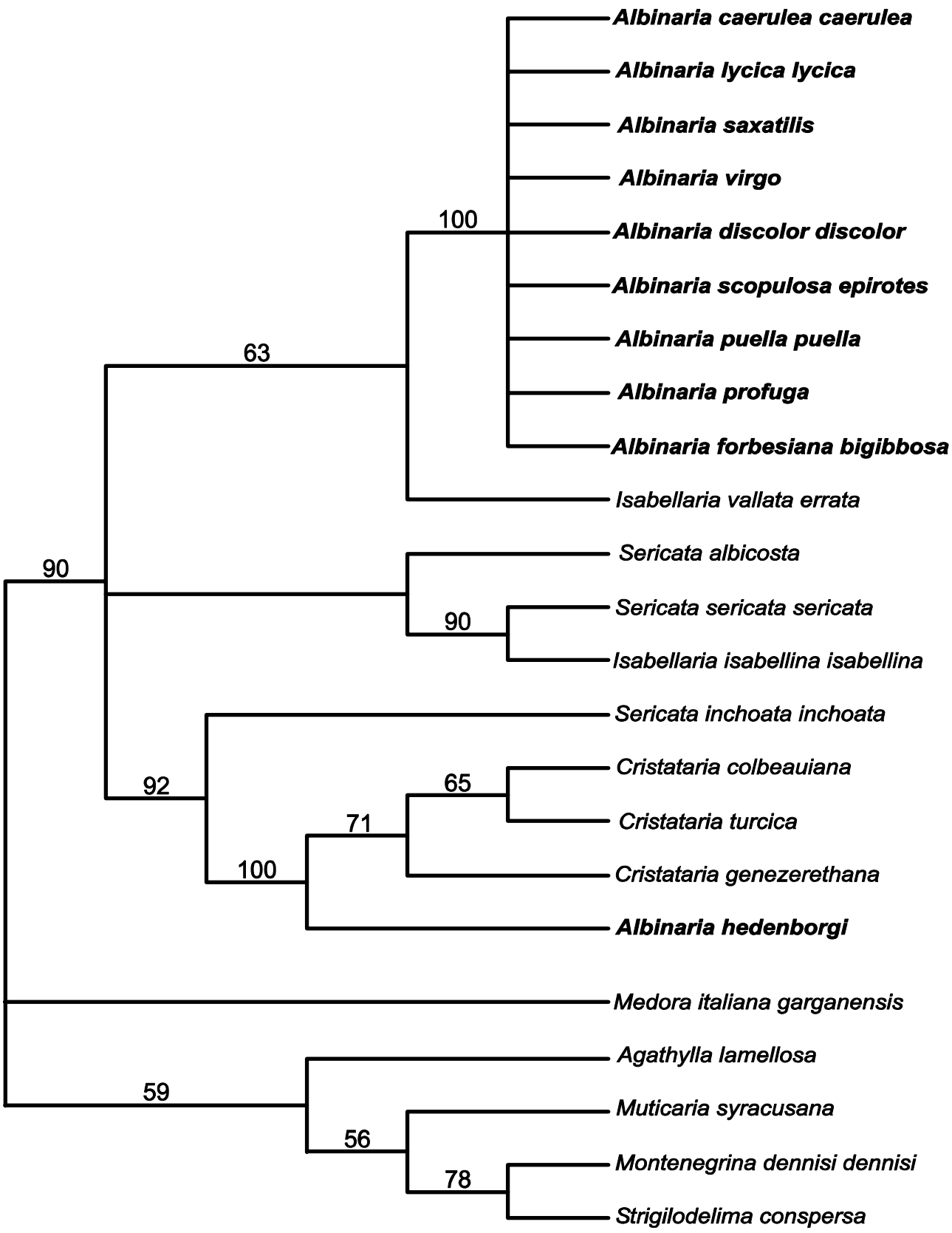


Figure 3.6. Strict consensus cladogram of the 12 MP trees from the combined 12S and ITS data set. Numbers indicate parsimony bootstrap values.

The grouping of *A. hedenborgi* with *Cristataria* necessitates a reappraisal of the conchological and anatomical characters, which have led systematists astray (but see Bar, 1977). It is also relevant for the historical biogeography of the taxa involved.

Re-evaluation of the morphological characters

Differences in diverticulum length between *Albinaria* and *Cristataria* are gradual, if consistently present at all (see Nordsieck, 1997: Table 2), and only a limited number of samples and species have been studied to support the classification of *A. hedenborgi* with *Albinaria* (Nordsieck, 1977a). More accurate, quantified, data on this character are not available, which hampers its interpretation. In *A. hippolyti*, for example, the relative length of the diverticulum varies as a subspecific character state from not longer to clearly longer than the bursa reaches (Schilthuizen & Lombaerts, 1995; Schilthuizen, 1995). Diverticulum length appears to be more variable and consequently less informative about deeper-level phylogenetic relationships than previously acknowledged.

The basal keel on the shell, which is considered a synapomorphy for the *munda* group in *Albinaria* (Nordsieck, 1997), is at the same time characteristic for many species of the genus *Cristataria* (Nordsieck, 1971: 238). Therefore, the presence of a basal keel in *A. hedenborgi* and in the systematically allied *A. nadimi* does not conflict with the position of these species in the *Cristataria*-clade, nor would it in *A. staudingeri*.

Taxonomic implications

We conclude that *'A.' hedenborgi,* and consequently the allied *A. nadimi* too, should be classified with *Cristataria*. Pending the availability of evidence to the contrary, the presence of *Albinaria* species in Lebanon is rejected (see also Bar, 1977), and we propose to maintain the original name *Cristataria staudingeri* for the third Lebanese *Albinaria* species. From now on we will use the names *Albinaria* and *Cristataria* in conformity with these views.

Biogeographic patterns

The ranges of *Albinaria* and *Cristataria*, as defined now, are separated on the mainland by a region extending from Silifke (Seleucia) on eastward, in which another clausiliid species, viz. *Armenica bicarinata*, occurs (Neubert, 1992). The Cyprian species, which occupy a geographic borderline, clearly belong to *Albinaria*. The occurrence of *Albinaria alajana* in southern Anatolia as well as in Cyprus (Nordsieck, 1993: 22) indicates that dispersal between Anatolia and Cyprus occurred relatively recently.

Our results lend some support to the hypothesis that the *Cristataria* species "came from Balkan stock" (Bar, 1977). All genera within the tribe Medorini, with the exception of *Albinaria* and *Cristataria*, are restricted to south-eastern Europe. In fact, *Albinaria* and *Cristataria* are the easternmost distributed genera within the subfamily Alopiinae (see Nordsieck, 1979). The combined analysis of the ITS and 12S sequences demonstrates that these two genera are nested separately among SE-European taxa. These results imply that *Cristataria* indeed descended from a, most probably, SE-European ancestor, as did *Albinaria*.

Although the *Cristataria* species are found on the African plate, in contrast to all other Medorini, which are located on the Eurasian plate (see Robertson, 1998), *Cristataria*'s

relatively easterly distribution cannot be attributed to tectonic events. Such an explanation would be problematic, since the SE-European landmasses, where the sister group and the clades basal to *Cristataria* occur, have been widely separated from the Levant from at least early Cretaceous onward (Robertson & Dixon, 1984; Dercourt *et al.*, 1992). This separation predates even the earliest European fossils of Clausiliidae known (see Nordsieck, 2000). Alternatively, it can be hypothesized that the ancestors of *Cristataria* spread from south-eastern Europe through Asia Minor towards the current generic range. If so, it has to be accepted that they became extinct in Asia Minor, to be replaced there by *Albinaria* species.

In this light, it is critical whether the Anatolian species that were placed in the *Albinaria munda* group with '*A.*' *hedenborgi*, '*A.*' *nadimi* and '*A.*' *staudingeri* (Nordsieck, 1997; 1999) are part of the *Albinaria*-clade indeed, or relic species most closely related to *Cristataria*. Particularly uncertain is the systematic position of the supposedly closely related species (Nordsieck, 1993) *Albinaria schuetti*, *A. supercarinata*, *A. pellucida* and *A. monocristata,* which are all characterized by a very prominent basal keel. These species are distributed in Asia Minor near the eastern border of the range of *Albinaria*. Except for *A. schuetti,* their small ranges are located within or at the border of the ranges of other undisputed *Albinaria* species (Nordsieck, 1993). The latter species group might exemplify a process of eastward dispersal of *Albinaria* species along the coast of Asia Minor, resulting in the subsequent replacement of the earlier local species related to *Cristataria*, but not completely so in the regions that were most recently invaded. On the other hand, the basal keel and other conchological characters observed in *A. schuetti*, *A. supercarinata*, *A. pellucida* and *A. monocristata* could also be seen as indicative of a close relationship with *A. virgo* (Nordsieck, 1993), occurring in the northern part of Cyprus opposite the ranges of the four Anatolian species. This would imply that *A. schuetti*, *A. supercarinata*, *A. pellucida* and *A. monocristata* are part of a subclade in *Albinaria* to which *A. virgo* belongs (see Fig 3.6). We consider this option the most plausible alternative.

Future research

Neither the *Albinaria*- nor the *Cristataria*-clade can be diagnosed unequivocally by conchological or genital-anatomical characters. As a consequence, only further studies on DNA sequences of *A. schuetti*, *A. supercarinata*, *A. pellucida* and *A. monocristata*, as well as of the now reclassified *C. staudingeri*, could resolve their systematic positions. To be able to reconstruct the historical biogeography of the *Albinaria*-clade in more detail, in particular in relation to the transgression of the Aegean Sea and the colonization of Cyprus, many additional eastern taxa should be investigated.

ACKNOWLEDGEMENTS

H. Nordsieck collected the *Cristataria colbeauiana* and *Cristataria turcica* samples; H.-J. Niederhöfer from the Staatliches Museum für Naturkunde Stuttgart kindly donated foot tissue of these samples to us. All sequences of *Albinaria virgo*, *Cristataria genezerethana* and *Strigilodelima conspersa*, as well as the 12S sequences of *Albinaria forbesiana bigibbosa*,

Albinaria profuga, *Albinaria puella puella*, *Albinaria scopulosa epirotes* and *Albinaria virgo*, were determined by D.S.J. Groenenberg.

Chapter 4

WIDESPREAD POLYPHYLY AMONG ALOPIINAE SNAIL GENERA: WHEN PHYLOGENY MIRRORS BIOGEOGRAPHY MORE CLOSELY THAN MORPHOLOGY

ABSTRACT

Consider a group of species that is evenly divided by an easily identifiable complex morphological character. Most biologists would assume that this character should provide better phylogenetic information than, say, the spatial distribution of these species over a fairly continuous 500-km radius area. Paradoxically, this is not the case among terrestrial snail genera in the clausiliid subfamily Alopiinae. A phylogenetic analysis using the nuclear markers ITS1/ITS2 and mitochondrial markers COI/12S reveals widespread homoplasy in the clausilial apparatus (a complex aperture-closing mechanism), and concomitant extensive polyphyly among *Carinigera*, *Isabellaria* and *Sericata*. In contrast, phylogenetic relationships as revealed by molecular data are highly congruent with biogeography at a relatively small scale. A combination of extremely low vagility and extremely high morphological convergence has conspired to produce this unexpected result. Implications as to the function of the clausilial apparatus are discussed.

INTRODUCTION

A unique apomorphic closing mechanism, the clausilial apparatus (CA), characterizes the shells of pulmonate snails in the family Clausiliidae, and modifications of it are used in intra-family classification. The CA is a door-like structure, consisting of a moveable plate and associated lamellae, located in the ultimate whorl of the shell (Fig. 4.1a). Two types of CA are recognized: (1) the N-type (Fig. 4.1b) and (2) the G-type (Fig. 4.1c). In the N-type, the plate cannot close off the aperture entirely, and always leaves open a by-pass canal, supported by two parallel lamellae. In the G-type these lamellae are absent and the moveable plate can seal off the aperture completely. The N-type is considered the plesiomorphic condition, from which the G-type is thought to have evolved several times in parallel (Nordsieck, 1963, 1982). Despite the homoplasious nature of this character, the G-type is used as a diagnostic character at the genus level.

Both types of CA are found in a monophyletic (Uit de Weerd & Gittenberger, Chapter 2, this thesis) group of limestone-dwelling genera: *Isabellaria* Vest, 1867, *Carinigera* Moellendorf, 1873 , *Sericata* O. Boettger, 1878, *Albinaria* Vest, 1867 and *Cristataria* Vest, 1867. The genus *Isabellaria* (sensu Gittenberger, 1998a) is, among others, defined as having a G-type CA, in *Albinaria* (sensu Uit de Weerd & Gittenberger, Chapter 3, this thesis) both types are encountered, whereas all other genera by definition possess an N-type CA. Of the two genera containing G-type species, *Albinaria* is best studied. Despite its polymorphic CA,

Dennis R. Uit de Weerd, Edmund Gittenberger and William H. Piel. Widespread polyphyly among Alopiinae snail genera: when phylogeny mirrors biogeography more closely than morphology.

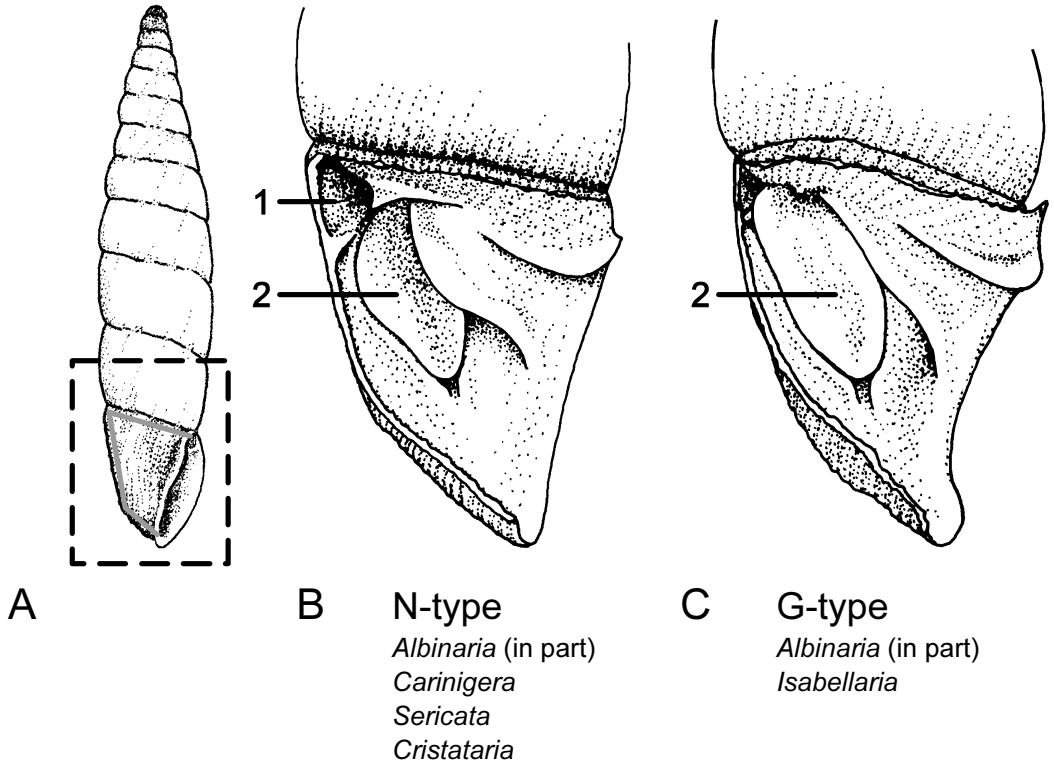


Figure 4.1. The two types of CA, and their position in the ultimate whorl of the shell. **A** Side-view of the shell. The palatal shell-wall located within the grey lines was removed to reveal internal shell structures; **B** N-type CA with by-pass canal (1) next to the clausilial plate (2); **C** G-type CA, with complete sealing of the aperture by the clausilial plate (2). Modified after van Moorsel *et al*., 2000.

Albinaria is clearly monophyletic, as demonstrated by two molecular studies (Douris *et al*., 1998b; van Moorsel *et al.*, 2000).

The monophyly of *Isabellaria*, on the other hand, is controversial. In its present definition, *Isabellaria* can be roughly recognized by its putatively apomorphic G-type CA in combination with a mostly greyish-brown shell surface (Gittenberger, 1998a). Both molecular and morphological studies, however, indicate close affinities between some *Isabellaria* and *Sericata* species (Douris *et al*., 1998b; van Moorsel *et al.*, 2000). Furthermore, recent molecular studies suggest that *Carinigera* species are closely related to *Isabellaria* species (Uit de Weerd & Gittenberger, Chapter 2, this thesis). Apart from their presumably plesiomorphic N-type CA, the genera *Sericata* and *Carinigera* cannot be distinguished from *Isabellaria* conchologically. *Carinigera* has been set apart from *Isabellaria* and *Sericata* on the basis of some putatively apomorphic genital-anatomical characters, which previous molecular studies, however, revealed as homoplastic (Uit de Weerd & Gittenberger, Chapter 2).

Using the present, largely CA-based, criterion for their classification, the so-called *Isabellaria* species show a somewhat disjunct distribution (Fig. 4.2). Their combined range extends from the north-eastern Peloponnese in Greece up northwards into the country of Macedonia. In almost this entire area, the ranges of the individual *Isabellaria* species are interspersed with those of so-called *Sericata* and *Carinigera* species (Fig. 4.3). *Sericata,* as a whole, has a range roughly coincident with that of *Isabellaria*, whereas *Carinigera* has a more

Figure 4.2. Schematic distribution of the G-type species that are classified with the genus *Isabellaria*. Grey lines indicate borders of distribution areas, and are based on the literature and data in the National Museum of Natural History in Leiden, the Netherlands.

Figure 4.3. Schematic distribution of the N-type species, which are classified with the genera *Carinigera* and *Sericata*. Grey lines indicate borders of distribution areas, and are based on the literature and data in the National Museum of Natural History in Leiden, the Netherlands.

north-eastern, albeit overlapping, distribution, occurring in the region from central Greece into Thracia and even into Serbia. The species hardly ever occur sympatrically, presumably because of competitive exclusion (Nordsieck, 1974), which may be indicative of poor niche differentiation among taxa.

The curious incongruence between distribution patterns and morphology extends to species pairs: neighbouring species are often highly similar in shell morphology, yet are placed in different genera because of differences in the clausilial apparatus. Four such species pairs, consisting of *Sericata* and *Isabellaria* species, are referred to by Nordsieck (1974, 1977b, 1984) (Fig. 4.4): *S. stussineri & I. lophauchena; S. bathyclista & I. riedeli; S. parnassia & I. thermopylarum*; *S. lutracana & I. isabellina*. Two hypotheses have been proposed to explain these occurrences. The species within a species pair are supposed not to be closely related and similarities in external shell morphology exist because of either (a) introgression of these features through hybridization (Nordsieck, 1984; 1997) or (b) convergent evolution (Nordsieck, 1974: 132). As an alternative explanation, it was hypothesized that the species in a species pair are, in fact, sister species, requiring either (a) recurrent independent origin of a G- from an N-type CA (van Moorsel *et al.*, 2000) or (b) xenologous transfer of the genetic basis for a G-type CA (Gittenberger, 1998a, 1998b, 2000).

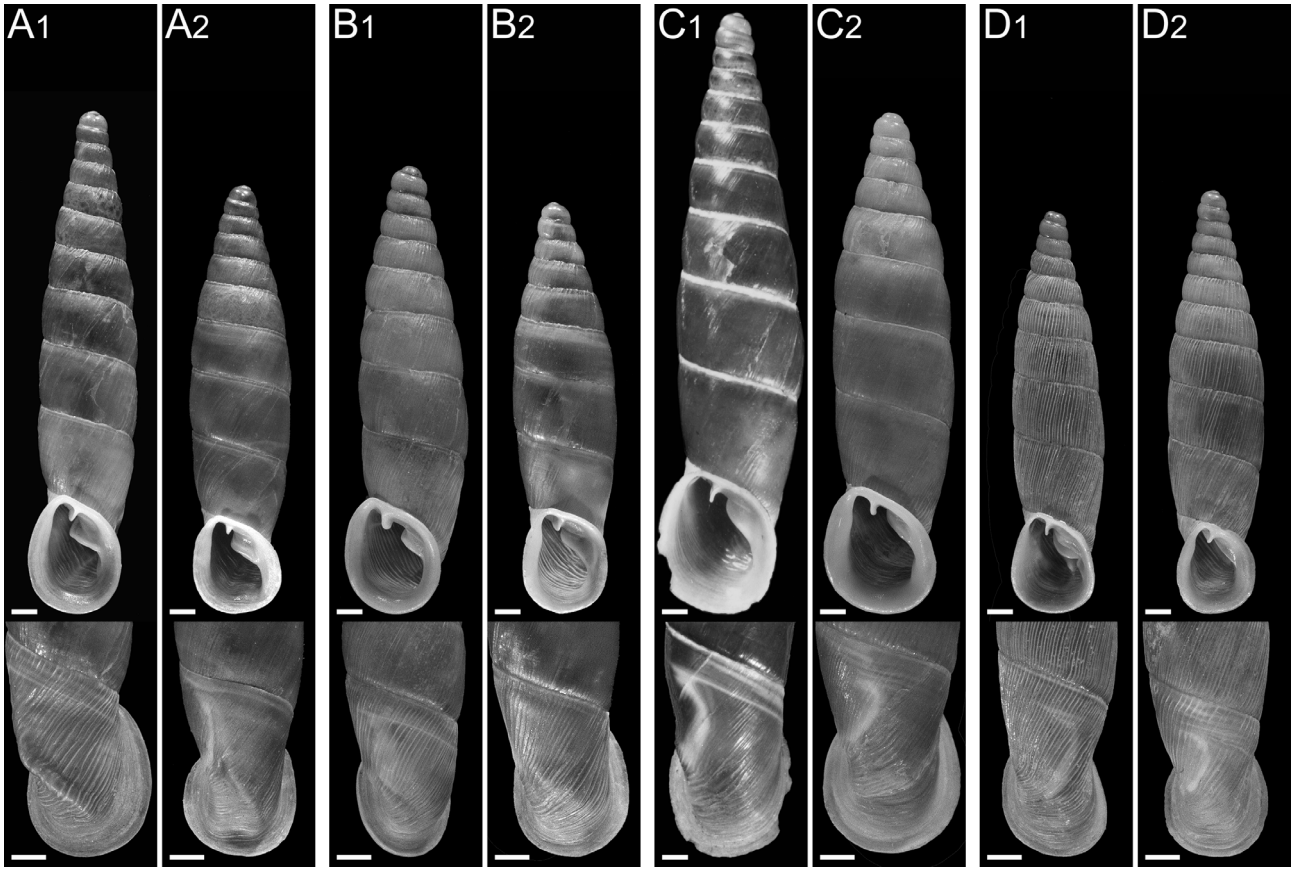


Figure 4.4. A-D reported N/G-type *Sericata-Isabellaria* species pairs. **A1** *Sericata stussineri*, **A2** *Isabellaria lophauchena*; **B1** *Sericata bathyclista*, **B2** *Isabellaria riedeli*; **C1** *Sericata parnassia*, **C2** *Isabellaria thermopylarum*; **D1** *Sericata lutracana*, **D2** *Isabellaria isabellina*. All photographs except C1 (after Brandt, 1962) by A 't Hooft (IBL, Leiden).

Molecular analyses indicate that the species in comparable N/G-type species pairs within *Albinaria* are closely related, thereby demonstrating that recurrent transformations in CA-type are possible (van Moorsel *et al.*, 2000). The evolution of the CA in *Albinaria* could not be reconstructed unequivocally (see van Moorsel *et al.*, 2000), however. Neither can the possibility of transspecific introgression of the G-type CA (Gittenberger, 1998a, 1998b, 2000) be excluded, since the G-type *Albinaria* species, placed in different clades together with N-type ones, inhabit a more or less continuous combined range in the eastern Peloponnese. In this respect, these G-type *Albinaria* species differ from the G-type *Isabellaria* species, which have a much more disjunct distribution (see Fig. 4.2).

To study the phylogenetic relationships between species placed in *Isabellaria*, *Carinigera* and *Sericata* independently from the disputed morphological characters, we used DNA sequence data, both nuclear and mitochondrial. The nuclear data set consists of the ribosomal internal transcribed spacer 1 (ITS1) and 2 (ITS2), which have proved informative with respect to the relationships between species of *Albinaria*, *Isabellaria* and *Sericata* (van Moorsel *et al.*, 2000). Of the mitochondrion we sequenced fragments of two genes, viz. the small ribosomal subunit (12S) and the cytochrome *c* oxidase subunit I (COI).

MATERIALS AND METHODS

Selection of taxa

All species sampled and sources of their sequences are listed in Table 4.1. The localities of the samples are shown in Figures 4.5 and 4.6. We included all species presently placed in the genera *Isabellaria* (14 species), and nearly all from the genera *Carinigera* (11 species) and *Sericata* (14 species), with the exception of *Sericata calabacensis* Westerlund, 1892, *Sericata parnassia* Boettger, 1888 and *Carinigera pellucida* Dedov & Neubert, 2002. Of the two *Sericata* species, to our knowledge, no living specimens have ever been collected. The third species, *Carinigera pellucida*, had not yet been described at the time of the laboratory analyses. *Isabellaria vallata* is represented by both of its subspecies, since these are widely separated geographically, and so is *Isabellaria clandestina*. *Isabellaria clandestina subsuturalis* was originally described as a separate species, and was only recently and guardedly placed in *Isabellaria clandestina* (Nordsieck, 1974). The *Albinaria* clade and the *Cristataria* clade (see Uit de Weerd & Gittenberger, Chapter 3, this thesis) are represented by six and four species, respectively. For outgroup rooting, we used four genera that belong together with the ingroup species in the tribe Medorini. In addition, *Montenegrina* represents the related tribe Montenegrinini.

Table 4.1. (next page →). Species sampled in this study, with their locality codes and the source of ITS1, ITS2 and 12S sequences used, when obtained from previous studies: (1) van Moorsel *et al.* (2000); (2) van Moorsel *et al.*(2001d); (3) Hatzoglou *et al*. (1995); (4) Uit de Weerd & Gittenberger, Chapter 2 of this thesis; (5) Uit de Weerd & Gittenberger, Chapter 3 of this thesis. (*) Different specimen from the same population. (**) Different specimen from another population, not shown on the map.

sample information				**Origin sequence**		
No	Species.	co-ordinates	UTM	ITS1	ITS2	12S
1.	*Albinaria caerulea caerulea*	unknown	GH	2	2	3
2.	*Albinaria discolor discolor*	36º41'N 23º03'E	FF8362	1**	1**	5
3.	*Albinaria forbesiana bigibbosa*	36º39'N 29º06'E	PA85	2	2	5
4.	*Albinaria profuga*	37º38'N 22º44'E	FG5367	5	5	5
5.	*Albinaria puella puella*	37º52'N 27º16'E	NB29	2	2	5
6.	*Albinaria scopulosa epirotes*	38º59'N 21º23'E	EJ31	1**	1**	5
7.	*Carinigera buresi nordsiecki*	40°54'N 24°14'E	KF6731	4	4	
8.	*Carinigera drenovoensis*	41°25'N 21°54'E	EL78	4	4	
9.	*Carinigera eximia*	43°19'N 22°08'E	EN99	4	4	
10.	*Carinigera hausknechti alticola*	38°57'N 21°48'E	EJ6910	4	4	
11.	*Carinigera megdova tavropodensis*	38°57'N 21°41'E	EJ5910	4	4	
12.	*Carinigera octava*	41°42'N 21°46'E	EM61	4	4	
13.	*Carinigera pharsalica*	39°29'N 22°37'E	FJ3871	4	4	
14.	*Carinigera schuetti*	41°07'N 23°38'E	GL2554	4	4	
15.	*Carinigera septima*	41°24'N 22°15'E	FL08	4	4	
16.	*Carinigera superba*	41°13'N 23°54'E	GL4266	4	4	
17.	*Cristataria colbeauiana*	36º14'N 36º07'E	BA40	4	4	5
18.	*Cristataria genezerethana*	32º48'N 35º31'E	YB35	4	4	5
19.	*Cristataria hedenborgi*	34º17'N 35º54'E	YC69	5	5	5
20.	*Cristataria turcica*	36º07'N 35º56'E	YF60	5	5	5
21.	*Isabellaria almae*	38°24'N 22°16'E	FH0949			
22.	*Isabellaria chelidromia chelidromia*	39°08'N 23°43'E	GJ353349			
23.	*Isabellaria clandestina clandestina*	39°12'N 23°12'E	FJ9042	4	4	
24.	*Isabellaria clandestina subsuturalis*	37°53'N 22°29'E	FG334957			
25.	*Isabellaria isabellina isabellina*	37º50'N 23º02'E	FG8190	4	1*	5
26.	*Isabellaria leucoraphe*	39°11'N 23°29'E	GJ142407			
27.	*Isabellaria lophauchena*	40°32'N 22°05'E	EK9188			
28.	*Isabellaria perplana perplana*	38°29'N 22°31'E	FH3261	4	1*	
29.	*Isabellaria praecipua praecipua*	40°29'N 22°12'E	FK08	1*	1*	
30.	*Isabellaria praestans*	39°07'N 23°42'E	GJ332327			
31.	*Isabellaria riedeli*	38°48'N 23°28'E	GH148979	1*	1*	
32.	*Isabellaria saxicola aperta*	37°56'N 23°47'E	GH4504	1*	1*	
33.	*Isabellaria thermopylarum faueri*	38°32'N 22°23'E	FH2066	1*	1*	
34.	*Isabellaria thessalonica crassilabra*	40°23'N 23°10'E	FK8371			
35.	*Isabellaria vallata errata*	39º07'N 22º03'E	EJ93	4	4	5
36.	*Isabellaria vallata vallata*	39°42'N 20°51'E	DJ89	1*	1*	
37.	*Sericata abyssoclista*	37°40'N 23°08'E	FG8872			
38.	*Sericata albicosta*	40º05'N 22º25'E	FK2038	4	4	5
39.	*Sericata bathyclista*	38°46'N 23°19'E	GH019933			
40.	*Sericata dextrorsa*	40°58'N 21°55'E	EL7635			
41.	*Sericata inchoata inchoata*	39º09'N 20º41'E	DJ7233	4	4	5
42.	*Sericata liebegottae*	39°07'N 23°59'E	GJ582346			
43.	*Sericata lutracana*	38°02'N 22°51'E	FH6311	1*	1*	
44.	*Sericata regina*	39°17'N 20°51'E	DJ8647	4	4	
45.	*Sericata sericata sericata*	38º35'N 23º50'E	GH4774	4	1*	5
46.	*Sericata stussineri stussineri*	39°53'N 22°38'E	FK3916			
47.	*Sericata tantilla*	39°42'N 22°14'E	FJ0596			
48.	*Sericata torifera*	39°41'N 21°41'E	EJ5891			
	outgroup					
49.	*Agathylla lamellosa*	42º39'N 18º05'E	BN62	4	4	5
50.	*Medora italiana garganensis*	41º40'N 15º53'E	WG71	4	4	5
51.	*Montenegrina dennisi*	40º01'N 21º17'E	EK2429	4	4	5
52.	*Muticaria syracusana*	37º04'N 15º18'E	WB20	4	4	5
53.	*Strigilodelima conspersa*	39º40'N 20º57'E	DJ9591	4	4	5

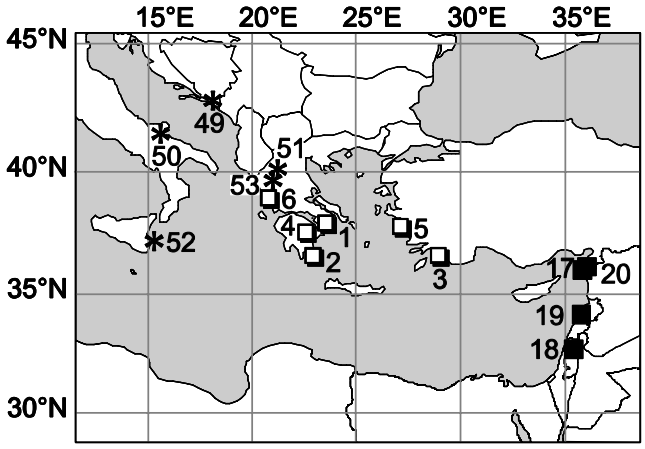


Figure 4.5. (↑) Collection sites of included *Albinaria* species (open squares), *Cristataria* species (closed squares) and outgroup species (asterisks).

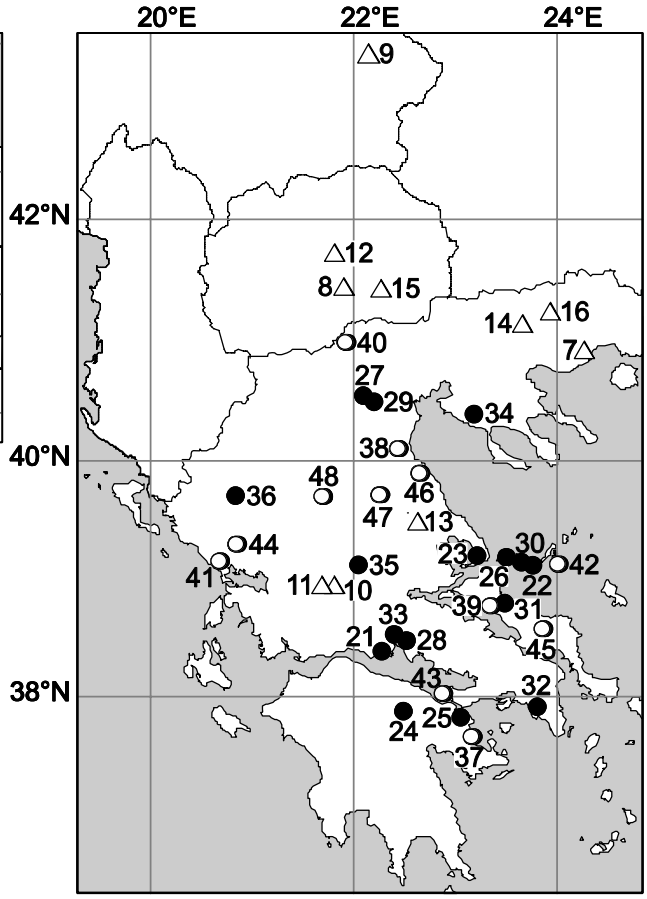


Figure 4.6. (→) Collection sites of included *Carinigera* species (triangles), *Isabellaria* (dots) and *Sericata* species (open circles).

Sequences and alignment

DNA was extracted from frozen and ethanol-preserved tissue using CTAB. For frozen and fresh tissue, the protocol by Schilthuizen *et al.* (1995) was followed. All tissue that had been stored in ethanol for more than one year was extracted using a modified protocol (see Uit de Weerd & Gittenberger, Chapter 2, this thesis).

The ITS1, ITS2, 12S and COI regions were amplified in 35 PCR cycles, using the primer sets listed in Table 4.2. The ITS 1 and 2 primer sets each amplify the respective spacer as well as adjacent regions of the rRNA genes. The 12S primers span nearly the total length of 12S, with the exception of 79 positions at its 3' side, and also amplify 24 bases at the 3' end of the methionine tRNA. The COI primers span 708 bases in the 5' half of cytochrome *c* oxidase subunit I. PCR product was pooled, gel purified using spin columns (Qiaquick® Gel Extraction Kit by Qiagen®) and dye-terminator cycle-sequenced (Big Dye™ by PE Biosystems®) in both directions. Electrophoresis was performed on an ABI 377 automated sequencer (PE Biosystems®). Forward and reverse sequences were assembled and edited in Sequencher (Gene Codes Corp.®).

All sequences were imported in MegAlign 4.03© (DNASTAR Inc., 1999), and aligned using the Clustal V method with default settings. The ITS and 12S alignments were further adjusted manually in MacClade 4.0 (Maddison & Maddison, 2000). A secondary structure model of domain III of 12S of *Albinaria caerulea* was calculated using the RNAlign Server (Page, 2000), to be used in alignment refinements and in the selection of positions to be

included in the analyses. Since secondary structure models can be misleading for ITS, at least in the group studied here (Armbruster, 2001; Uit de Weerd & Gittenberger, Chapter 2, this thesis), we instead compared the alignment to previously identified conserved regions that had been shown to be informative (van Moorsel *et al.*, 2001d; Uit de Weerd & Gittenberger, Chapter 2). All regions within ITS1, ITS2 and 12S that were ambiguously aligned within the ingroup were excluded. The COI data matrix was checked for gaps and stop codons. The resulting alignments were deposited with TreeBASE (http://www.treebase.org).

Bayesian analyses

Since rates and modes of evolution were expected to differ between ITS, 12S and COI, and even between sites within COI (see Li, 1997: 177-193), we decided to use a model-based approach to reconstruct the phylogeny of the group. We performed Bayesian inference, using MrBayes 3.0 (Ronquist & Huelsenbeck, 2003), as this approach allows optimization of parameters within datapartitions, while at the same time assuming a similar underlying phylogenetic tree for each partition. We recognized six data partitions: ITS1, ITS2, 12S, COI position 1, COI position 2, and COI position 3. Using PAUP* 4.0b10, we calculated for each datapartition the likelihood scores of different models superposed on a JC69 NJ tree calculated for that partition. These likelihood scores were subsequently evaluated using MrModeltest 1.1b (Nylander, 2002). Whenever the likelihood ratio test and the Akaike information criterion favoured different models, we chose to use a more inclusive model that combines both models.

All Bayesian analyses were performed twice, starting with random trees. Four Markov chains were run $4x10^6$ generations, and sampled once every 100 generations. We used three incrementally (T=0.20) heated chains and a cold one. All analyses were started under default priors. Likelihood settings were changed to the preferred substitution model, and unlinked across partitions.

Bayesian inference was first performed independently for two subsets: (1) mitochondrial (12S and the three codonpositions of COI) and (2) nuclear (ITS1&2) sequences. We determined the burn-in period by graphically checking for stationarity. When the outcomes of the two parallel Bayesian analyses within each subset were consistent, the trees of both analyses of each

Table 4.2. Primers used for amplification of the DNA regions analysed.

region	primer (5' to 3')	origin	T
ITS1	Forward: CAC ACC GCC CGT CGC TAC TAC C Reverse: ATG CGT TCA AGA TGT CGA TGT TCA A	Modified from Hillis & Dixon, 1991; see Chapter 2.	61°C
ITS2	Forward: GGC GGC CTC GGG TCC ATC C Reverse: TTC CCG CTT CAC TCG CCG TTA CTG	Newly developed; see Chapter 2.	61°C
COI (5' half)	Forward: ACT CAA CGA ATC ATA AAG ATA TTG G Reverse: TAT ACT TCA GGA TGA CCA AAA AAT CA	Gittenberger *et al.* (2004), modified from Folmer *et al.* (1994)	47°C
3' $tRNA^{Met}$ & 5' 12S rRNA	Forward: TAA GCT GTA GGG CTC ATA AC Reverse: GAG AGT GAC GGG CGA TTT G	Newly developed, see Chapter 3.	47°C

data set were combined, discarding those from within the burn-in period. Tree and bipartition probabilities of these combined trees were calculated in MrBayes 3.0. The 95% credibility intervals of trees obtained from the mitochondrial and the ITS data set were checked for overlap, and we compared the probabilities of the individual clades. This was done (1) by identifying clades supported in the majority rule consensus tree of both data sets, (2) by discriminating within each data set between clades significantly (>95%) and those not significantly supported by the other data set, and comparing the probabilities of both groups (one-tailed Mann-Whitney U-test, SPSS), and (3) by tracing incompatible clades, significantly supported by each data set. Depending on their congruence, all data partitions were analysed together in MrBayes 3.0, using the same procedure as before with parameter settings for each partition that were identical to those in the previous analyses.

Reconstruction of character evolution

Since the reconstruction of CA-type evolution is based both on the topology inferred as well as on the states of the terminal nodes, we randomized each parameter to test for significant deviations. The trees obtained through Bayesian inference allowed a probabilistic inference of character evolution. Of all 76,000 trees of a respective data set, the minimum number of CA-type transformations required was calculated in MacClade 4.0 (Maddison & Maddison, 2000). These numbers were then compared to the number of transformations required in an equal number of random trees generated in PAUP* 4.0b10 (Swofford, 2002). The distribution of states on the terminal nodes, given the 50% majority rule consensus tree, was tested using a T-PTP test as implemented in PAUP* 4.0b10 (Swofford, 2002).

Apart from the total number, we also investigated the inferred relative contribution of each type of transformation, i.e. from N-type to G-type and reverse, using both ACCTRAN and DELTRAN optimization. As different equally parsimonious reconstructions are possible for the same tree, we counted unambiguous changes only, and did so over all trees from a given Bayesian analysis.

RESULTS

Sequences and alignment

The alignment of the sequences was not problematic. The secondary structure calculated for domain III of 12S matched that of *Aplysia cervina* (Medina *et al.*, 2001). Nearly all positions included in our study were situated in regions marked as conserved by these authors, mostly — though not exclusively — in stem regions. Our ITS1 and 2 alignment contained all previously identified conserved cores (van Moorsel *et al.*, 2001d). No gaps or stop codons were found in the COI data set. Since positional homology within the COI sequences was beyond question, even the most variable positions could be aligned among ingroup species. These COI positions represent the larger part of the variation in the mitochondrial data set (67%), since only relatively slowly evolving, and therefore less variable, regions could be unambiguously aligned between the 12S sequences. In the ITS data set many of the most variable positions also had to be excluded due to alignment difficulties. Consequently, in the ingroup a larger sequence

divergence, both uncorrected and GTR corrected, was observed within COI than within 12S and within ITS1&2 (one-tailed Wilcoxon Signed Ranks Test, P<0.001). The pairwise uncorrected and GTR-corrected sequence divergence within each mitochondrial gene was larger than in ITS (one-tailed Wilcoxon Signed Ranks Test, P< 0.001) for the ingroup.

Comparison of the nuclear and mitochondrial tree

Based on the outcome of MrModeltest 1.1b, the GTR+I+Γ model was used for all data partitions in the MrBayes 3.0 analyses. This model was selected both by the likelihood ratio test and by the Akaike information criterion for all datapartitions except COI position 2 (LRT: F81+Γ; AIC: GTR+I) and COI position 3 (LRT: GTR+Γ; AIC: GTR+I+Γ). In both cases the GTR+I+Γ model was chosen for further analyses, being the least inclusive model to combine both alternatives. The likelihood scores within all subsequent Bayesian analyses levelled off within 200,000 generations.

Although their 95% credibility intervals did not overlap, the trees obtained through separate Bayesian analysis of nuclear (ITS) and mitochondrial (12S and COI) DNA often supported

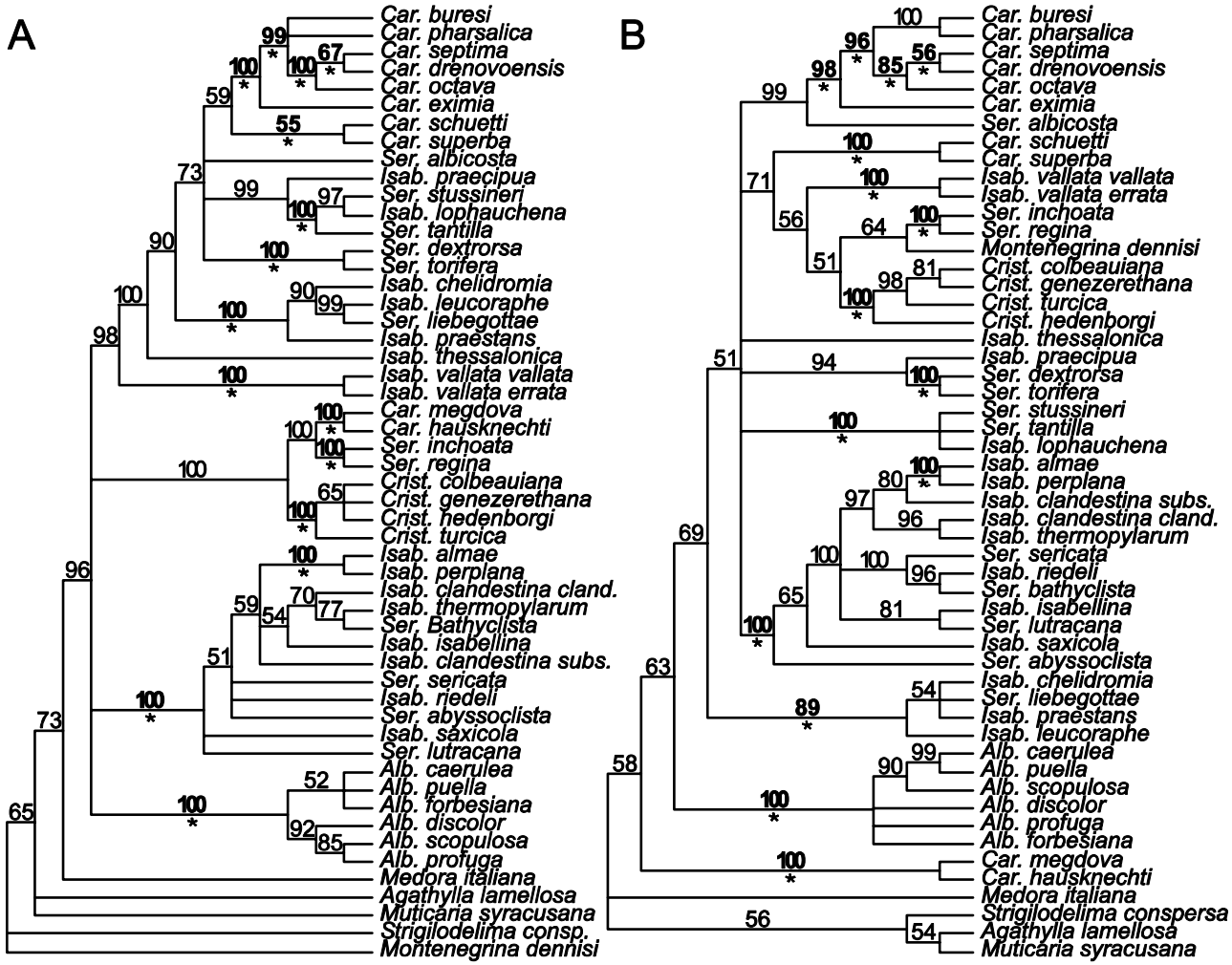


Figure 4.7. The 50% majority rule consensus trees from the Bayesian analysis of the separate data sets. Numbers represent posterior probabilities; asterisks indicate clades supported by a majority of trees in both data sets. **A** Tree obtained from the ITS1&2 data set. **B** Tree obtained from the mitochondrial data set, viz., 12S and COI.

identical clades (Fig. 4.7). Still, since such a comparison ignores clades that are less than 50% supported by either one of the data sets, it provides a rather conservative estimate of the degree of congruence between both data sets. Overall, well-supported clades in one data partition are also supported in the other. Clades significantly (>95%) supported by mitochondrial DNA had an average support of 60% in the ITS analyses, whereas the not significantly supported clades from the mitochondrial analyses had an average support of 0.04% in the ITS analyses (one-tailed Mann-Whitney U-test, $P<0.001$). Similarly, the average probabilities were, respectively, 64% and 0.05% for the mitochondrial analyses (one-tailed Mann-Whitney U-test, $P< 0.001$). As a third measure of degree of topological congruence, we traced conflicting signal between the two data sets, i.e. significantly (>95%) supported clades found in one data set result that are incompatible with significantly supported clades from the other. No such conflicting clades were found, although the data sets place *Isabellaria praecipua* in different highly supported clades. The ITS data set groups this species with *Sericata tantilla*, *Sericata stussineri* and *Isabellaria lophauchena* (99% supported), whereas the mitochondrial data set places *I. praecipua* with *Sericata dextrorsa* and *Sericata torifera* (94% supported).

Most of the topological differences between the mitochondrial and the ITS tree are found near the base of each tree. The two data sets differ in their degree of resolution of apparently deeper divergences. These can be highly supported by ITS, but always receive low probabilities in the mitochondrial data set. Thus in the analysis of the mitochondrial data set even the monophyly of the ingroup is poorly supported. In 64% of the trees, *Montenegrina* is placed as a sister group to *Sericata inchoata* and *Sericata regina*, whereas only 28% of the trees group *Montenegrina* with the other outgroup taxa.

Since both data sets often similarly resolved relatively recent divergences, but were less informative with respect to older divergences, we pooled them to extract any remaining phylogenetic signal that could resolve these early divergences. The observation that both data sets are largely congruent with respect to the more shallow divergences, where both show good resolution, in our opinion justifies this concatenation of the data sets.

The analyses of both data sets combined, with a graphically checked burn-in of 200,000 generations, produced a well-resolved tree (Fig. 4.8), even with respect to most of the basal ingroup divergences. This increased clade support of the combined data set demonstrates that the separate data sets are not incongruent but rather are inconclusive with respect to deeper divergences. The concordance of the inferred deeper divergences with distributional data provides further support for the tree inferred from the combined data set. We consequently based inferences of phylogenetic relationships and character evolution on this tree.

Phylogenetic relationships

Three well-supported main clades are found within the ingroup (Fig. 4.8): (1) an *Albinaria* clade, with both CA-types; (2) a clade consisting purely of N-type species, viz. the genus *Cristataria* together with its sister clade (clade 4), consisting of *Sericata inchoata*, *Sericata regina*, *Carinigera hausknechti* and *Carinigera megdova*; (3) a clade with the remaining *Carinigera* and *Sericata* species together with the *Isabellaria* species, thus consisting of both N- and G-type species. The interrelationships between these three main clades are poorly resolved: 44% of the trees support a sister group relation between clade 1 and 2, 27% between clade 1

and 3, whereas 29% of the trees show clade 2 and 3 as sister groups. Clade 3 is confined to central and northern Greece, Bulgaria, Macedonia and Serbia; the other two clades have a larger, more southern, area of distribution. Our tree thus places all *Isabellaria* species in a single mixed clade (clade 3), but distributes *Sericata* and *Carinigera* species across two clearly separated clades, viz. clade 3 and clade 4. Both clades are more or less geographically delimited. Clade 4 inhabits the western part of the Greek mainland (Fig. 4.9a), whereas the remaining species placed in clade 3 are found in the eastern part (Fig. 4.9b-d).

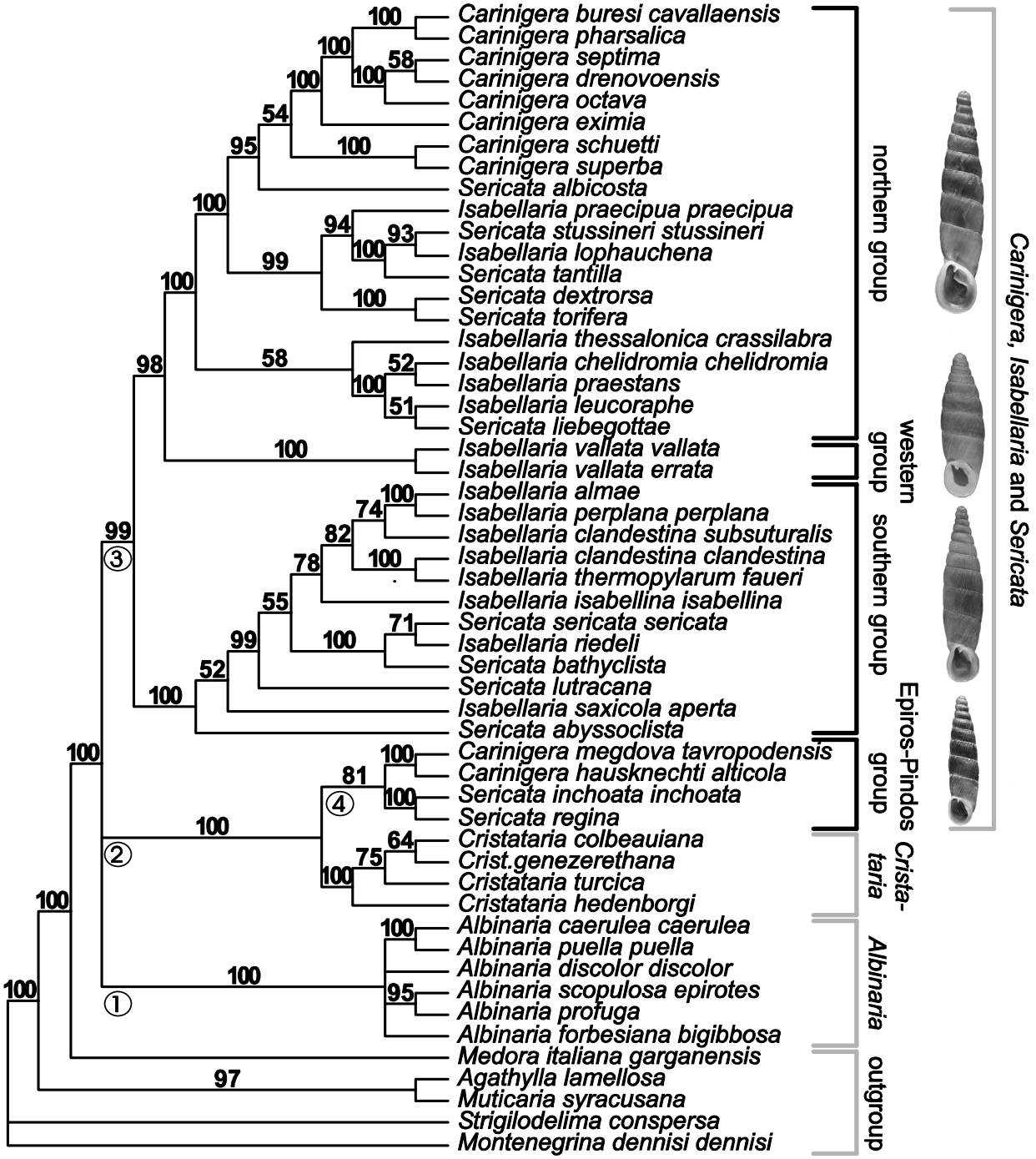


Figure 4.8. The 50% majority rule consensus tree of the trees from the Bayesian analysis of the combined data set. Numbers above branches represent posterior probabilities; the encircled numbers indicate clades referred to in the text.

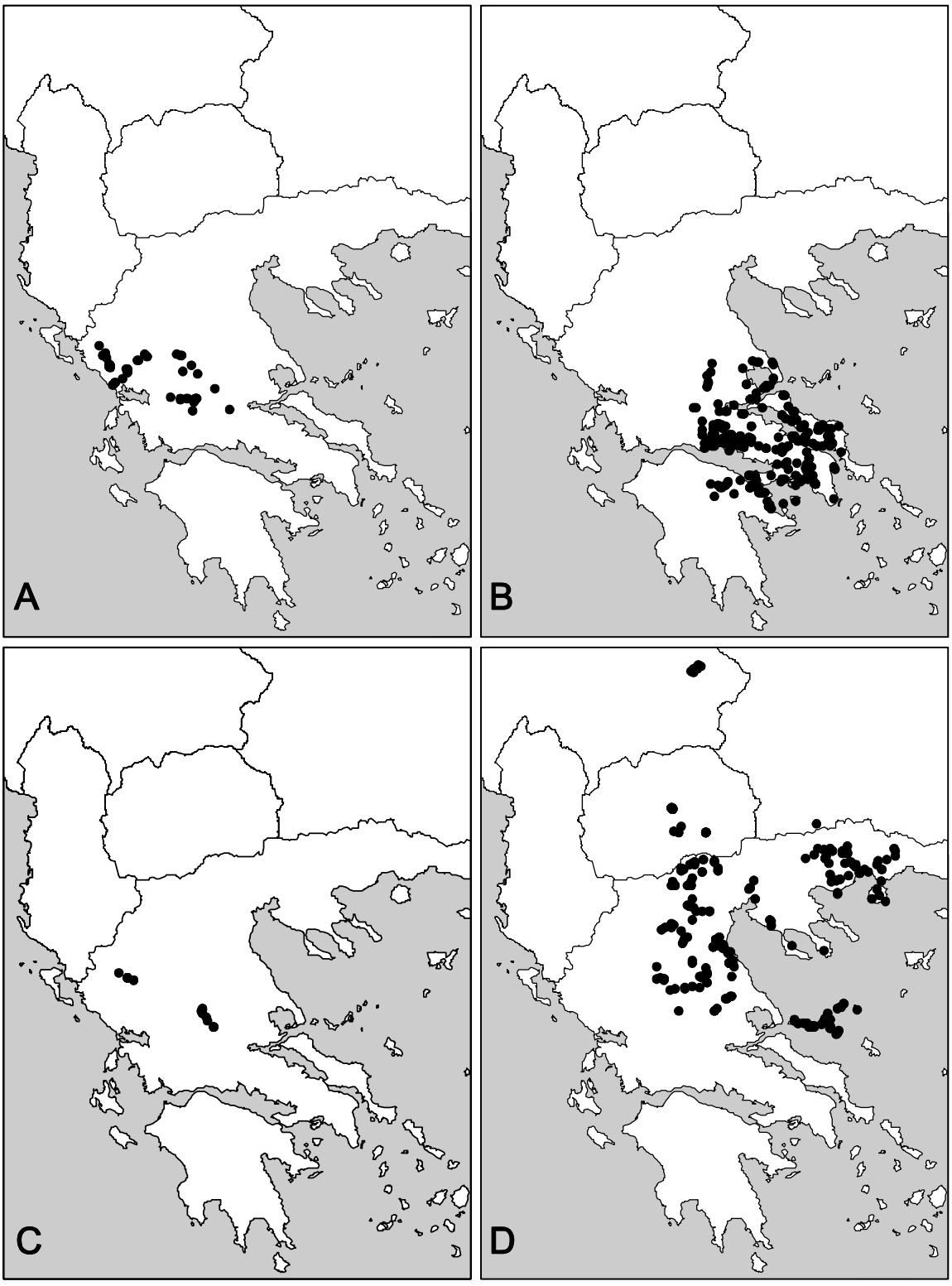


Figure 4.9. The geographical distribution of the clades that were identified by the Bayesian analysis of the combined data set: **A** clade 4, from Epiros and the Pindos mountains; **B** the southern group within clade 3; **C** the western group, viz. *Isabellaria vallata*, within clade 3; **D** the northern group within clade 3. Localities are based on the literature and data in the National Museum of Natural History in Leiden, the Netherlands. Localities of species that remain unstudied molecularly, i.e. *C. pellucida*, *S. calabacensis* and *S. parnassia* are not shown.

The inferred relationships within clade 3 show a strong geographic pattern, but do not correspond to the CA-based classification. Three well-supported geographically confined subclades are found, each containing so-called *Isabellaria* species: a southern group (Fig. 4.9b), a western group (Fig. 4.9c) and a northern group (Fig. 4.9d), each separated by the plains of Thessaly. The southern subclade consists of both *Isabellaria* and *Sericata* species, the western subclade is formed by a single *Isabellaria* species, viz. *I. vallata*, and its sister group, the northern subclade contains *Isabellaria* and *Sericata* species, as well as a *Carinigera* clade. Relationships within each of the subclades again follow a geographic pattern. For instance, all species from the Northern Sporades, viz. *Isabellaria chelidromia*, *I. leucoraphe*, *I. praestans* and *Sericata liebegottae*, constitute a well-supported clade.

CA-type evolution

Our results demonstrate that transformations in CA-type have occurred frequently. The consistency index for CA-type, when plotted on the majority rule consensus tree of all data partitions combined (Fig. 4.10), has a value of only 0.11 (re-scaled CI: 0.056). At least eight instances of parallelism and one reversal are required under DELTRAN optimization. When using ACCTRAN optimization, seven cases of parallelism and two reversals are found. Nevertheless, the G- and N- types are clearly not randomly distributed across the terminal nodes (T-PTP test: $P<0.05$). Taking into account uncertainties in tree topology, our results are still significantly more concordant with the distribution of G- and N- type species than would be expected by chance. All 76,000 DNA-derived trees required fewer than 12 transformations in CA-type, compared to 2% of the random trees ($P<0.02$). Although species within each of the three sampled species pairs (*S. stussineri* & *I. lophauchena*; *S. bathyclista* & *I. riedeli; S. lutracana* & *I. isabellina*) are shown as relatively closely related in the 50% majority rule consensus tree, only *S. stussineri* and *I. lophauchena* are identified as sister species.

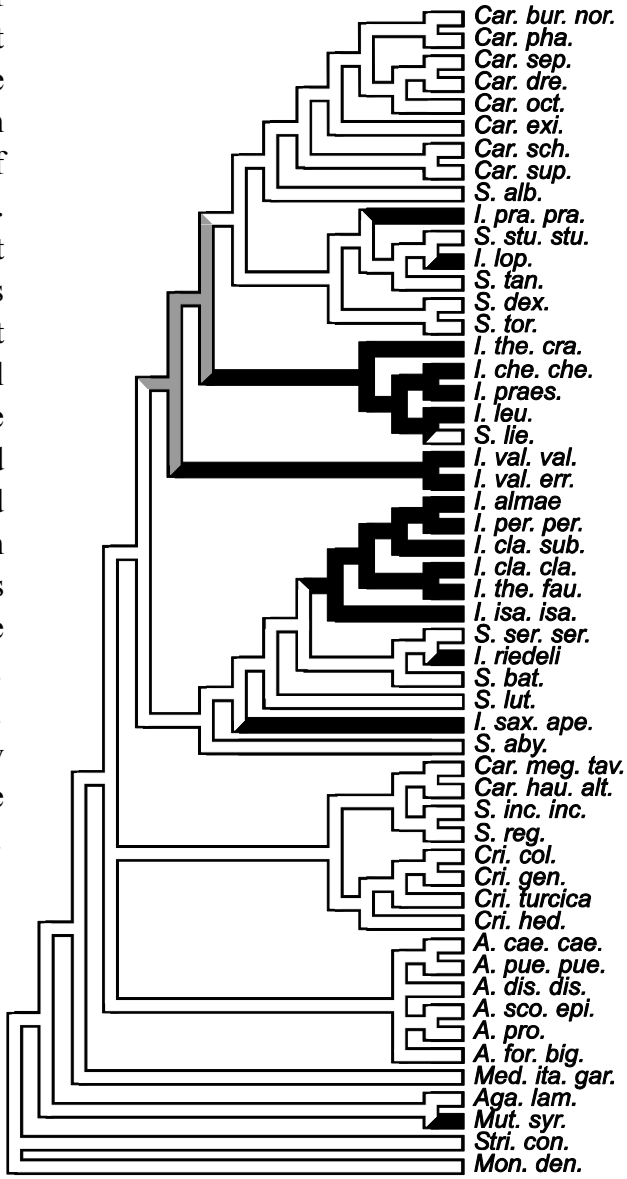


Figure 4.10. Reconstruction of CA-type evolution, plotted on the Bayesian tree of the combined data set (Fig. 4.9). White branches represent an inferred N-type, black lines an inferred G-type. The grey-coloured branches indicate an equivocal reconstruction, ACCTRAN optimization preferring the G-type, DELTRAN optimization the N-type.

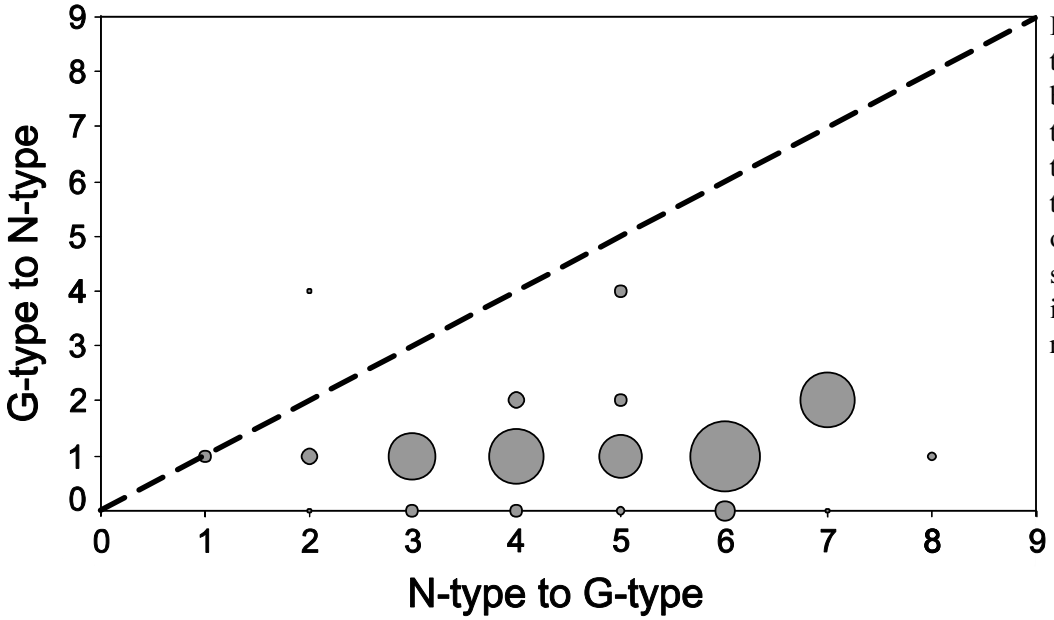


Figure 4.11. Distribution of unambiguous transformations in the 76,000 trees obtained from the Bayesian anaysis of the combined data set. The hatched line indicates the 1:1 relationship.

The transformations from N-type to G-type were found more common than the reverse. In 99% of the trees from the Bayesian analysis, the number of inferred N-type to G-type transformations exceeds that of the inferred G-type to N-type transformations (see Fig. 4.11).

DISCUSSION AND CONCLUSIONS

Our results confirm the hypothesis that the CA-type is a highly homoplasious character at the systematic level addressed in this study. In contrast to the CA-type, biogeographic patterns and the external conchological features are more indicative of relatedness. The T-PTP test and comparisons with random trees demonstrate that, nevertheless, the CA-type is informative, but only at a low, i.e. intrageneric, systematic level. Even at relatively recent divergences, for which the mitochondrial and nuclear tree are largely congruent, 'mixed clades' are found.

Mitochondrial compared to nuclear DNA evolution

The mitochondrial sequences examined lend high support to relatively recent divergences only. The lack of deep-level phylogenetic resolution in the mitochondrial data set is probably due to saturation effects, since the less divergent ITS sequences resolve older divergences reasonably well. Even though 12S and COI rank among the most conserved regions within the mitochondrion (Palumbi, 1996: 235-236), mitochondrial DNA generally has a higher evolutionary rate than has nuclear DNA (Li, 1997: 192), and mitochondrial evolution may even have accelerated within land snails. Comparatively high rates of mitochondrial DNA evolution have been inferred within the land snail genus *Mandarina* (Chiba, 1999a), and a high diversity of mitochondrial DNA has been observed even at species level in land snails (e.g. Thomaz *et al*., 1996; Hayashi & Chiba, 2000; Watanabe & Chiba, 2001). The comparatively high divergence between COI sequences probably further contributed to the noise in the mitochondrial data set.

The increased resolution of the combined data set relative to the separate data sets nevertheless demonstrates that at least some phylogenetic information, even for the oldest divergences, is still present in the mitochondrial DNA. Studies incorporating the inferred amino acid sequences of all mitochondrial protein-coding genes of *Albinaria caerulea* show that deep-level phylogenetic information can indeed be retrieved from mitochondrial DNA, given sufficient sampling (see Grande *et al*., 2002; see Tomita *et al*., 2002).

Distribution

In finding geographically confined clades, our tree demonstrates the limited dispersal abilities of these snails. A previous molecular study on *Albinaria* in Crete revealed a comparable geographic coherence of clades (Douris *et al*., 1998a). Direct measurements on dispersal velocity, carried out on *Albinaria* in Crete, confirm that this is low for at least this genus and this region. Field observations on marked *Albinaria* specimens suggest a mean dispersal velocity of 1-2 m/y per snail, with dispersal mainly following limestone boulders or outcrops (Schilthuizen and Lombaerts, 1994). Dispersal appears to be similarly restricted in *Cristataria* (see Bar, 1977). The spread of a species as a whole may be even lower, especially in areas already inhabited by other species (Welter-Schultes, 2000b). Based on its presumed date of introduction and its present distribution, Welter-Schultes (2000b: 79) estimates the dispersal of *Albinaria praeclara* at the Knossos ruins at 0.5-0.6 m/y. On a larger spatial scale, across the area of distribution of *Isabellaria*, *Carinigera* and *Sericata*, suitable habitats, viz. limestone and marble outcrops, at least presently have a highly fragmented distribution (see Bornovas & Rondogianni-Tsiambaou, 1983), which should hamper dispersal even further. Our results suit the observations of slow dispersal better than does the traditional classification, which implies either many instances of long-distance dispersal or extinction following extensive spread of ancestral taxa.

The most notable exception to the pattern of geographically confined clades is *Carinigera pharsalica*, which is one of the southernmost distributed species of the northern clade. Our analyses group this species among the other *Carinigera* species of the northern clade, all of which occur far more to the north, near the Greek-Bulgarian border, in Macedonia, and in Serbia. Further studies (Uit de Weerd *et al.*, Chapter 5, this thesis) indicate that the southern distribution of *C. pharsalica* can only be explained as a relatively recent long-distance dispersal event. The other southerly distributed clade within the so-called northern group, viz. the species of the Northern Sporades (*I. chelidromia, I. leucoraphe, I. praestans, and S. liebegottae*), may have colonized these islands from the nearby Chalkidiki peninsula, where their supposed sister group, viz. *Isabellaria thessalonica,* still occurs (Fig. 4.2).

CA-type evolution

Our results refute the hypothesis that *Isabellaria* is monophyletic, and demonstrate that instead the G-type CA was acquired several times in parallel. Although we made no *a priori* assumptions about character state polarity within the CA-type, both the ACCTRAN and DELTRAN reconstruction (Fig. 4.10) show the N-type as ancestral, which is in conformity with the unanimous view in the literature. Several lineages independently acquired the G-type CA, which subsequently reversed to the N-type in one lineage or possibly two lineages. It has been

hypothesized that such transformations in CA-type may be caused by a relatively simple change early in CA ontogeny (Gittenberger, 2000).

The inferred polarity of the CA-type appears to be insensitive to the limited sampling of *Albinaria* species. While the vast majority of *Albinaria* species have an N-type CA, in some a G-type is present. None of the latter species is represented in this study. The large number of *Albinaria* species and their relatively rapid divergence complicate phylogeny reconstruction within this genus (van Moorsel *et al.*, 2001c). However, even when all *Albinaria* species were coded as G-type, in a nearly significant 94% of the trees the transformations to the G-type exceeded those to the N-type, whereas only 0.9 % favoured the transformations to the N-type.

The previous study by van Moorsel *et al.* (2000), which included only a small subset of the species placed in clade 3, markedly differs from ours in the character reconstruction within this clade and consequently in its conclusions. That study showed (1) the G-type as ancestral to this clade, and (2) only limited homoplasy in the form of a parallel development of the N-type CA. Our results refute both these findings. It is our opinion that these different inferences are due to a severe underrepresentation of N-type *Sericata* and *Carinigera* species in the study by van Moorsel *et al.* (2000), with only two of these N-type species being included.

The inferred parallel evolution of the G-type CA is concordant with the view that the G-type is a last step in a process of increased sealing of the aperture (Wagner, 1919: 88, 89; Nordsieck, 1982). Clearly, in the family Clausiliidae as a whole, the G-type evolved several times in parallel, long after the main groups had diverged. Fossils of N-type clausiliid snails are known from the Palaeocene onwards (Nordsieck, 2000), whereas the first known G-type fossils date from the upper Pliocene (Nordsieck, 1982, 2000). Our data confirm this pattern of evolution, and show that this parallel evolution occurs even at much lower systematic levels than previously thought. Moreover, our results are concordant with the postulated post-Miocene parallel evolution of the G-type CA within the Clausiliidae. The split between the *Albinaria* clade and *Isabellaria saxicola* is estimated to have occurred minimally 9.3 MYA, early in late Miocene (van Moorsel *et al.*, 2001c), and clearly predates the evolution of a G-type CA within clade 3.

The recurrent evolution of a G-type clausilium within clade 3 is suggestive of local selective advantages conferred by this type of CA. These supposed advantages apparently outweigh the improved ventilation through the by-pass canal when the snail is retracted (von Vest, 1867; Rees, 1964; Gittenberger, 1996). Selection in favour of the G-type may still be operating today, reducing the ranges of N-type species. In the southern part of the Greek mainland in particular, ranges of *Sericata* species are relatively small as compared to those of the *Isabellaria* species, and are more or less surrounded by G-type species. At its type locality, where it was collected in 1887 (Boettger, 1888), *Sericata parnassia* is nearly absent, whereas the G-type species *Isabellaria thermopylarum* and *Idyla bicristata* appear to be thriving there (Uit de Weerd & Gittenberger, pers. obs.).

Two advantages of a G-type CA have been proposed. The G-type, in lacking the by-pass canal, may (1) limit evaporation (von Vest, 1867; Nordsieck, 1982) or (2) more effectively protect the snail against predation (Schmidt, 1868; Gittenberger & Schilthuizen, 1996; Gittenberger, 1997). According to the first view (von Vest, 1867; Nordsieck, 1982), the G-type clausilium is an adaptation to promote survival in a dry climate. So far, studies on the effect of

the presence (Cristelow, 1992) or shape (Warburg, 1972) of the clausilial plate on evaporation have been inconclusive, however. The impact of a G-type CA during aestivation is questioned by Gittenberger & Schilthuizen (1996), who state that G- and N-type species alike effectively seal off the aperture by 'gluing' their shell to the substratum. Evaporation through the apertural region is impossible then, and the permeability of the entire shell wall becomes the dominant factor. Indeed, differences in shell thickness may have contributed to differential survival during aestivation of two *Albinaria* species (Giokas *et al*., 2000). In addition, studies on the genus *Cristataria*, which dwells in a relatively arid environment, suggest that behavioural adaptations such as aestivation in aggregates in crevices may be more crucial than the clausilium in preventing desiccation (Heller & Dolev, 1994; Arad *et al.*, 1995). A G-type may, however, be advantageous in winter, when the snails are most active and cannot as easily escape desiccation by other means. Precipitation in winter is comparatively low in the eastern half of the Greek mainland, where all G-types occur (see Nellestijn & Dekker, 1998).

The second hypothesis states that the G-type serves as a protection against predation, from — most notably — *Drilus* larvae. *Drilus* predation varies between localities, and can exceed 50% in *Albinaria* populations (Welter-Schultes, 2000b: 126,127). The larvae presumably attack the snails when these are glued to the rock during aestivation (Schilthuizen *et al.*, 1994) by boring holes in the ultimate or the penultimate whorl of the shell (Roth, 1855; see also Gittenberger, 1999). Van Moorsel *et al.* (2001a) found a majority of these drilid entrance holes located in front of the clausilium. The small drilid larvae may subsequently pass the clausilial barrier through the by-pass canal in N-type species, whereas in G-type species the clausilial plate blocks the entrance to the shell (Gittenberger & Schilthuizen, 1996; Gittenberger, 1997). The presence of *Drilus* larvae inside N-type shells with only a hole in front of the clausilium, suggests that the by-pass canal can be used by the predators indeed (A. & E. Gittenberger, pers. obs.). Even so, the occurrence of drill-holes through the clausilial plate (A. & E. Gittenberger, pers. obs.) in G-type specimens indicates that, even in these snails, the apertural devices are not completely impenetrable.

Ideally, a hypothesis on the adaptive value of the G-type should explain the relatively recent occurrence of the G-type CA, after the divergence of the main geographic clades. If the evaporation hypothesis is correct, the evolution of a G-type CA could have been triggered by a progressively dryer climate. Palynological and palaeobotanical studies indeed indicate a more humid climate in north-eastern Greece in late Miocene (±6.5 MYA) than at present (Velitzelos and Gregor, 1986; Kloosterboer-van Hoeve *et al.*, 2000a: 81). It is around that time that a general gradual change towards a dryer climate is first observed (Karistineos & Ioakim, 1989), albeit with superimposed cyclical fluctuations (Kloosterboer-van Hoeve *et al.*, 2000b). This timescale matches the time span of 9.3 MYA, in which the G-type is thought to have evolved. Alternatively, one can speculate that the iterative acquisition of a G-type CA within separate lineages from the eastern Greek mainland was facilitated by an increase in *Drilus* predation there. This defence mechanism may not yet have evolved and subsequently been selected for in other regions, presumably more recently invaded by *Drilus*. This hypothesis is concordant with studies on *Drilus* larvae in *Albinaria*, which appear to be in an intermediate state in a process of host specialization (Örstan, 1999).

Asymmetric though the transformation rate may be, our results indicate that the transformations to a G-type are not irreversible. Such irreversibility had been assumed (Nordsieck, 1982), because the transformation from N- to G-type involves the loss of two lamellae. One should bear in mind, however, that these lamellae are formed by folds of the mantle, which can fold only in a restricted number of positions because of internal organs. Moreover, this transformation may be directed by the position of a single lamella earlier in the development of the shell (Gittenberger, 2000). Interestingly, the only unambiguous reversal inferred occurs in the ancestor to *S. liebegottae*, a species that is found only on a few small remote islands in the Aegean Sea. As *Drilus* females are flightless (Lawrence, 1991), it would be interesting to know whether these islands are inhabited by *Drilus.*

Until selective forces acting on the G- and N-type CA have been better identified, we can only speculate about the causes of the parallel evolution of a G-type CA in the past. Obviously, comparative field studies on *Drilus* predation and desiccation pressures should be conducted. Candidate species for such studies are *Isabellaria lophauchena*, and *Sericata dextrorsa*, two species shown as closely related in this study, having large overlapping areas of distribution, that offer various sample sites where both G- and N- type snails occur syntopically.

Geography and morphological evolution

Our data suggest that in land snails even supposedly complex characters may arise in parallel in geographically confined clades, probably as a result of a common selection pressure. A similar phenomenon has been reported in several land snail genera from oceanic islands, viz. *Samoana* (Johnson *et al*., 2000), *Samoana* and the allied genus *Partula* (Goodacre & Wade, 2001a) and the genus *Mandarina* (Chiba, 1999a). Molecular studies of these genera revealed clades, confined to islands or island groups, in which a similar suite of characters had evolved independently, in presumably ecologically adapted morphotypes. Even in the absence of geographical barriers, the limited range of dispersal of land snails may prevent selectively advantageous traits from spreading rapidly, leaving opportunity for parallel evolution of these traits as a response to changing or highly localized selective regimes. Given that isolation by distance is observed in many land snails (e.g. Pfenninger *et al*., 1996; Ross, 1999; Schilthuizen *et al*., 1999b), such iterative parallel evolution of morphological traits may be much more common than currently thought. Here it remained undetected because these traits were used in systematics as diagnostic characters.

Note on the generic nomenclature

The taxonomic consequences of our finds will be addressed in a separate study, which will aim at a shell-morphological definition of clade 3 and clade 4. A re-evaluation of shell-morphological characters may help to elucidate the systematic position of the rare species *Carinigera pellucida*, *Sericata calabacensis* and *Sericata parnassia* in the continued absence of information on other characters.

ACKNOWLEDGEMENTS

We are grateful to J. Heller, W.J. Moes, H. Nordsieck, and T.A.C. Reydon, who helped us to obtain samples from outside Greece, and to H.C. den Bakker for advice on the Bayesian analyses. H. Nordsieck collected the samples of *Cristataria colbeauiana* and *Cristataria turcica*; foot tissue of these samples was kindly donated by H.-J. Niederhöfer from the Staatliches Museum für Naturkunde Stuttgart. D.S.J. Groenenberg determined all DNA sequences of *Cristataria genezerethana* and *Strigilodelima conspersa* and the COI sequences of *Cristataria colbeauiana* and *Cristataria turcica*. D. Schneider isolated DNA and obtained COI sequences of *Carinigera octava* and *Carinigera eximia*.

Chapter 5

MOLECULAR PHYLOGEOGRAPHY OF *CARINIGERA PHARSALICA* AND ITS RELATIVES: EVIDENCE OF RECENT LONG-DISTANCE DISPERSAL WITHIN A GROUP OF LOWLY VAGILE CLAUSILIID SNAILS (GASTROPODA, PULMONATA)

ABSTRACT

The Greek land snail species *Carinigera pharsalica* can be readily distinguished from related *Carinigera* species by conchological characters and by its distribution in Thessaly, which is far south of the ranges of these other species. Nevertheless, analyses of mitochondrial cytochrome *c* oxidase I sequences reveal that the sequences of *C. pharsalica* nest as a monophyletic clade among those of *C. buresi* from north-eastern Greece and adjacent Bulgaria. This result is in line with the comparatively low genetic divergence between the *C. pharsalica* sequences as compared to the *C. buresi* sequences. Since this nested position can hardly be explained as resulting from ancestral mtDNA polymorphisms or introgression of mtDNA, we conclude that *C. pharsalica* descended from specimens of *C. buresi* that dispersed into Thessaly, and that it should therefore be classified as a subspecies with *C. buresi*. Additional support for this conclusion comes from conchological similarities between *C. pharsalica* and *C. buresi*. Given the low vagility of the snails in question and the approximately 200-km separation by land and sea between the ranges of *C. pharsalica* and *C. buresi*, passive dispersal of the *C. pharsalica* ancestors is most likely. We discuss the possibility of human-aided dispersal during the transportation of limestone.

INTRODUCTION

Among the proverbially slowly dispersing land snails, the genus *Albinaria* and its relatives rank among the least vagile ones, with an estimated dispersal in the order of 1-2 metres per year (Schilthuizen and Lombaerts, 1994; Welter-Schultes, 2000b: 79) or even per lifetime (Giokas *et al*, 2000). Their calcicoly puts even further restrictions on the long-term dispersal of these snails, especially in areas in which limestone or marble outcrops are widely scattered. Dispersal of *Albinaria* is stepping-stone-wise at a very small spatial scale, with restricted migration between adjacent boulders (Schilthuizen and Lombaerts, 1994). This slow mode of dispersal has left its imprint on the present day genealogical structure within *Albinaria* and allied species. Instead of with supposedly congeneric species, species classified with the genera *Carinigera*, *Isabellaria* and *Sericata* cluster with geographically close species in molecular phylogenetic analyses (Chapter 4, this thesis).

One exception to this geographic pattern is *Carinigera pharsalica*. Occurring in central Greece, it is separated nearly 200 km by land and sea from, according to molecular phylogenetic analyses, its closest relative *C. buresi* from NE Greece and adjacent Bulgaria

Dennis R. Uit de Weerd, Dennis Schneider, and Edmund Gittenberger. Molecular phylogeography of *Carinigera pharsalica* and its relatives: evidence of recent long-distance dispersal within a group of lowly vagile clausiliid snails (Gastropoda, Pulmonata).

(Fig. 5.1). Limestone areas in the intermediate area are widely scattered (see Bornovas & Rondogianni-Tsiambaou, 1983), thus limiting dispersal possibilities for these slowly dispersing calcicole snails. Molecular phylogenetic analyses (see Chapter 4, this thesis) nest both species within a so-called northern *Carinigera*-clade, further consisting of *C. eximia*, *C. drenovoensis*, *C. octava* and *C. septima* from the southern Balkan. The systematic position of a fifth species, *C. pellucida* from Macedonia, remains elusive, as no sequences of this species have been determined. The analyses further demonstrate that *C. pharsalica* is only distantly related to the *Carinigera* species nearest geographically, namely *Carinigera hausknechti* and *Carinigera megdova* from the Pindos mountains and southern Thessaly. These two species constitute a second, southern, *Carinigera* clade not directly related to other so-called *Carinigera* species, except perhaps *C. pellucida* (Dedov & Neubert, 2002). Taken together, these inferences suggest that *C. pharsalica* originated from NE Greece or the southern Balkans.

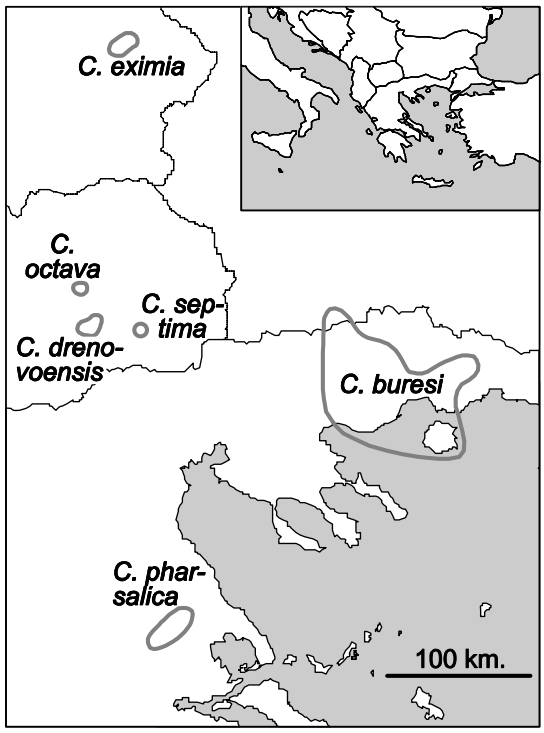


Figure 5.1. Schematic distribution of species from the northern *Carinigera* clade, based on the literature and data in the National Museum of Natural History in Leiden.

Critical for the phylogeography of *C. pharsalica* is its systematic position with respect to the subspecies of the highly diverse *Carinigera buresi*. While *C. pharsalica* does not vary much conchologically and can easily be recognized (Nordsieck, 1974), *C. buresi* is very heterogeneous (Fig. 5.2) and it lacks a morphological definition. Acknowledging this conchological diversity, no less than nine subspecies are recognized within *C. buresi* (Nordsieck, 1977b; Gittenberger & Uit de Weerd, in press): *C. buresi buresi*, *C. buresi cavallaensis*, *C. buresi conciliatrix*, *C. buresi dramaensis*, *C. buresi damjanovi, C. buresi insularis*, *C. buresi militis*, *C. buresi nordsiecki* and *C. buresi polimilitis.* Several of these taxa were originally considered separate species (see Brandt, 1962: 135-138; see Nordsieck, 1972: 9-10), all closely — but not equally closely — related to *C. pharsalica* (see Nordsieck, 1974). More extensive sampling in NE Greece revealed that intermediate forms indicative of hybridization connect most of these former so-called species. Therefore, they were dealt with as subspecies, while the geographically isolated *C. pharsalica* retained its species status (Nordsieck, 1977b).

In this study, we have investigated the relationship within the northern *Carinigera*-clade, in particular between *C. pharsalica* and the subspecies of *C. buresi*. As an independent marker we used mitochondrial cytochrome *c* oxidase subunit I (COI) nucleotide sequences at the species, subspecies and population level. As a mitochondrially encoded gene, COI can be used for phylogeny reconstruction and phylogeography at both the intraspecific (Avise *et al.*, 1987) and the interspecific level.

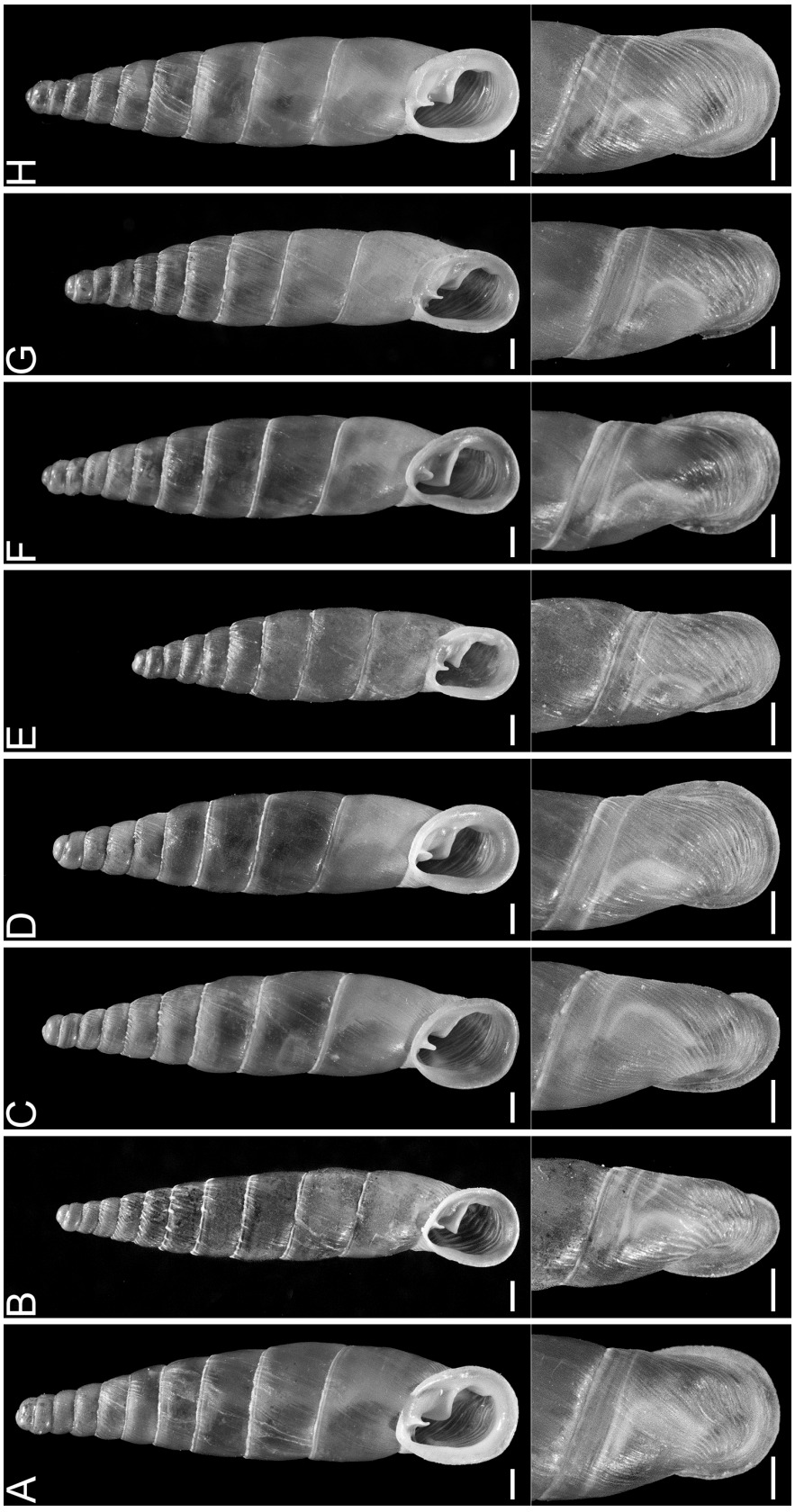


Figure 5.2. Frontal (above) and dorsal view (below) of the shells of *C. pharsalica* (**A**) and *C. buresi* subspecies: (**B**) *C. buresi buresi*, (**C**) *C. buresi cavallaensis*, (**D**) *C. buresi conciliatrix*, (**E**) *C. buresi polimilitis*, (**F**) *C. buresi dramaensis*, (**G**) *C. buresi insularis*, (**H**) *C. buresi nordsiecki*. Not shown here are *C. buresi damjanovi* and *C. buresi militis*. The scale bar represents 1.0 mm.

MATERIALS AND METHODS

Representatives of all five species from the northern *Carinigera*-clade are included in this study. In addition we obtained tissue from six out of nine subspecies of *C. buresi*, three of which were sampled from several localities. When possible, we sampled multiple individuals per population, to be able to recognize ancestral polymorphisms or possible introgression events among populations. Species, sub-species and collection sites are listed in Table 5.1, and the distribution of sampling sites is shown in Figure 5.3. *Carinigera pharsalica* itself is represented by two populations from opposite parts of its range.

Tissue of the specimens sampled was either kept frozen at –80 °C after collection or had been stored in 70% ethanol (see Table 5.1). DNA from fresh tissue was extracted using the protocol described by Schilthuizen *et al.* (1995), whereas a modified protocol was used to reduce loss of DNA from older ethanol-stored tissue (see Chapter 2 and 3, this thesis). Extracted DNA was amplified in a PCR reaction consisting of 35 cycles with an annealing temperature of 47°C using the forward primer (ACT CAA CGA ATC ATA AAG ATA TTG G) and the reverse primer (TAT ACT TCA GGA TGA CCA AAA AAT CA) (Gittenberger *et al.*, 2004). PCR product was gel-purified using spin columns (Qiaquick® Gel Extraction Kit by Qiagen®) and dye-terminator cycle-sequenced in both directions (Big Dye™ by PE Biosystems®). The cycle-sequenced samples were subsequently run on an ABI 377 automated sequencer (PE Biosystems®). Sequences thus obtained were assembled and edited in Sequencher (Gene Codes Corp.®). All sequences were aligned in MegAlign 4.03© (DNASTAR Inc., 1999), compared to previously published *Albinaria* COI sequences, and checked for stop codons and gaps.

Tests for data quality and phylogenetic analyses were performed in PAUP* 4.0b10 (Swofford, 2002). We checked for deviations in base frequencies for each codon position separately using a χ^2 test. The presence of a phylogenetic signal was tested using a 1000 replicate permutation test with 10 random addition replicates, TBR, and steepest descent, within each replicate (Archie, 1989; Faith & Cranston, 1991). In addition a g_1 value was calculated from the distribution of 10,000

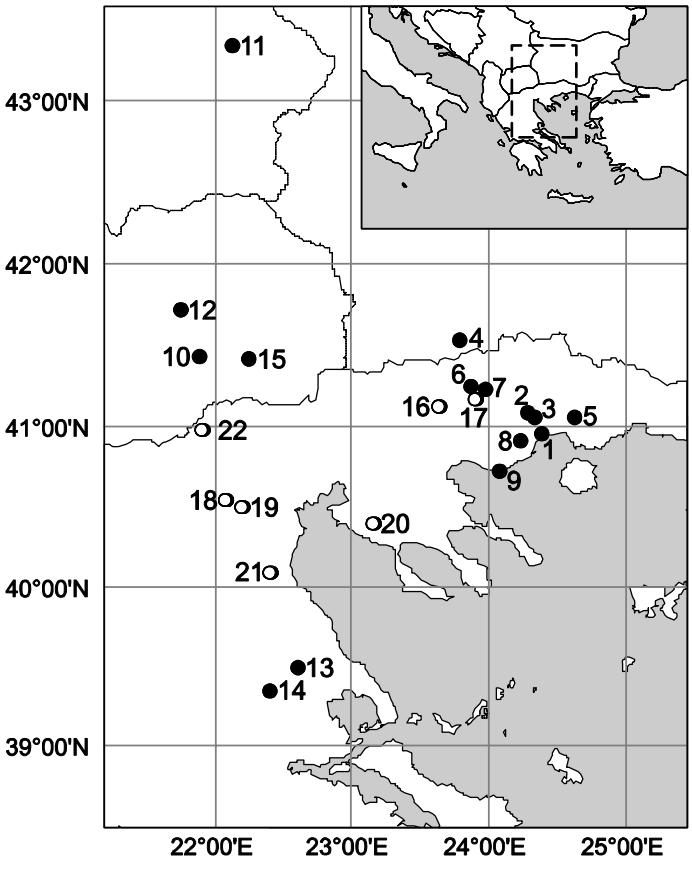


Figure 5.3. Distribution of sampling sites. Black dots refer to the localities of the ingroup samples; open circles to those of outgroup samples.

random trees (Hillis and Huelsenbeck, 1992). Phylogenetic analyses were based on both maximum parsimony and maximum likelihood approaches. Maximum parsimony heuristic searches were performed using 1000 random addition replicates, TBR and steepest descent. Maximum parsimony bootstrap analyses consisted of 10,000 bootstrap replicates (1 addition per bootstrap replicate, TBR and steepest descent). The maximum likelihood search, with 10 random addition replicates (TBR, without steepest descent), was carried out using the GTR+I+Γ

Table 5.1. Samples included in the analyses and their locality information.

Species	locality	co-ordinates	UTM code	initial state
Ingroup				
Carinigera buresi cavallaensis 191	1	40°56'N 24°24'E	KF8234	frozen
Carinigera buresi conciliatrix 343	2	41°04'N 24°17'E	KF7149	frozen
Carinigera buresi conciliatrix 344	2	41°04'N 24°17'E	KF7149	frozen
Carinigera buresi conciliatrix 1126	2	41°04'N 24°17'E	KF7149	frozen
Carinigera buresi conciliatrix 1115	3	41°02'N 24°20'E	KF74	70% ethanol
Carinigera buresi damjanovi 1172	4	41°31'N 23°48'E	GM2706	70% ethanol
Carinigera buresi polimilitis 1138	5	41°02'N 24°38'E	LF0145	70% ethanol
Carinigera buresi polimilitis 1140	5	41°02'N 24°38'E	LF0145	70% ethanol
Carinigera buresi dramaensis 188	6	41°13'N 23°54'E	GL4266	frozen
Carinigera buresi dramaensis 194	6	41°13'N 23°54'E	GL4266	frozen
Carinigera buresi dramaensis 192	7	41°13'N 23°59'E	GL4967	frozen
Carinigera buresi dramaensis 978	7	41°13'N 23°59'E	GL4967	frozen
Carinigera buresi nordsiecki 337	8	40°54'N 24°14'E	KF6731	frozen
Carinigera buresi nordsiecki 934	8	40°54'N 24°14'E	KF6731	frozen
Carinigera buresi nordsiecki 330	9	40°42'N 24°05'E	KF5312	frozen
Carinigera buresi nordsiecki 345	9	40°42'N 24°05'E	KF5312	frozen
Carinigera drenovoensis 1134	10	41°25'N 21°54'E	EL78	70% ethanol
Carinigera drenovoensis 1163	10	41°25'N 21°54'E	EL78	70% ethanol
Carinigera eximia 1104	11	43°19'N 22°08'E	EN99	70% ethanol
Carinigera eximia 1114	11	43°19'N 22°08'E	EN99	70% ethanol
Carinigera octava 1103	12	41°42'N 21°48'E	EM61	70% ethanol
Carinigera octava 1113	12	41°42'N 21°48'E	EM61	70% ethanol
Carinigera pharsalica 195	13	39°29'N 22°37'E	FJ3871	frozen
Carinigera pharsalica 933	13	39°29'N 22°37'E	FJ3871	frozen
Carinigera pharsalica 1125	13	39°29'N 22°37'E	FJ3871	frozen
Carinigera pharsalica 1136	14	39°20'N 22°25'E	FJ25	70% ethanol
Carinigera pharsalica 1141	14	39°20'N 22°25'E	FJ25	70% ethanol
Carinigera septima 1137	15	41°24'N 22°15'E	FL08	70% ethanol
Carinigera septima 1142	15	41°24'N 22°15'E	FL08	70% ethanol
Outgroup				
Carinigera schuetti 1235	16	41°07'N 23°38'E	GL2554	frozen
Carinigera superba 193	17	41°13'N 23°54'E	GL4266	frozen
Isabellaria lophauchena 268	18	40°32'N 22°05'E	EK9188	frozen
Isabellaria praecipua praecipua 844	19	40°29'N 22°12'E	FK08	frozen
Isabellaria thessalonica crassilabra 266	20	40°23'N 23°10'E	FK8371	frozen
Sericata albicosta 307	21	40°05'N 22°25'E	FK2038	frozen
Sericata dextrorsa 760	22	40°58'N 21°55'E	EL7635	frozen

model, selected by MrModeltest 1.1b (Nylander, 2002) after running the standard modelblock in PAUP* 4.0b10. Apart from the base frequencies, for which empirical values were used, all other parameters were estimated during the maximum likelihood analysis. Identical parameter settings were subsequently used in a maximum likelihood bootstrap analysis consisting of 100 bootstrap replicates (1 random addition per bootstrap replicate, TBR, without steepest descent). For computational efficiency, all populations identified as monophyletic in the ML analysis were constrained to be monophyletic in the ML bootstrap analysis, as was the ingroup. In order to further speed up the analysis, we restricted the maximum number of rearrangements per bootstrap replicate to 250.

RESULTS

We were able to determine the bases of 633-657 positions of CO1 for the samples included in this study. The data set did not contain gaps or stop codons. For none of the codon positions were significant deviations in base frequencies detected ($P>0.95$). Both the permutation test ($P=0.001$) and the g1 value ($P<0.01$) indicated a highly significant phylogenetic signal within the data set. The total data set contained 243 variable positions, 218 of which were potentially parsimony informative. Within the ingroup, 228 positions were variable, the vast majority (97.8%) containing synonymous variation. Only five positions harboured non-synonymous variation. The number of potentially parsimony informative positions within the ingroup was 203.

The six most parsimonious trees (score: 977; CI: 0.432; rescaled CI: 0.293, see Fig. 5.4 for strict consensus) and the maximum likelihood tree (GTR+I+Γ; -lnL 4900.31308; proportion of invariable sites: 0.58649587 and α: 0.967911, see Fig. 5.5) agreed with respect to the position of the *C. pharsalica* sequences as a clade nested among *C. buresi* sequences. In fact, the MP strict consensus and the ML tree are identical with respect to the phylogenetic relationships among the *C. pharsalic*a and the most closely related *C. buresi* sequences in clade A. Four successive branches with *C. buresi* subspecies separate *C. pharsalica* from the other *Carinigera* species sampled, according to the MP trees. Besides these, an additional most basal *C. buresi* branch is identified in the maximum likelihood analysis. In particular, a sister group relationship between *C. pharsalica* and the *C. buresi conciliatrix* population 2 is highly supported (MP bootstrap 94%, ML bootstrap 91%). In contrast, there is low support for the monophyly of *C. buresi*, including *C. pharsalica*, as a whole. The monophyly of this group as found in the maximum likelihood analysis is supported by only two of the six most parsimonious trees; the four other trees group the *C. buresi* populations 3, 4 and 9 with *C. octava*. The ML bootstrap support for the monophyly of *C. buresi* plus *C. pharsalica* is only 6%. All populations included are found to be monophyletic in the maximum parsimony analysis and all except one, viz. number 7, in the maximum likelihood analysis. The sequences of this population are paraphyletic with respect to population 6. However, the short terminal branches leading to these two sequences from locality 7 and the short internal branch separating them suggest their paraphyly may have been spuriously inferred, a conclusion supported by the ML bootstrap analysis, which supports the monophyly of population 7 by 53%.

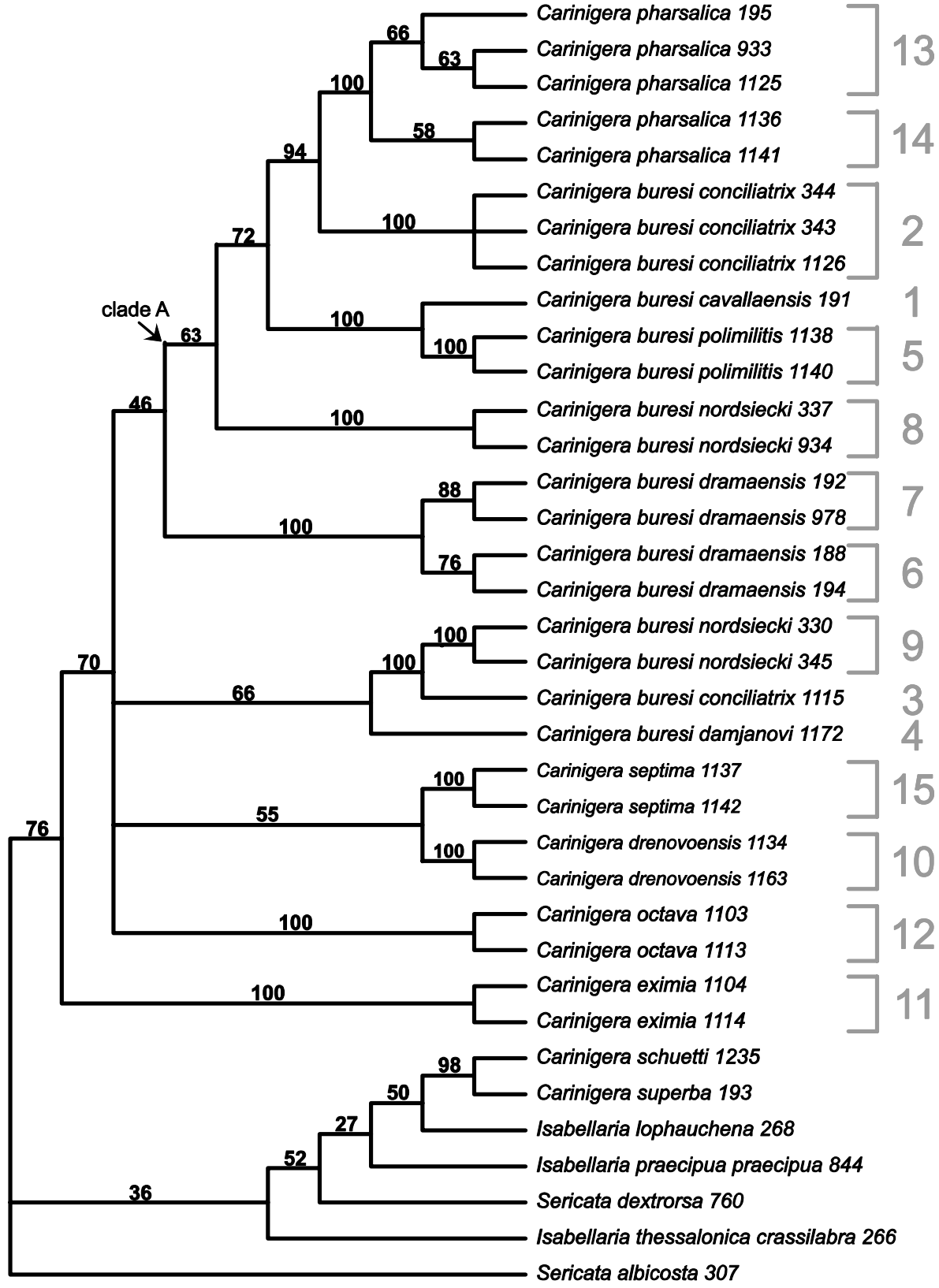


Figure 5.4. Strict consensus of the six most parsimonious cladograms of the COI sequences. Numbers above branches represent bootstrap values derived from 10,000 bootstrap replicates. Grey numbers refer to the populations listed in Table 5.1.

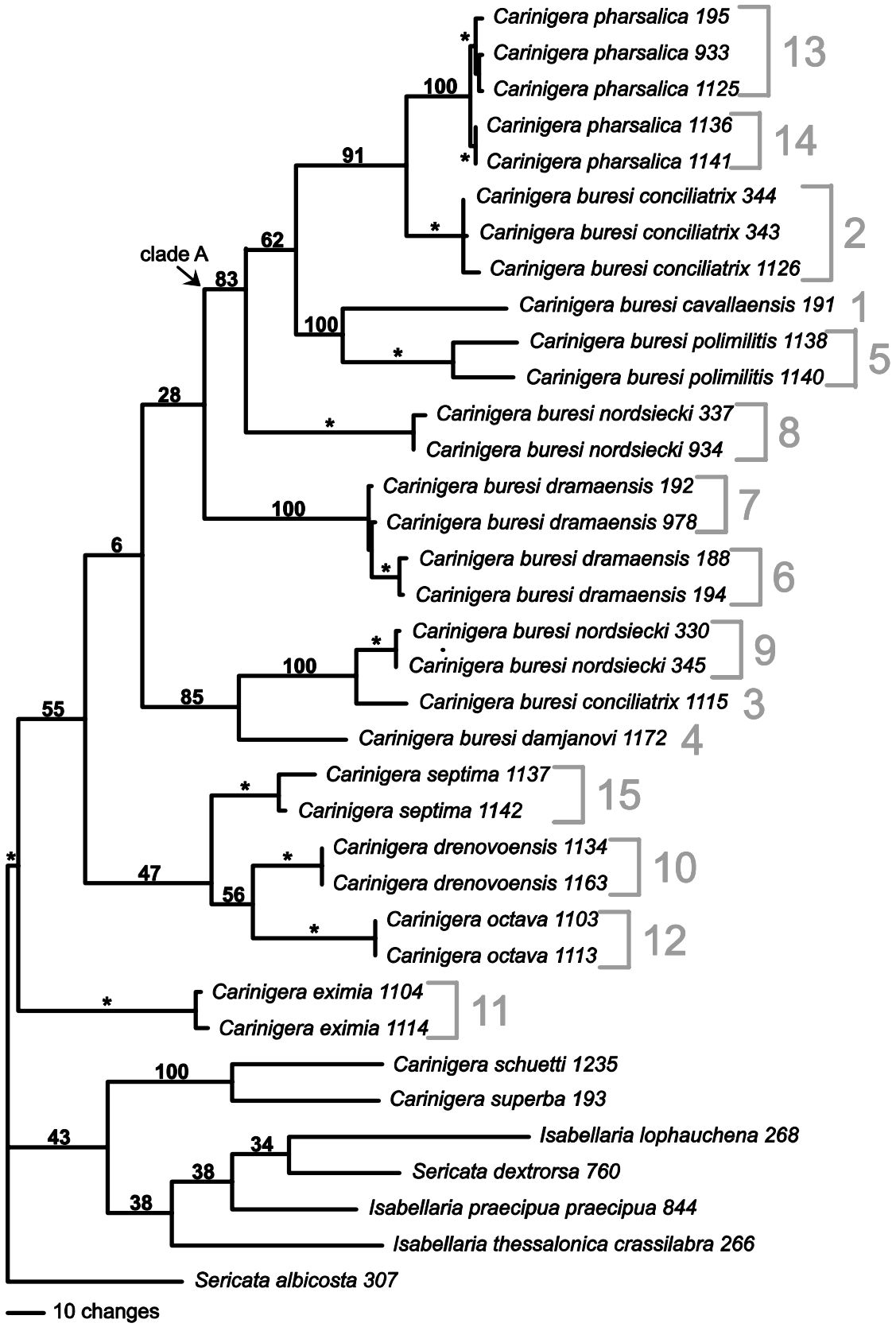


Figure 5.5. Maximum likelihood tree, based on a GTR model with gamma distribution and a proportion of invariable sites (see text). Numbers above branches represent ML bootstrap values derived from 100 bootstrap replicates with an upper limit of 250 rearrangements. Asterisks indicate clades constrained in the ML bootstrap analyses. Grey numbers refer to the populations listed in Table 5.1.

In order to compare amounts of sequence divergence within *C. pharsalica* and related *C. buresi* populations, we first checked for rate constancy in clade A. Both MP and ML analyses provide fairly high bootstrap values for this clade, and identify a highly similar and thus robust topology, with the only differences residing within the *C. buresi conciliatrix* population (No. 2). Using a likelihood ratio test, we compared the likelihood values of clade A under GTR+I+Γ, with and without the assumption of rate constancy (Felsenstein, 1988). Under the molecular clock constraint, the two *C. buresi nordsiecki* samples were used as a monophyletic outgroup to the remaining sequences within this clade A, in line with the inferred topology within this clade. The test did not refute rate constancy within this clade, neither for the six MP topologies nor for the ML topology (df.=11: $0.30<P<0.60$).

In view of the rate constancy within clade A, the genetic divergence within *C. pharsalica* is remarkably low compared to that within *C. buresi* from this clade. Taking into account all *C. pharsalica* sequences, seven sites are variable and no more than four nucleotides differ between any pair of sequences. Given this low number of sites variable between the *C. pharsalica* sequences and the clustering of these sequences in the tree, multiple hits are highly unlikely. Hence we estimated the amount of divergence by calculating uncorrected distances only. The sequences of the two individuals from the southernmost *C. pharsalica* population (No. 14) are identical, whereas intrapopulational divergence ranges from 0.31 to 0.62% in the three sequences from the northern population (No. 13). The divergence between both *C. pharsalica* populations is 0.62%. This interpopulational divergence within *C. pharsalica* lies within the range of 0.15 to 0.77% observed within the *C. buresi* populations of clade A. The maximum uncorrected distance within *C. buresi* as a whole amounts to 14.84%, and is found between the samples from population 1 and 3.

DISCUSSION AND CONCLUSIONS

Our analyses unambiguously nest the *C. pharsalica* sequences between the sequences of *C. buresi*, a position not only supported by high bootstrap values but also by the extremely small divergence between the *C. pharsalica* sequences as compared to those of *C. buresi*.

From gene tree to species tree

In nesting the COI sequences of *C. pharsalica* as a clade among those of the more northerly species *C. buresi*, our tree is discordant with geography (Fig. 5.1). The observed paraphyly of the *C. buresi* sequences can be explained in three ways: (1) as arising from ancestral polymorphisms, (2) as caused by introgression events, or (3) as due to the paraphyly of the 'species' itself. We will first consider the first two explanations, in particular since they appear to apply to the mitochondrial genealogy of other land snail species (e.g. Goodacre & Wade, 2001b; Douris *et al*., 1998a; van Moorsel *et al*., 2001c).

The nested phylogenetic position of *C. pharsalica* sequences with respect to those of *C. buresi* can hardly be attributed to retention of ancestral polymorphisms dating from their common ancestor. Since the *C. pharsalica* mitochondrial lineage is grouped with *C. buresi* lineages in at least four successively nested clades (Fig 5.4, Fig 5.5), this would imply: (1) four

mitochondrial lineages in their common ancestor, (2) retention of these lineages within *C. buresi*, and (3) loss of three lineages in *C. pharsalica*. Generally, lineage sorting takes place quite rapidly, with the expected fixation time, measured as the number of generations, of a mitochondrial lineage in a hermaphroditic population equalling the effective population size (Avise *et al.*, 1984). The monophyly of all populations sampled in this study, as well as that of *C. pharsalica*, suggests that lineage sorting has indeed been fairly effective at erasing mitochondrial lineages, even within *C. buresi*. Such a completely population-wise clustering of mitochondrial variants is highly exceptional in land snails (cf. Ross, 1999; Davison & Clarke, 2000; Watanabe & Chiba, 2001; Goodacre, 2002; Holland & Hadfield, 2002; Hugall *et al.*, 2002; Pfenninger & Posada, 2002). Theoretically, ancestral polymorphisms could persist over a far longer time span within a species as a whole than they do in populations or demes (Thomaz *et al.*, 1996; Edwards & Beerli, 2000), if populations or demes conform to a stepping stone model such as observed in *Albinaria* (Schilthuizen & Lombaerts, 1994). However, as far as the sampling of ingroup specimens overlaps, the mitochondrial trees are nearly completely congruent with a previously determined nuclear ITS based phylogenetic tree (see Chapter 4, this thesis: Fig. 4.7) incorporating a single specimen per ingroup species. Taking all observations together, we consider retention of ancestral polymorphisms as a too far-fetched hypothesis to explain the patterns observed here.

Introgression of mitochondrial DNA from *C. buresi* to *C. pharsalica* provides an equally unlikely explanation for the nested position of *C. pharsalica* sequences among those of *C. buresi*. True enough, such interspecific introgression has been reported from *Albinaria* (Douris *et al*., 1998a; van Moorsel *et al*., 2001c), a genus closely related to the *Carinigera* species of this study. However, both these cases concern species that are known to hybridize in the field. To our knowledge, only one possible case has been described in land snails of past hybridization between two presently widely disjunct species leaving its imprint in the distribution of mtDNA lineages (Shimizu & Ueshima, 2000). Those species are not as widely separated, and occur in a geologically much more dynamic region, which is thought to have induced local extinctions and migrations (see also Hayashi & Chiba, 2000). The wide geographic separation between *C. pharsalica* and *C. buresi* makes past secondary contact between *C. pharsalica* and *C. buresi* highly unlikely. We therefore also dismiss interspecific introgression of DNA as an explanation of the inferred position of the *C. pharsalica* sequences, and conclude that *C. pharsalica* shares a common ancestor with a subdivision of *C. buresi*.

Congruence between the molecular and conchological data

The inferred descent of *C. pharsalica* from *C. buresi* is supported by the congruence of patterns revealed by our DNA analysis with conchological data. First, our analyses show that the morphological diversity within *C. buresi* (see Nordsieck, 1977b) is matched by a comparably high amount of genetic divergence, whereas COI sequences for the two populations of the conchologically far less variable *C. pharsalica* are highly similar. Second, both the morphological (Nordsieck, 1974) and our molecular characters question the reciprocal monophyly of *C. pharsalica* and *C. buresi*, combining *C. pharsalica* with a subgroup of *C. buresi*. The mitochondrial lineages do not coincide with the morphologically defined subspecies of *C. buresi*. This is hardly surprising, given the many intermediate forms between most of

these subspecies (Nordsieck, 1977b), suggesting extensive hybridization with many opportunities for introgression of mtDNA. In accordance with the evidence now available, we propose classifying *C. pharsalica* as a subspecies of *C. buresi* also, thus extending the earlier revision of *C. buresi* (Nordsieck, 1977b). We will refer to it as *C. buresi pharsalica* from now on.

Dispersal into Thessaly

The geographic distribution of *C. buresi pharsalica* is clearly discordant with its phylogenetic position. Our data strongly suggest that *Carinigera buresi pharsalica* originates from the eastern part of the Greek province Macedonia or from Thracia, since it is nested in a *C. buresi* clade distributed in that region. In the genus *Albinaria*, similar disjunct patterns of distribution have been attributed either to dispersal by rafting across sea (Douris *et al.*, 1998a) or to human-aided dispersal (Boettger, 1891: 62; Liebegott, 1986: 18; Welter-Schultes, 2000b: 70-75).

Dispersal by sea is unlikely in this particular case. The areas of distribution of *C. buresi pharsalica* and the other subspecies of *C. buresi* are separated by a Neogene (Higgins & Higgins, 1996: 91) more-or-less-continuous mountain chain, consisting of the Olympos, Ossa, Mavrovouni and Pilio mountains, and precluding a direct connection by sea in the recent past. Moreover, the possibility of long-distance rafting has recently been questioned (Welter-Schultes, 2000b: 78).

Human-aided dispersal of *C. buresi*, on the other hand, offers a more plausible a priori explanation. Human-aided dispersal of eastern Mediterranean land snails is supported by archaeological finds of land snail shells among the cargo of a 3300-year-old shipwreck (Welter-Schultes, 2001), and may have had an impact on their present-day distribution patterns (Mylonas, 1984; Glaubrecht, 1990, unpublished MSc thesis). The snails studied here dwell on marble and limestone rocks, which have often been used as building or sculpturing blocks in the past. Marble in particular was transported across large distances in the eastern Mediterranean area (e.g. Tykot & Ramage, 2002). Marble blocks quarried were often transported in a raw form and then finished at the site of destination (Waelkens *et al*., 1988b; Déroche *et al.*, 1989; Herrmann & Barbin, 1993). Consequently, snails may have been transported along with the rocks. Such a mode of dispersal has been inferred to explain distribution patterns within the related genus *Albinaria* (Pfeiffer, 1955; Pfeiffer, 1956; Liebegott, 1986: 18; Welter-Schultes 1998). The hypothesis that *C. buresi* has been artificially dispersed was previously advanced by Frank (1988). She reports specimens of *Carinigera buresi cavallaensis* from the ancient temple of Zeus in Athens (Frank, 1987; 1988), a find that has never been confirmed, however.

We know of two regions in NE Greece within the home range of *C. buresi*, containing well-documented marble quarries dating from antiquity, viz. an area near Filippi (Waelkens *et al*., 1988a) and the island of Thasos (Dworakowska, 1975). At least some of the Filippi quarries were situated above the town of Lidia (Waelkens *et al*., 1988a: note 93), which places them within four kilometres of collection site 2, where the sister group of *C. buresi pharsalica* is found (see fig. 5.6). Thus the ancestors of *C. buresi pharsalica* may have been transported from one of these quarries to Thessalia. The island of Thasos is inhabited by *C. buresi insularis*, which could not be sequenced. Thasian marble has been studied far better than marble from

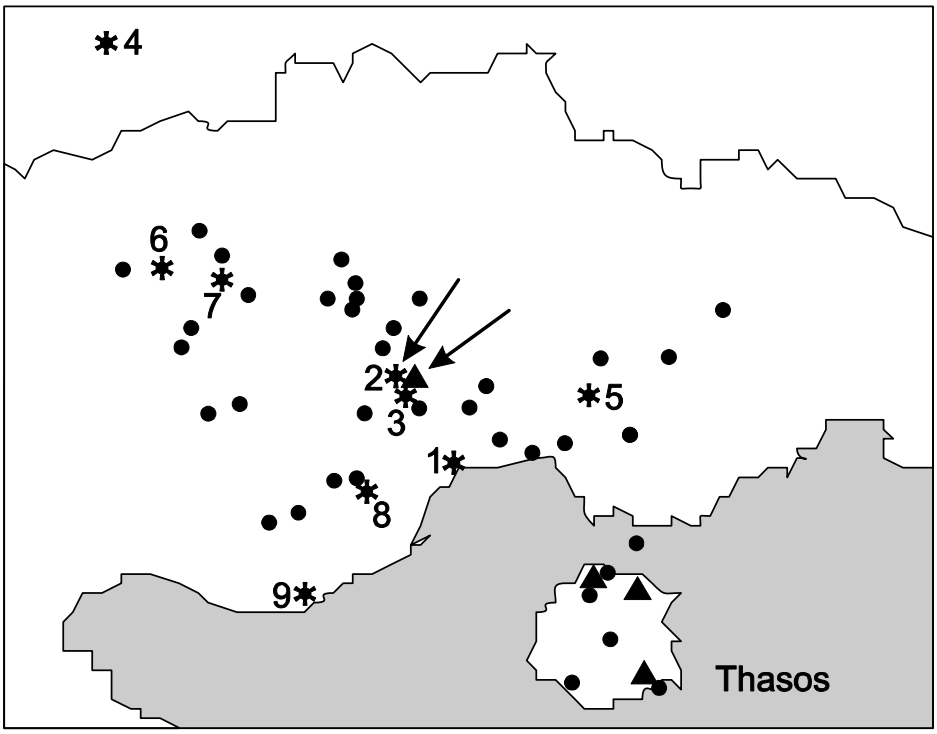


Fig. 5.6. Coincidence of ancient marble quarries (triangles) and known locations of *C. buresi* in NE Greece and adjacent Bulgaria. Asterisks refer to *C. buresi* populations sampled in this study. The locations of other *C. buresi* populations (dots) are based on the literature and data in the National Museum of Natural History in Leiden, the Netherlands. Locations of the marble quarries are based on Waelkens *et al*. (1988a) and Dworakowska (1975). The arrows mark the sister group of *C. buresi pharsalica* and the Filippi quarries.

Filippi, and it has been identified from archaeological sites throughout the eastern and central Mediterranean area (e.g. Maniatis *et al.*, 1988; Herrmann, 1992; Tykot *et al*., 2002). It was transported as far as ancient Delphi, situated beyond the present range of *C. buresi pharsalica*, as early as in the sixth century BC (Déroche *et al.*, 1989). Large blocks of marble were far more easily transported across water than across land (Tykot & Ramage, 2002), and long-distance transport of marble from the Filippi quarries, which are located 10-15 kilometres further inland than those of Thasos, may have been more problematic. Nevertheless, several devices for marble transport across land, such as four-wheeled carts, are known from antiquity (Wurch-Kozelj, 1988).

In order to evaluate the possibility of human-aided dispersal, divergence time can only be tentatively estimated, bearing in mind that these estimations rest on uncorroborated assumptions and may therefore be flawed. We can arrive at only a crude estimate of the minimal time span from the deepest divergences within the *C. buresi pharsalica* clade, based on the range of previously reported mitochondrial divergence rates. The highest mitochondrial sequence divergence so far reported within land snails, calculated from mitochondrial lrDNA and srDNA, amounted to 10% pairwise divergence per million years (Chiba, 1999a). This is markedly higher than the estimations in *Albinaria*, which range from 1-1.2% (Douris *et al.*, 1998a) to 5% (Douris *et al*., 1995). Considering the maximum pairwise difference between *C. buresi pharsalica* sequences of 0.62%, the uppermost estimate of 10% would imply that the two populations shared a common ancestor at the very least approximately 62,000 years BP. This minimum estimate outdates marble quarrying in Macedonia and Thracia by more than a factor ten. However, we note three caveats. First, all inferred mitochondrial divergence rates within land snails so far have been based on ribosomal DNA, not on protein-coding genes harbouring synonymous variation. Second, the low number of maximally four sites, variable between *C.*

buresi pharsalica sequence pairs, may introduce a large stochastic error. Finally, the divergence of the mitochondrial lineages present within *C. buresi pharsalica* may predate the dispersal into Thessaly. In fact, when assuming passive dispersal through marble transport, it is highly likely that numerous snails would be transported at once. Provided that complete lineage sorting has not been accomplished since then, several of these ancestral lineages may have been sampled.

At this point, we consider transport of the ancestors of *C. buresi pharsalica* with Filippi marble to Thessaly the most plausible explanation for its erratic distribution. Still, a denser sampling of *C. buresi* populations from NE Greece is needed to locate the provenance of the ancestors of *C. buresi pharsalica* with greater confidence. Further studies on the genetic variation within *C. buresi pharsalica* may help to pinpoint its site of introduction in Thessalia. These results can then be compared with archaeological data.

ACKNOWLEDGEMENTS

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Chapter 6

REPRODUCTIVE CHARACTER DISPLACEMENT BY INVERSION OF COILING IN CLAUSILIID SNAILS (GASTROPODA, PULMONATA)

ABSTRACT

In land snails, a change in the direction of coiling, being associated with a shift in the position of the genital apparatus, may act as a barrier against hybridization between sympatric species. Putative reproductive character displacement by an inversion in chirality has been reported in only a few land snails, based on observations in the field and interbreeding experiments. In this study, we present a new case of possible reproductive character displacement in the direction of coiling, viz. in the closely related clausiliid snails *Isabellaria lophauchena* and *Sericata dextrorsa*. The latter species is dextrally coiled, in contrast to all of its nearest relatives, including *I. lophauchena*, which share the putatively plesiomorphic sinistral coiling. The ranges of these two oppositely coiled species overlap and they are often found syntopically.

In this study, we have analysed mitochondrial cytochrome *c* oxidase subunit I (COI) sequences of five populations of *I. lophauchena* and six *S. dextrorsa* populations, four of either species being syntopic. Although the average K2P-corrected and LogDet genetic distance between *S. dextrorsa* and *I. lophauchena* was smaller than that between reputedly interbreeding *Albinaria* species, we found no evidence of introgression of mitochondrial DNA between the species, which may be indicative of a prezygotic isolating mechanism. Sequences of two populations of the sinistral species *Sericata torifera*, which is allopatrically distributed with respect to other related sinistral species, were nested among *S. dextrorsa* sequences. This observation implies that a shift in direction of coiling occurred at least two times, depending on the presence or absence of sympatric related sinistral species. This result is in line with the hypothesis of genetic isolation by reproductive character displacement, although other mechanisms possibly contribute to the observations as well.

INTRODUCTION

Reproductive character displacement, the divergence in mating-associated traits that enhances prezygotic reproductive isolation between species in their area of sympatry, is considered one of the most fascinating, yet at the same time controversial, issues in evolutionary biology (for an overview see Howard, 1993). Reproductive character displacement between species can evolve in an area of sympatry when (1) heterospecific mating results in an overall decrease of reproductive success, and (2) divergence in a heritable trait between the species reduces the frequency of such heterospecific mating. A continuing point of criticism has been the lack of empirical evidence for this hypothesis (Howard, 1993). One of the best supported (Howard, 1993) and most elegant of all presumed examples of reproductive character displacement concerns the shift in chirality in land snails. Snails can be dextrally (right-handedly) or sinistrally (left-handedly) coiled. Coiling direction is determined by a single locus and is expressed by a female on its eggs. Hence a female's genotype is manifested only in its offspring (Boycott *et al*., 1930; Degner, 1952; Murray & Clarke, 1966). This genetically simple

Dennis R. Uit de Weerd, Dick S. J. Groenenberg, Edmund Gittenberger and Menno Schilthuizen. Reproductive character displacement by inversion of coiling in clausiliid snails (Gastropoda, Pulmonata).

trait can have great consequences for reproduction in snails. Male and female reproductive organs of these generally hermaphroditic animals open on only one side of the body: on the dextral side in dextral snails, and on the sinistral side in sinistral snails. A different position of the genital apparatus may prevent or complicate the exchange of gametes between snails of opposite coil.

So far, two possible cases of character displacement in direction of coiling have been reported from land snails, viz. in the prosobranch genus *Diplommatina* (Peake, 1973) and in the pulmonate genus *Partula* (Clarke and Murray, 1969; Murray & Clarke, 1980). The phenomenon was extensively studied in the latter genus only. The initial evidence of reproductive character displacement within *Partula* came from three observations (see Clarke and Murray, 1969; see Murray & Clarke, 1980): (1) the species of interest are apparently closely related, (2) their ranges touch or overlap and (3) only in these contact zones the species have an opposite direction of coiling. The potential role of direction of coiling as an isolating mechanism within this genus is demonstrated by the partial reproductive isolation even between conspecific dextral and sinistral forms of *Partula suturalis* (Clarke & Murray, 1969; Lipton & Murray, 1979), caused by attempts at intromission in the wrong place (Lipton & Murray, 1979; Johnson, 1982).

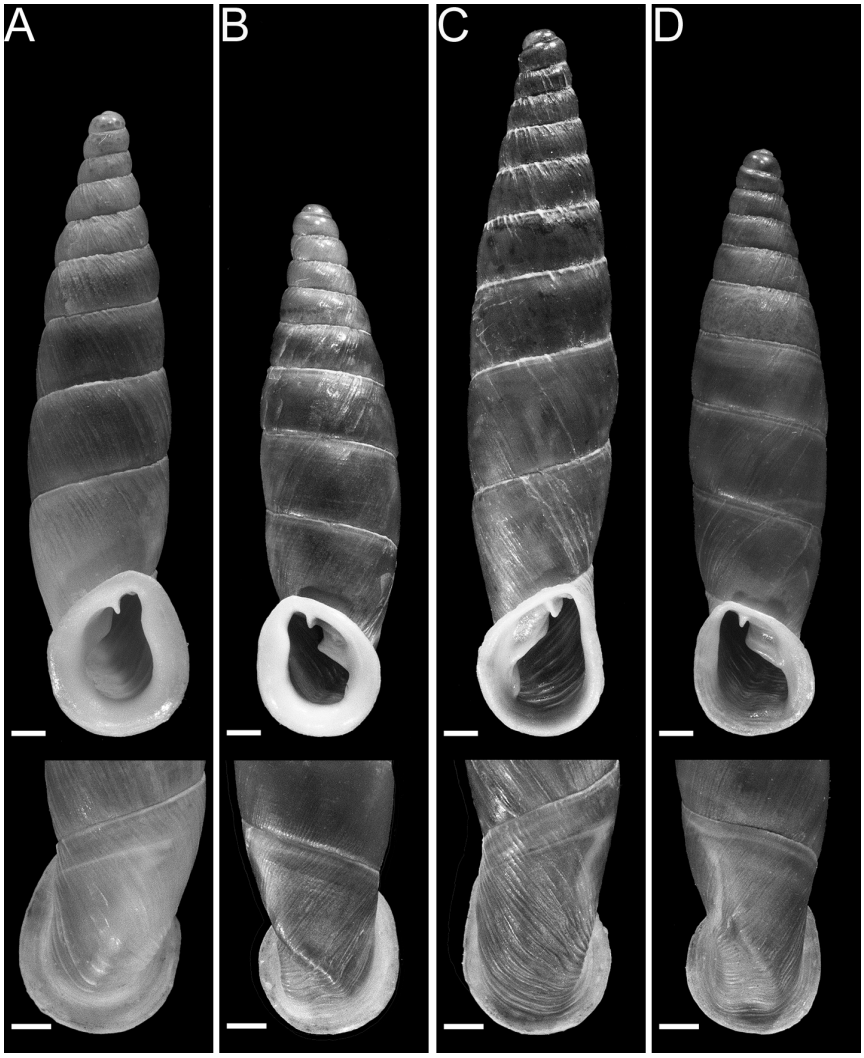

Figure 6.1. Syntopic *S. dextrorsa* and *I. lophauchena* samples from two localities. Locality numbers refer to Figure 6.2. Above: ventral aspect; below: cervix, dorsal aspect. Scale bar 1.0 mm. **A** *S. dextrorsa* from Theodoraki (locality 1); **B** *I. lophauchena* from Theodoraki (locality 1); **C** *S. dextrorsa* from locality 2; **D** *I. lophauchena* from locality 2.

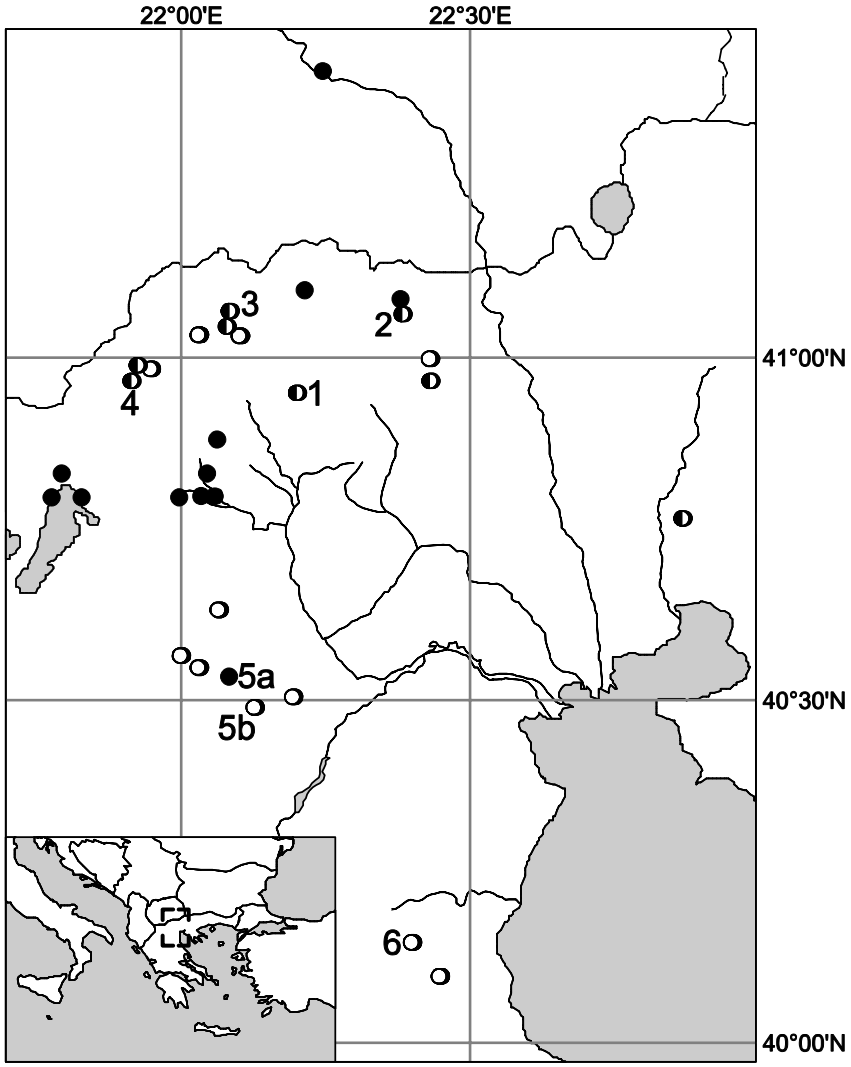

Figure 6.2. Distribution of *Sericata dextrorsa* and *Isabellaria lophauchena*, based on the literature and data in the National Museum of Natural History in Leiden. Open circles indicate *S. dextrorsa*, closed circles *I. lophauchena*. Localities where both species were found syntopically are marked with half-filled circles. Numbers refer to the sample sites shown in Table 6.1.

Recent fieldwork in northern Greece, and molecular analyses revealed a distribution of dextral and sinistral forms within the family Clausiliidae reminiscent of that in *Partula*. These dextral and sinistral forms are placed in *Sericata dextrorsa* and *Isabellaria lophauchena*, respectively (Fig. 6.1). DNA analysis shows that these species, although conchologically placed in different genera, are actually closely related (see Chapter 4, this thesis). Among the species currently placed in *Isabellaria* and *Sericata*, *S. dextrorsa* is uniquely dextrally coiled. While the sinistral *Sericata* and *Isabellaria* species have a mosaic-like distribution (see Nordsieck, 1974: 131-132), *S. dextrorsa* is found in sympatry with *I. lophauchena* in most of the northern part of its range, which extends from the Olympos Mountain to the Greek-Macedonian border (Fig. 6.2). In the southern part of its range, where *I. lophauchena* is absent, *S. dextrorsa* is found in parapatry to *Sericata albicosta*. Although *Sericata dextrorsa* and *Isabellaria lophauchena* often coexist syntopically within their area of sympatry, no hybrids between *S. dextrorsa* and *I. lophauchena* have been found so far. A possible indication of gene flow between these species comes from the syntopic *S. dextrorsa* and *I. lophauchena* population found in Theodoraki (locality 1). Both these populations have a tumid thick-walled shell, and in general appearance they more closely resemble each other than conspecific populations from other localities (Fig.

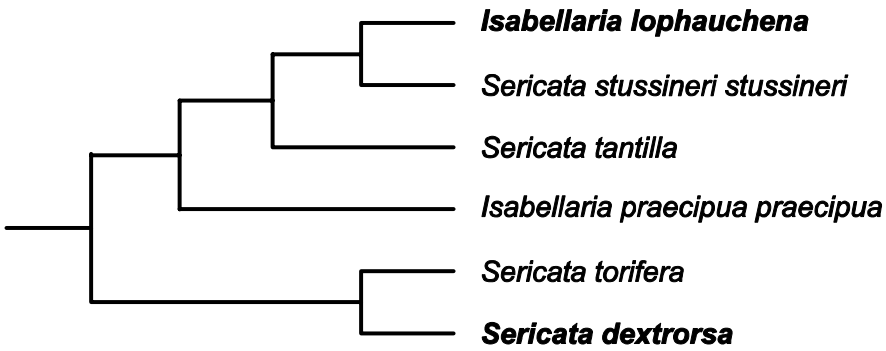

Figure 6.3. The phylogenetic position of *Sericata dextrorsa* and *Isabellaria lophauchena* among related species, as inferred from Bayesian inference (Chapter 4, this thesis).

6.1). Such similarities may result from interspecific introgression of genes for advantageous shell traits (Chiba, 1993). Phylogenetic analyses of combined ITS1, ITS2, 12S and COI sequences (see Chapter 4, this thesis) reveal that *S. dextrorsa* and *I. lophauchena* are part of a single clade, in which *S. dextrorsa* is grouped with *Sericata torifera* (Fig. 6.3). The position of *S. dextrorsa* and *I. lophauchena* within this clade, isolated from each other, and nested among more south-westerly distributed species (Fig. 6.4), suggests that the contact between *S. dextrorsa* and *I. lophauchena* is secondary.

Selection of this difference in direction of coiling as a premating isolating mechanism is a possible explanation for the patterns observed among these clausiliid snails. As in other snails, chirality is determined by two alleles in the Clausiliidae (Degner, 1952), the sinistral allele being dominant over the dextral one in this family. The overwhelming majority of clausiliid species is sinistral (Zilch, 1959: 377), and sinistral coiling is consequently assumed to be the plesiomorphic condition within the family. The position of *S. dextrorsa*, nested among sinistral species, supports the notion that dextral coiling evolved secondarily within the family. Copulation of clausiliid snails can be simultaneously reciprocal (Nordsieck, 1969; Schilthuizen & Lombaerts, 1995), i.e. both partners transfer spermatophores. At least partial premating reproductive isolation may arise from differences in chirality in clausiliid snails, despite the observation that copulation between individuals of opposite chirality is physically possible within the Clausiliidae (Gittenberger, 1988; Asami *et al*., 1998). Studies on *Partula* show that, even if copulation between snails of opposite coil is possible, such copulations occur less frequently and result in fewer offspring than those between snails with the same direction of coil (Johnson, 1982).

As a first examination of the hypothesis of reproductive character displacement, we investigated whether molecular phylogenetic analyses join syntopic and adjacent *S. dextrorsa* and *I. lophauchena* populations with conspecific populations rather than with each other, thus indicating an absence of gene flow. This outcome would be expected if character displacement has been successful. Should we, on the other hand, find mixed clades containing sequences of both species, then direction of coiling is ineffective in establishing reproductive isolation, making the hypothesis of reproductive character displacement far less likely. Since this approach relies on the detection of past introgression events between the species, we tried to maximize the chances to trace these. Thus we focussed in particular on the two highly similar populations from Theodoraki. In addition, we used a mitochondrial gene, viz. cytochrome *c* oxidase subunit I (COI). The unlinked nonrecombining nature of mtDNA allows for the detection of hybridization even after long time spans (see Shimizu & Ueshima, 2000).

MATERIALS AND METHODS

Nearly all samples of *S. dextrorsa* and *I. lophauchena* came from localities where both species coexist syntopically, with only two exceptions, both from the southern part of their range. In this area, which was less adequately sampled, we did not locate syntopic populations of both species. Therefore we included *I. lophauchena* and *S. dextrorsa* samples from two localities 6 kilometres apart, numbered 5a and 5b, respectively. The conchologically highly similar populations from Theodoraki, site 1, were represented by five individuals each, all other populations by one. An additional *S. dextrorsa* sample (not sympatric with *I. lophauchena*) and a *S. albicosta* sample (No. 753) from adjoining populations on Mount Olympos (locality 6) were also included. The selection of additional ingroup and outgroup species was based on previous phylogenetic inferences (see Chapter 4, this thesis). Thus the ingroup consisted, apart from *S. dextrorsa* and *I. lophauchena*, of the closest relatives of either species, viz. *I. praecipua*, *S. stussineri*, *S. tantilla* and *S. torifera*. For outgroup rooting we used two samples of *Sericata albicosta*, including the sample from Mount Olympos, *Isabellaria thessalonica* and *Carinigera septima*. The additional ingroup and outgroup species surround the range of *S. dextrorsa* and *I. lophauchena* (Fig. 6.4). To estimate the effect of genetic compatibility on gene flow between *S. dextrorsa* and *I. lophauchena* independently from direction of coil, we compared their genetic divergence to that between interbreeding (Mylonas *et al.*, 1988) species of the closely related genus *Albinaria*. To this end, we also determined COI sequences of four *Albinaria* species, viz. *A. brevicollis, A. discolor, A. grisea* and *A. puella,* that were reported to interbreed. The COI sequence of a fifth species used in those studies, viz. *A. caerulea*, was determined by Hatzoglou *et al*. (1995) and obtained from GenBank (accession number X83390).

Total genomic DNA was extracted from frozen tissue, collected 1-3 years before, using the protocol described by Schilthuizen *et al*. (1995). A 708-base-pair fragment of the 5' half of the COI gene was PCR-amplified with an annealing temperature of 47°C, using the forward primer L1490-Alb (5'-ACT CAA CGA ATC ATA AAG ATA TTG G-3') and the reverse primer H2198-Alb (5'-TAT ACT TCA GGA TGA CCA AAA AAT CA-3') (see Gittenberger *et al*., 2004). PCR fragments were gel-purified using spin columns (Qiaquick® Gel Extraction Kit by Qiagen®). Sequences of forward and reverse dye-terminator (Big Dye™ by PE Biosystems ®) cycle-sequenced PCR products were determined on an ABI 377 automated sequencer (PE Biosystems®). The forward and reverse sequences were assembled in the program Sequencher (Gene Codes Corp.®), and aligned using the Clustal V method implemented in MegAlign 4.03© (DNASTAR Inc., 1999). All sequences were checked for stop codons and missing bases.

Prior to the phylogenetic inferences, we tested the quality of our data set by calculating the g1 value from a distribution of 10,000 random trees (Hillis & Huelsenbeck, 1992) and by performing a permutation test (Archie, 1989; Faith & Cranston, 1991) with 1000 replicates (5 random addition replicates, TBR, steepest descent), both implemented in PAUP* 4.0b10 (Swofford, 2002). The amount of sequence divergence between *S. dextrorsa* and *I. lophauchena* relative to the sequence divergence within *Albinaria* was determined using a Kimura's two-parameter model (K2P) to correct for multiple hits, as well as a LogDet model to correct for unequal base frequencies (Swofford *et al*., 1996: 459-461). The K2P model was chosen to facilitate comparisons with previous studies using K2P corrected divergences between

Albinaria sequences (van Moorsel *et al.*, 2001c; Douris *et al*., 1998a). Only a single sequence from each of the two Theodoraki populations sampled, i.e. Nos. 779 and 761, was used in these calculations, because otherwise this single population might dominate in the analysis.

Phylogenetic inference was based on maximum parsimony heuristic searches (1000 random addition replicates, TBR, steepest descent) using PAUP* 4.0b10 (Swofford, 2002). Bootstrap support was calculated using 10,000 replicates (1 random addition per replicate, TBR and steepest descent). All trees within two steps of the most parsimonious tree were obtained through a second heuristic search with 100,000 replicates, and their likelihood scores were calculated using a GTR+I+Γ model selected by MrModeltest 1.1b after running the standard model block in PAUP*. A larger area of tree space was sampled rather than that consisting of

Table 6.1. Sample information.

localities	Species	number	co-ordinates	UTM
1	*Sericata dextrorsa*	761	40°57'N 22°12'E	FL0034
1	*Sericata dextrorsa*	1355	40°57'N 22°12'E	FL0034
1	*Sericata dextrorsa*	1356	40°57'N 22°12'E	FL0034
1	*Sericata dextrorsa*	1357	40°57'N 22°12'E	FL0034
1	*Sericata dextrorsa*	1358	40°57'N 22°12'E	FL0034
2	*Sericata dextrorsa*	762	41°04'N 22°23'E	FL1547
3	*Sericata dextrorsa*	756	41°03'N 22°05'E	EL9144
4	*Sericata dextrorsa*	760	40°58'N 21°55'E	EL7635
5b	*Sericata dextrorsa*	296	40°29'N 22°08'E	EK9582
6	*Sericata dextrorsa*	755	40°09'N 22°24'E	FK1946
1	*Isabellaria lophauchena*	779	40°57'N 22°12'E	FL0034
1	*Isabellaria lophauchena*	1359	40°57'N 22°12'E	FL0034
1	*Isabellaria lophauchena*	1360	40°57'N 22°12'E	FL0034
1	*Isabellaria lophauchena*	1361	40°57'N 22°12'E	FL0034
1	*Isabellaria lophauchena*	1362	40°57'N 22°12'E	FL0034
2	*Isabellaria lophauchena*	782	41°04'N 22°23'E	FL1547
3	*Isabellaria lophauchena*	1363	41°03'N 22°05'E	EL9144
4	*Isabellaria lophauchena*	776	40°58'N 21°55'E	EL7635
5a	*Isabellaria lophauchena*	268	40°32'N 22°05'E	EK9188
7	*Isabellaria praecipua praecipua*	844	40°29'N 22°12'E	FK08
8	*Sericata stussineri stussineri*	302	39°53'N 22°38'E	FK3916
9	*Sericata tantilla*	300	39°42'N 22°14'E	FJ0596
10	*Sericata torifera*	304	39°41'N 21°41'E	EJ5891
11	*Sericata torifera*	813	39°41'N 21°35'E	EJ5092
12	*Carinigera septima*	1137	41°24'N 22°15'E	FL08
13	*Isabellaria thessalonica crassilabra*	266	40°23'N 23°10'E	FK8371
14	*Sericata albicosta*	307	40°05'N 22°25'E	FK2038
6	*Sericata albicosta*	753	40°09'N 22°24'E	FK1946
-	*Albinaria brevicollis brevicollis*	697	36°51'N 28°16'E	PA17
-	*Albinaria discolor discolor*	675	36°44'N 22°54'E	FF6967
-	*Albinaria grisea akrocurta*	674	36°53'N 22°48'E	FF5983
-	*Albinaria puella puella*	700	37°52'N 27°16'E	NB29

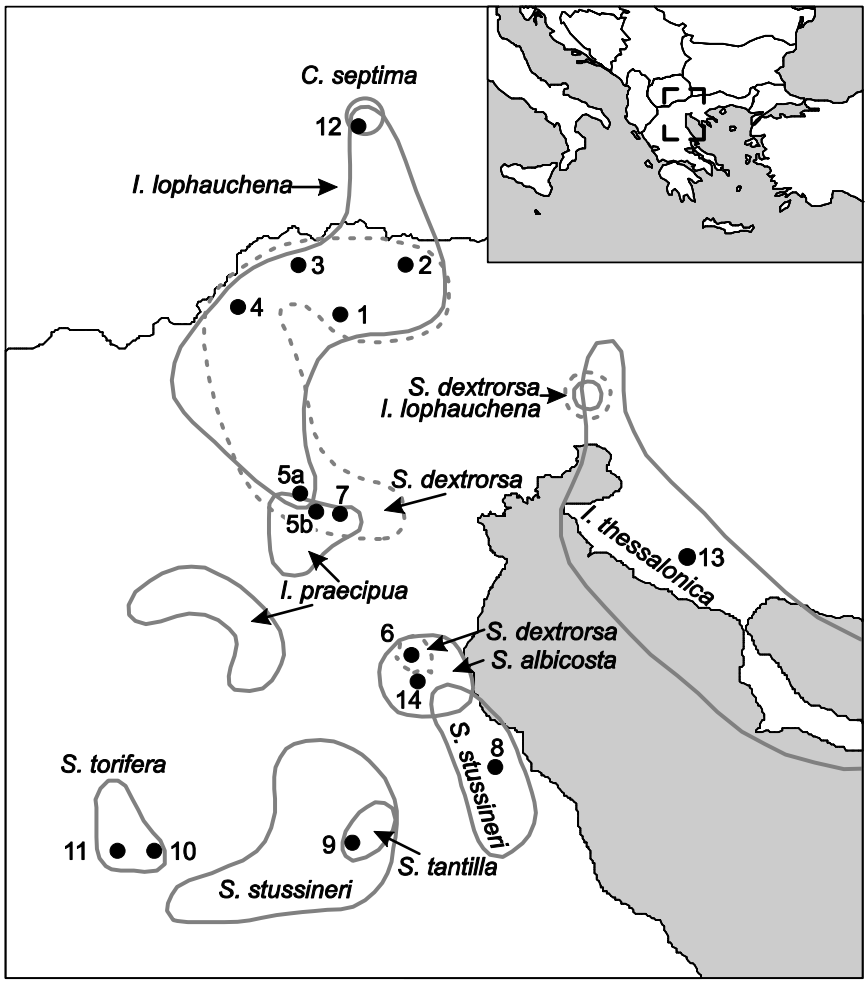

Figure 6.4. Schematic distribution of the species included in this study, based on the literature and data in the National Museum of Natural History in Leiden. The approximate borders of the range of *S. dextrorsa* are shown by hatched lines, those of the other, sinistral, species by uninterrupted lines. Numbers refer to the sample sites shown in Table 6.1.

the most parsimonious trees, because these most parsimonious trees do not necessarily represent the trees with maximum likelihood scores. The tree with the highest likelihood score was re-evaluated using a molecular clock constraint, and both likelihood values were compared in a likelihood ratio test (Felsenstein, 1988) to check for rate constancy.

RESULTS

Of the amplified region, 651 bases could be identified for all *S. dextrorsa*, *I. lophauchena* and *Albinaria* samples. Base frequencies at codon positions 1 and 2 were not significantly inhomogeneous across taxa (P=1.00), whereas significant inhomogeneity was detected at codon position 3 (P<0.001). These differences appear to be correlated with the phylogenetic tree inferred from position 3. Thus the species with the highest percentage of adenosine, viz. *I. praecipua*, *S. torifera* and all included *S. dextrorsa* samples, all clustered within a single clade. We therefore tested whether this compositional bias at position 3 had introduced a systematic error into the phylogenetic inferences. To that end, the phylogenetic signal inferred from position 3 was compared in a partition homogeneity test (1000 replicates, heuristic search, 5 random additions per replicate, TBR, steepest descent) to that of the unbiased positions 1 and 2 combined, excluding all uninformative positions. No evidence was found for significantly

Table 6.2. Kimura's 2-parameter corrected and LogDet genetic distance between samples.

distances between *Albinaria* samples		
Samples	K2P	LogDet
A. disc. discolor 675 – *A. brev. brevicollis* 697.	0.19094	0.196559
A. puella puella 700 – *A. brev. brevicollis* 697	0.154556	0.163869
A. puella puella 700 – *A. disc. discolor* 675	0.177611	0.189392
A. caerulea -*A. brev. brevicollis* 697	0.154831	0.165477
A. caerulea - *A. disc. discolor* 675	0.189712	0.198261
A. caerulea - *A. puella puella* 700	0.154556	0.16828
A. grisea akrocurta 674 - *A. brev. brevicollis* 697	0.178775	0.181407
A. grisea akrocurta 674 – *A. disc. discolor* 675	0.188528	0.194067
A. grisea akrocurta 674 – *A. puella puella* 700	0.179249	0.185701
A. grisea akrocurta 674 – *A. caerulea*	0.189559	0.195716
Average within *Albinaria*	0.175832	0.183873
Average distances of the *S. dextrorsa* and *I. lophauchena* samples		
Samples	K2P	LogDet
Within *S. dextrorsa*	0.055241	0.062214
Within *I. lophauchena*	0.100326	0.107319
S. dextrorsa – I. lophauchena	0.168584	0.176944
S. dextrorsa – Albinaria species	0.187705	0.189568
I. lophauchena – Albinaria species	0.216102	0.220428

contradicting phylogenetic signal between position 3 and position 1 and 2 combined (P=0.12). We also compared the parsimony scores of the LogDet NJ tree of all positions, which is corrected for compositional bias (Swofford *et al*., 1996: 459-461), with those of the MP trees for all positions. MP trees were calculated using heuristic search, 1000 random addition replicates, TBR and steepest descent. Four most parsimonious trees were found with a score of 680. In spite of superimposed differences between the two methods with respect to the type of data used and the tree-building process (Page & Holmes, 1998: 178-193), the LogDet NJ tree required only two additional transformations, which is a non-significant difference (Templeton test: 0.6374<P<0.6547; Winning sites test: 0.8145<P<0.8238). Consequently, we included the third codon position in all subsequent analyses.

Average pairwise divergence (Table 6.2) between sequences of *S. dextrorsa* and *I. lophauchena* was slightly lower (K2P: 0.168584; LogDet: 0.176944) than the average estimated divergence between the *Albinaria* sequences (K2P: 0.175832; LogDet: 0.183873). These roughly similar divergences cannot be attributed to saturation effects, as the K2P and LogDet pairwise divergences between *S. dextrorsa* and *I. lophauchena* populations were lower on average than those of either genus to *Albinaria*, indicating that a maximum divergence level has not yet been reached between *S. dextrorsa* and *I. lophauchena*. Genetic divergence within *S. dextrorsa* was significantly lower than within *I. lophauchena* (two-tailed Mann-Whitney U-test: P<0.001). This difference can not be attributed to a denser sampling of genetic variation within *S. dextrorsa*, since a comparison of an equal number of *I. lophauchena* samples and adjoining *S. dextrorsa* samples, viz. those of localities 1 to 5, likewise revealed significant differences (two-tailed Mann-Whitney U-test: P<0.005).

The permutation test (P=0.001) and the highly left-skewed length distribution of random trees (mean=1384.43, g1=-0.51, P<<0.01) indicated that the data set contained a highly significant phylogenetic signal under the parsimony criterion. The four most parsimonious trees, with a score of 680, differed only in the inferred relationships between the sequences from each population from locality 1 (Theodoraki). The strict consensus of these trees and the

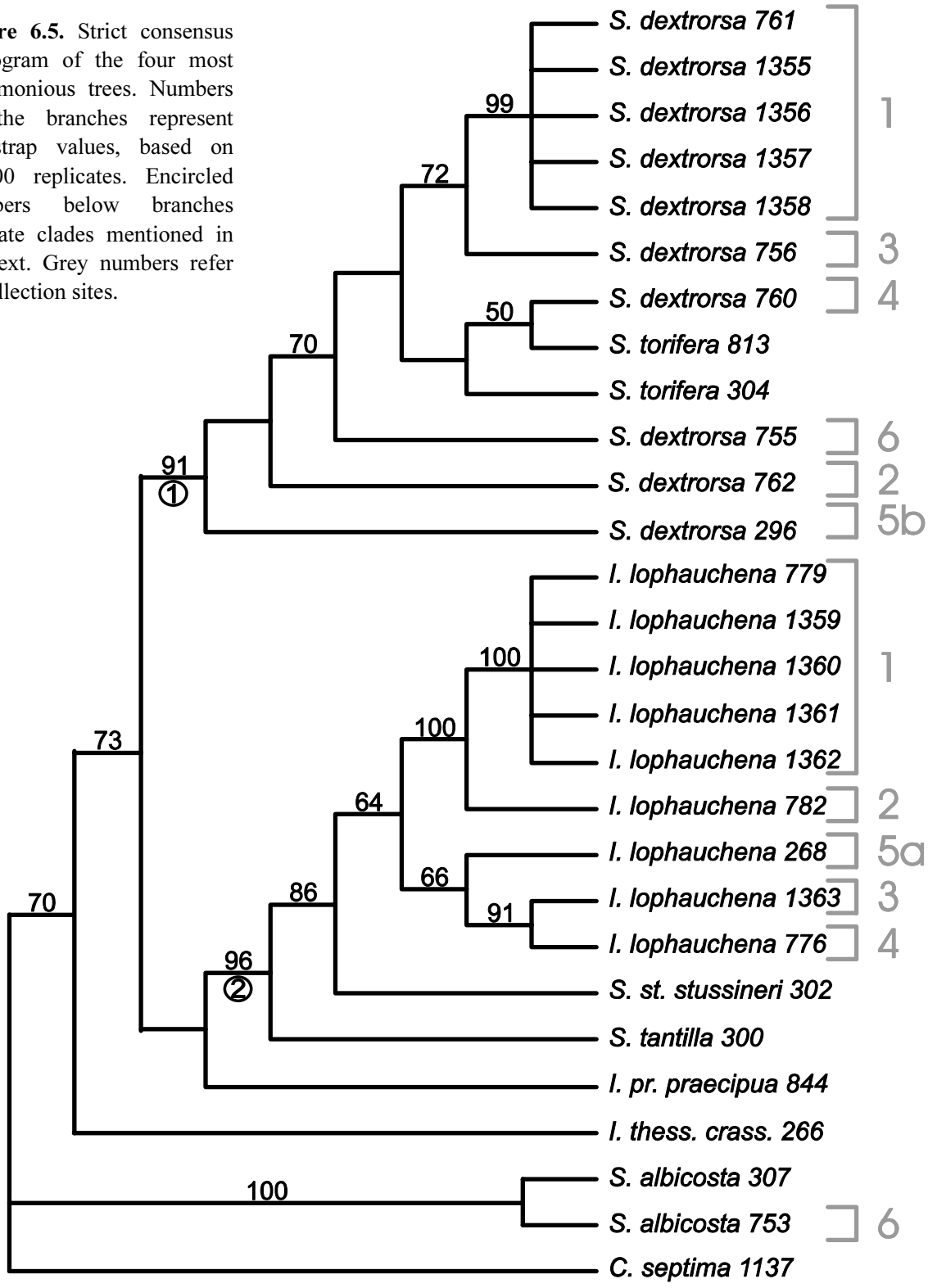

Figure 6.5. Strict consensus cladogram of the four most parsimonious trees. Numbers on the branches represent bootstrap values, based on 10,000 replicates. Encircled numbers below branches indicate clades mentioned in the text. Grey numbers refer to collection sites.

bootstrap support for its constituent clades are shown in Figure 6.5. The MP analyses placed the *S. dextrorsa* and the *I. lophauchena* sequences in different highly supported clades, numbered 1 and 2, respectively. Both these clades were previously identified using an independent, nuclear, marker, viz. ITS1&2 sequences. Those analyses moreover identified a topology within clade 2 identical to that found here. For the *I. lophauchena* sequences a monophyletic origin is inferred, whereas the *S. dextrorsa* sequences are para- or polyphyletic with respect to *S. torifera*. A relatively well-supported internode separates the *S. torifera* sequences and the *S. dextrorsa* sequences of localities 1, 3, 4 and 6 from the *dextrorsa* sequences of localities 2 and 5b. Other basal relationships within the *dextrorsa-torifera* clade are poorly bootstrap-supported. The samples of either species from locality 1 (Theodoraki) are monophyletic. The second MP search with 100,000 replicates retained 4598 trees with a score of 682 or less. Again, the same 4 most parsimonious trees with score 680 were found. The tree with the highest likelihood score (-ln L=3725.44) of all these trees had a significantly inconstant substitution rate (likelihood ratio test: $0.001<P<0.005$).

DISCUSSION

Our results demonstrate that *S. dextrorsa* and *I. lophauchena* are reproductively isolated. No evidence of past gene flow anywhere in the phylogeny of the two species was found. Even the specimens from site 1 (Theodoraki) cluster according to species. Studies on similarly sympatric non-interbreeding land snails demonstrate that such conchological similarities can be convergences (Solem, 1985; Emberton, 1995), an explanation supported by the co-occurrence of an unusually thick-shelled population of *Chondrula macedonica* at site 1. The apparent absence of past gene flow, even between sympatric *S. dextrorsa* and *I. lophauchena*, is consistent with the observation that the species do not hybridize in sympatry.

In spite of their apparent reproductive isolation, the genetic distance between *S. dextrorsa* and *I. lophauchena* is of the same order of magnitude as that within *Albinaria* species interbreeding in the laboratory. Nearly all these distances fall within the range of mtDNA genetic distances (11-18%) expected for congeneric species (Douris *et al*., 1998a). Assuming, on the basis of their similar genetic divergences (see Edmands, 2002), that *I. lophauchena* and *S. dextrorsa* are genetically as compatible as are the interbreeding *Albinaria* species, we conclude that their opposite direction of coiling may have contributed to their reproductive isolation.

The tree points to character displacement within the *dextrorsa-torifera* clade (clade 1). Within this clade either (1) dextral lineages, assigned to *S. dextrorsa*, evolved several times independently when in sympatry with sinistral species, or (2) dextrality evolved only once and a reversion to the sinistral '*S. torifera*' occurred when in allopatry to sinistral species. The deeper relationships within the *dextrorsa-torifera* clade are too poorly supported to decide between these alternatives. In fact, apart from the opposite direction of coil, no differences between the two supposed species have been described. The combination of an opposite direction of coiling and reproductive isolation in areas of sympatry of *S. dextrorsa* and *I. lophauchena* is suggestive of reproductive character displacement.

Niche differentiation in sympatry offers an unlikely alternative explanation of the character displacement observed. Admittedly, niche differentiation between related sympatric or syntopic species has been reported from other land snails (Murray *et al*., 1982; Ledergerber *et al*., 1997; Chiba, 1999b, 2002), and has been inferred for syntopic *Albinaria* species displaying differences in radula structure and in substrate usage (Kemperman, 1992: 117-119). Syntopic rock-dwelling clausiliid snails can even feed on different lichen species (Baur *et al*., 1994). Also feeding on lichens and bryophytes, *S. dextrorsa* and *I. lophauchena* could have developed a similar fine-patterned food partitioning that can be detected by very detailed studies only. Feeding-related differentiation into two mirror images has been observed in cichlid fish (Hori, 1993) and in crossbills (Benkman, 1988, 1996). However, these groups are asymmetric in their feeding apparatus, which is not the case in clausiliid snails (Kemperman, 1992: 85). Neither can we think of a way in which differences in chirality would facilitate a partitioning of their food resources. Similar studies on mirror types within *Partula* were likewise unable to relate these to ecology (Clarke & Murray, 1969). Although we cannot exclude some extent of niche differentiation between *S. dextrorsa* and *I. lophauchena*, this is unlikely to be the driving force behind the evolution of the mirror types.

Assuming that *S. dextrorsa* and *I. lophauchena* were capable of interbreeding initially, hybrid disadvantages, such as have been inferred in *Albinaria* (Giokas, *et al*. 2000; Schilthuizen, 1995; Schilthuizen & Lombaerts, 1995), may have driven the divergence in direction of coiling. Still, on basis of our data, we cannot exclude the possibility that reproductive isolation between *S. dextrorsa* and *I. lophauchena* arose prior to the character displacement between these species. Actual hybridization may not be a necessary condition for reproductive character displacement to evolve. For instance, even if not hybridizing, sympatric species may interfere in each others mating behaviour, thus reducing the number of successful copulations (Noor, 1999), or animals may suffer from higher mortality and lower fertility as a consequence of heterospecific mating (Servedio, 2001). Selection against heterospecific mating will be strongest in individuals of the rarer species. In the absence of assortative mating, most of their offspring consists of hybrids (Sawyer & Hartl, 1981; Johnson, 1982; Benedix & Howard, 1991), if such hybrids are produced at all. This selection against heterospecific mating, in particular in the rarer species, may overcome the initially reduced mating success with conspecifics that is associated with a change in chirality (Johnson, 1982) as well as the dominance of the sinistral allele, should such conspecific mating occur. Both these factors were found to hamper the spread of a recessive chirality allele in model studies (Johnson *et al*., 1990; van Batenburg & Gittenberger, 1996; but see Orr, 1991). Since the displacement in fact occurs in only one species, viz. *S. dextrorsa*, it is tempting to speculate that ancestors of either the *S. dextrorsa* lineages or the *dextrorsa-torifera* clade invaded the range of *I. lophauchena* and subsequently became dextral. However, the shift in chirality in *S. dextrorsa* could also have occurred under equal numbers of both species, provided that the dextral allele was absent in *I. lophauchena*, or was present in a lower frequency than in *S. dextrorsa* (see Howard, 1993). Some indirect support of an invasion of *S. dextrorsa* into the range of *I. lophauchena* comes from the significantly smaller intraspecific genetic divergence within *S. dextrorsa* as compared to *I. lophauchena*, which could indicate that *S. dextrorsa* is the younger species. The interpretation of these differences in genetic divergence is difficult, however, given the rate

heterogeneity within the tree, the yet inadequate sampling of both species, and possible effects of differing branching patterns.

Molecular surveys of polymorphic *Partula* species indicate that a difference in direction of coiling per se does not impose a barrier to gene flow in the long run (Murray *et al*., 1991; Johnson *et al*., 1987; Goodacre, 2002). Even the chirality alleles themselves appear to have introgressed at least once into an oppositely coiled species (Johnson *et al*., 1993). Moreover, even occasional hybridization between heterospecific dextral and sinistral *Partula* populations can have a large impact on the genetic composition of these populations, as demonstrated both by allozyme studies (Clarke, *et al*., 1996) and by the distribution of mitochondrial haplotypes (Goodacre, 2002). A similarly incomplete reproductive isolation between species distinguished on the basis of opposite chirality was inferred from molecular phylogenetic studies on the genus *Achatinella* (Thacker & Hadfield, 2000; Holland & Hadfield, 2002). It must be noted, however, that the *Partula* species studied are considered to be still in the process of speciation (Johnson *et al*., 1993; Murray & Clarke, 1980), and that the status of the *Achatinella* species in question is uncertain (Thacker & Hadfield, 2000; Holland & Hadfield, 2002).

The studies on *Partula* show that differences in chirality may be a large initial step towards premating reproductive isolation, and may thus facilitate the coexistence of two species, but that they do not preclude occasional mating. Similar results have been reported for the clausiliid genus *Alopia* (Nordsieck, 1978b), placed in the same subfamily Alopiinae as are *Sericata* and *Isabellaria*. That study consisted of two experiments, each with different *Alopia* species, and each with 15 individuals of a dextral and 15 of a sinistral species. Summed over the two experiments, three out of "about 20" copulations observed were between species of different coil, the remaining between conspecific individuals.

Complete prezygotic reproductive isolation between *S. dextrorsa* and *I. lophauchena* may be accomplished along additional pathways. For instance, increased assortative mating in sympatry has been observed between congeneric species of pulmonate snails (Wullschleger *et al*., 2002). Mate recognition in pulmonate land snails may be based on body and shell shape, courtship behaviour or chemical cues. Courtship behaviour of pulmonate stylommatophoran land snails generally is very elaborate (Leonard, 1991), and can show fixed differences even between closely related species (Adamo & Chase, 1988; Lipton & Murray, 1979). Chemical cues on the other hand may play a role in trail following, which appears to be directed to conspecific mucus trails only (Chase *et al*., 1978; but see Cook, 1977), and they may serve as pheromones (Takeda & Tsuruoka, 1979; Tomiyama, 1994). A study on *Albinaria hippolyti* (Schilthuizen & Lombaerts, 1995) could not demonstrate trail following, but a role of pheromones in the formation of aggregates of conspecific snails could not be excluded. In addition, the differences in chirality between snails could also serve as the substrate for a mate recognition system (Tomiyama, 1996). It has been hypothesized that shell structure, in particular in the cervical area, in *Albinaria* has been subject to sexual selection (Schilthuizen, 2003). Should snails be able to differentiate between conspecifics on the basis of relatively small conchological differences, then a mate recognition system based on direction of coil could certainly evolve. Even without a mate recognition system, some extent of premating reproductive isolation between *S. dextrorsa* and *I. lophauchena* could exist simply by a separation of their reproductive activities in time or space. Thus different but nonetheless

overlapping mating periods were reported for sympatric species of the land snail *Mastus* in Greece (Parmakelis & Mylonas, 2002), and differences in spatial distribution caused by habitat preferences may have contributed to premating reproductive isolation between *Mandarina* species (Chiba, 1996; 2002). Although studies on *Albinaria* show that mating of different species is triggered by similar weather conditions and is consequently often highly synchronous (Giokas & Mylonas, 2002; Schilthuizen, 1995), this does not necessarily have to apply to *S. dextrorsa* and *I. lophauchena*. So far, no data are available on the distribution of individuals or the timing of reproduction of either species. It is clear that future studies are needed on both ecology and mating behaviour of *S. dextrorsa* and *I. lophauchena*, to find out to what extent other mechanisms besides the opposite direction of coiling contribute to the reproductive isolation between these species.

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APPENDIX: SAMPLE INFORMATION

Origin of the sequences:
A: determined by van Moorsel *et al.* (2000)
B: determined by van Moorsel *et al.* (2001d)
C: determind by Hatzoglou *et al.* (1995)
Superscript numbers indicate newly obtained sequences and refer to the relevant chapters in this thesis.
*: Sequence from different specimen than the newly determined sequences.
**: Sequence from another population, not given in the table.

sample information				GenBank accession numbers			
species	voucher	co-ordinates	UTM	ITS1	ITS2	12S	COI
Agathylla lamellosa lamellosa	838	42º39'N 18º05'E	BN62	AY382123 [2]	AY382098 [2]	AY382081 [3]	AY425594 [4]
Albinaria brevicollis brevicollis	697	36°51'N 28°16'E	PA17	-	-	-	AY438419 [6]
Albinaria caerulea caerulea	-	unknown	GH	not subm. [B]	not subm. [B]	X83390 [C]	X83390 [C]
Albinaria discolor discolor	687	36º41'N 23º03'E	FF8362	AF254589 [A]**	AF254589 [A]**	AY382064 [3]	AY425547 [4]
Albinaria discolor discolor	675	36°44'N 22°54'E	FF6967	-	-	-	AY438420 [6]
Albinaria forbesiana bigibbosa	1031	36º39'N 29º06'E	PA85	not subm. [B]	not subm. [B]	AY382065 [3]	AY425548 [4]
Albinaria grisea akrocurta	674	36°53'N 22°48'E	FF5983	-	-	-	AY438421 [6]
Albinaria lycica lycica	1165	36º17'N 29º58'E	QA61	AY382086 [3]	AY382087 [3]	AY382066 [3]	-
Albinaria profuga	702	37º38'N 22º44'E	FG5367	AY382088 [3]	AY382089 [3]	AY382067 [3]	AY425549 [4]
Albinaria puella puella	700	37º52'N 27º16'E	NB29	not subm. [B]	not subm. [B]	AY382068 [3]	AY425550 [4]
Albinaria saxatilis	1164	34º46'N 32º25'E	VD44	AY382090 [3]	AY382091 [3]	AY382069 [3]	-
Albinaria scopulosa epirotes	698	38º59'N 21º23'E	EJ31	AF254588 [A]**	AF254588 [A]**	AY382070 [3]	AY425551 [4]
Albinaria virgo	1285	35º19'N 33º22'E	WE30	AY382092 [3]	AY382093 [3]	AY382071 [3]	-
Carinigera buresi cavallaensis	191	40°56'N 24°24'E	KF8234	-	-	-	AY438377 [5]
Carinigera buresi conciliatrix	343	41°04'N 24°17'E	KF7149	-	-	-	AY438378 [5]
Carinigera buresi conciliatrix	344	41°04'N 24°17'E	KF7149	-	-	-	AY438379 [5]
Carinigera buresi conciliatrix	1126	41°04'N 24°17'E	KF7149	-	-	-	AY438381 [5]
Carinigera buresi conciliatrix	1115	41°02'N 24°20'E	KF74	-	-	-	AY438380 [5]
Carinigera buresi damjanovi	1172	41°31'N 23°48'E	GM2706	-	-	-	AY438382 [5]
Carinigera buresi dramaensis	188	41°13'N 23°54'E	GL4266	-	-	-	AY438383 [5]

sample information				GenBank accession numbers			
species	voucher	co-ordinates	UTM	ITS1	ITS2	12S	COI
Carinigera buresi dramaensis	192	41°13'N 23°59'E	GL4967	-	-	-	AY438384 [5]
Carinigera buresi dramaensis	194	41°13'N 23°54'E	GL4266	-	-	-	AY438385 [5]
Carinigera buresi dramaensis	978	41°13'N 23°59'E	GL4967	-	-	-	AY438386 [5]
Carinigera buresi nordsiecki	330	40°42'N 24°05'E	KF5312	-	-	-	AY438387 [5]
Carinigera buresi nordsiecki	337	40º54'N 24º14'E	KF6731	AY382124 [2]	AY382099 [2]	AY425514 [4]	AY425552 [4]
Carinigera buresi nordsiecki	345	40°42'N 24°05'E	KF5312	-	-	-	AY438388 [5]
Carinigera buresi nordsiecki	934	40°54'N 24°14'E	KF6731	-	-	-	AY438389 [5]
Carinigera buresi polimilites	1138	41°02'N 24°38'E	LF0145	-	-	-	AY438390 [5]
Carinigera buresi polimilites	1140	41°02'N 24°38'E	LF0145	-	-	-	AY438391 [5]
Carinigera drenovoensis	1134	41º25'N 21º54'E	EL78	AY382125 [2]	AY382100 [2]	AY425515 [4]	AY425553 [4]
Carinigera drenovoensis	1163	41°25'N 21°54'E	EL78	-	-	-	AY438392 [5]
Carinigera eximia	1104	43º19'N 22º08'E	EN99	AY382126 [2]	AY382101 [2]	AY425516 [4]	AY425554 [4]
Carinigera eximia	1114	43°19'N 22°08'E	EN99	-	-	-	AY438393 [5]
Carinigera hausknechti alticola	766	38º57'N 21º48'E	EJ6910	AY382127 [2]	AY382102 [2]	AY425517 [4]	AY425555 [4]
Carinigera megdova tavropodensis	786	38º57'N 21º41'E	EJ5910	AY382128 [2]	AY382103 [2]	AY425518 [4]	AY425556 [4]
Carinigera octava	1103	41°42'N 21°48'E	EM61	-	-	-	AY438394 [5]
Carinigera octava	1113	41º42'N 21º48'E	EM61	AY382129 [2]	AY382104 [2]	AY425519 [4]	AY425557 [4]
Carinigera pharsalica	195	39º29'N 22º37'E	FJ3871	AY382130 [2]	AY382105 [2]	AY425520 [4]	AY425558 [4]
Carinigera pharsalica	933	39°29'N 22°37'E	FJ3871	-	-	-	AY438395 [5]
Carinigera pharsalica	1125	39°29'N 22°37'E	FJ3871	-	-	-	AY438396 [5]
Carinigera pharsalica	1136	39°20'N 22°25'E	FJ25	-	-	-	AY438397 [5]
Carinigera pharsalica	1141	39°20'N 22°25'E	FJ25	-	-	-	AY438398 [5]
Carinigera schuetti	1235	41º07'N 23º38'E	GL2554	AY382131 [2]	AY382106 [2]	AY425521 [4]	AY425559 [4]
Carinigera septima	1137	41º24'N 22º15'E	FL08	AY382132 [2]	AY382107 [2]	AY425522 [4]	AY425560 [4]
Carinigera septima	1142	41°24'N 22°15'E	FL08	-	-	-	AY438399 [5]
Carinigera superba	193	41º13'N 23º54'E	GL4266	AY382133 [2]	AY382108 [2]	AY425523 [4]	AY425561 [4]
Cristataria colbeauiana	1176	36º14'N 36º07'E	BA40	AY382134 [2]	AY382109 [2]	AY382072 [3]	AY425562 [4]
Cristataria genezerethana	1284	32º48'N 35º31'E	YB35	AY382135 [2]	AY382110 [2]	AY382073 [3]	AY425563 [4]

sample information				GenBank accession numbers			
species	voucher	co-ordinates	UTM	ITS1	ITS2	12S	COI
Cristataria hedenborgi	1068	34º17'N 35º54'E	YC69	AY382094 [3]	AY382095 [3]	AY382074 [3]	AY425564 [4]
Cristataria turcica	1177	36º07'N 35º56'E	YF60	AY382096 [3]	AY382097 [3]	AY382075 [3]	AY425565 [4]
Herilla bosniensis rex	1286	43º25'N 18º03'E	BP61	AY382136 [2]	AY382111 [2]	-	-
Isabellaria almae	627	38º24'N 22º16'E	FH0949	AY425486 [4]	AY425487 [4]	AY425524 [4]	AY425566 [4]
Isabellaria chelidromia chelidromia	247	39º08'N 23º43'E	GJ3534	AY425488 [4]	AY425489 [4]	AY425525 [4]	AY425567 [4]
Isabellaria clandestina clandestina	261	39º12'N 23º12'E	FJ9042	AY382137 [2]	AY382112 [2]	AY425526 [4]	AY425568 [4]
Isabellaria clandestina subsuturalis	1030	37º53'N 22º29'E	FG3395	AY425490 [4]	AY425491 [4]	AY425527 [4]	AY425569 [4]
Isabellaria isabellina isabellina	616	37º50'N 23º02'E	FG8190	AY382138 [2]	AF254618 [A]*	AY382076 [3]	AY425570 [4]
Isabellaria leucoraphe	238	39º11'N 23º29'E	GJ1440	AY425492 [4]	AY425493 [4]	AY425528 [4]	AY425571 [4]
Isabellaria lophauchena	268	40º32'N 22º05'E	EK9188	AY425494 [4]	AY425495 [4]	AY425529 [4]	AY425572 [4]
Isabellaria lophauchena	779	40°57'N 22°12'E	FL0034	-	-	-	AY438400 [6]
Isabellaria lophauchena	1359	40°57'N 22°12'E	FL0034	-	-	-	AY438401 [6]
Isabellaria lophauchena	1360	40°57'N 22°12'E	FL0034	-	-	-	AY438402 [6]
Isabellaria lophauchena	1361	40°57'N 22°12'E	FL0034	-	-	-	AY438403 [6]
Isabellaria lophauchena	1362	40°57'N 22°12'E	FL0034	-	-	-	AY438404 [6]
Isabellaria lophauchena	782	41°04'N 22°23'E	FL1547	-	-	-	AY438405 [6]
Isabellaria lophauchena	1363	41°03'N 22°05'E	EL9144	-	-	-	AY438406 [6]
Isabellaria lophauchena	776	40°58'N 21°55'E	EL7635	-	-	-	AY438407 [6]
Isabellaria perplana perplana	626	38º29'N 22º31'E	FH3261	AY382139 [2]	AF254614 [A]*	AY425530 [4]	AY425573 [4]
Isabellaria praecipua praecipua	844	40º29'N 22º12'E	FK08	AF254601 [A]*	AF254601 [A]*	AY425531 [4]	AY425574 [4]
Isabellaria praestans	244	39º07'N 23º42'E	GJ3332	AY425496 [4]	AY425497 [4]	AY425532 [4]	AY425575 [4]
Isabellaria riedeli	235	38º48'N 23º28'E	GH1497	AF254619 [A]*	AF254619 [A]*	AY425533 [4]	AY425576 [4]
Isabellaria saxicola aperta	614	37º56'N 23º47'E	GH4504	AF254613 [A]*	AF254613 [A]*	AY425534 [4]	AY425577 [4]
Isabellaria thermopylarum faueri	625	38º32'N 22º23'E	FH2066	AF254620 [A]*	AF254620 [A]*	AY425535 [4]	AY425578 [4]
Isabellaria thessalonica crassilabra	266	40º23'N 23º10'E	FK8371	AY425498 [4]	AY425499 [4]	AY425536 [4]	AY425579 [4]
Isabellaria vallata errata	633	39º07'N 22º03'E	EJ93	AY382140 [2]	AY382113 [2]	AY382077 [3]	AY425580 [4]
Isabellaria vallata vallata	634	39º42'N 20º51'E	DJ89	AF254604 [A]*	AF254604 [A]*	AY425537 [4]	AY425581 [4]
Macedonica pangaionica pang.	341	40º55'N 24º05'E	KF5532	AY382141 [2]	AY382114 [2]	-	-

sample information				GenBank accession numbers			
species	voucher	co-ordinates	UTM	ITS1	ITS2	12S	COI
Medora italiana garganensis	612	41º40'N 15º53'E	WG71	AY382142 [2]	AY382115 [2]	AY382082 [3]	AY425595 [4]
Montenegrina dennisi dennisi	821	40º01'N 21º17'E	EK2429	AY382143 [2]	AY382116 [2]	AY382084 [3]	AY425596 [4]
Muticaria syracusana	1071	37º04'N 15º18'E	WB20	AY382144 [2]	AY382117 [2]	AY382083 [3]	AY425597 [4]
Papillifera papillaris	787	39º10'N 20º59'E	EJ01	AY382145 [2]	AY382118 [2]	-	-
Sericata abyssoclista	619	37º40'N 23º08'E	FG8872	AY425500 [4]	AY425501 [4]	AY425538 [4]	AY425582 [4]
Sericata albicosta	307	40º05'N 22º25'E	FK2038	AY382146 [2]	AY382119 [2]	AY382078 [3]	AY425583 [4]
Sericata albicosta	753	40°09'N 22°24'E	FK1946	-	-	-	AY438417 [6]
Sericata bathyclista	313	38º46'N 23º19'E	GH0193	AY425502 [4]	AY425503 [4]	AY425539 [4]	AY425584 [4]
Sericata dextrorsa	760	40º58'N 21º55'E	EL7635	AY425504 [4]	AY425505 [4]	AY425540 [4]	AY425585 [4]
Sericata dextrorsa	761	40°57'N 22°12'E	FL0034	-	-	-	AY438408 [6]
Sericata dextrorsa	1355	40°57'N 22°12'E	FL0034	-	-	-	AY438409 [6]
Sericata dextrorsa	1356	40°57'N 22°12'E	FL0034	-	-	-	AY438410 [6]
Sericata dextrorsa	1357	40°57'N 22°12'E	FL0034	-	-	-	AY438411 [6]
Sericata dextrorsa	1358	40°57'N 22°12'E	FL0034	-	-	-	AY438412 [6]
Sericata dextrorsa	762	41°04'N 22°23'E	FL1547	-	-	-	AY438413 [6]
Sericata dextrorsa	756	41°03'N 22°05'E	EL9144	-	-	-	AY438414 [6]
Sericata dextrorsa	296	40°29'N 22°08'E	EK9582	-	-	-	AY438415 [6]
Sericata dextrorsa	755	40°09'N 22°24'E	FK1946	-	-	-	AY438416 [6]
Sericata inchoata inchoata	768	39º09'N 20º41'E	DJ7233	AY382147 [2]	AY382120 [2]	AY382079 [3]	AY425586 [4]
Sericata liebegottae	298	39º07'N 23º59'E	GJ5834	AY425506 [4]	AY425507 [4]	AY425541 [4]	AY425587 [4]
Sericata lutracana	615	38º02'N 22º51'E	FH6311	AF254609 [A]*	AF254609 [A]*	AY425542 [4]	AY425588 [4]
Sericata regina	788	39º17'N 20º51'E	DJ8647	AY382148 [2]	AY382121 [2]	AY425543 [4]	AY425589 [4]
Sericata sericata sericata	293	38º35'N 23º50'E	GH4774	AY382149 [2]	AF254612 [A]*	AY382080 [3]	AY425590 [4]
Sericata stussineri stussineri	302	39º53'N 22º38'E	FK3916	AY425508 [4]	AY425509 [4]	AY425544 [4]	AY425591 [4]
Sericata tantilla	300	39º42'N 22º14'E	FJ0596	AY425510 [4]	AY425511 [4]	AY425545 [4]	AY425592 [4]
Sericata torifera	304	39º41'N 21º41'E	EJ5891	AY425512 [4]	AY425513 [4]	AY425546 [4]	AY425593 [4]
Sericata torifera	813	39°41'N 21°35'E	EJ5092	-	-	-	AY438418 [6]
Strigilodelima conspersa	825	39º40'N 20º57'E	DJ9591	AY382150 [2]	AY382122 [2]	AY382085 [3]	AY425598 [4]

NAWOORD

Het onderzoek naar de fylogenie van Griekse landslakken omvat veel facetten. Enerzijds maakt dit het onderwerp erg boeiend en afwisselend, anderzijds blijft het een uitdaging je in de relatief korte tijd van een promotie-onderzoek in zeer uiteenlopende vakgebieden te verdiepen. Gedurende de verschillende fasen van het onderzoek hebben gelukkig velen mij met raad en daad bijgestaan. In de eerste plaats wil ik hier Coline van Moorsel, Hendrik-Jan Megens, Dirk Gassmann, Dick Groenenberg en Arjan Gittenberger noemen. Zij hebben mijn jaren als promovendus extra inhoud gegeven, zowel op het wetenschappelijke als op het persoonlijke vlak. Dick Groenenberg heeft daarnaast, toen het einde van mijn aanstelling in zicht kwam en een aantal interessante nieuwe vragen onbeantwoord dreigde te blijven, aanvullend laboratoriumwerk uitgevoerd, waardoor ik me op de analyses en het schrijfwerk kon richten.

Het veldwerk in Griekenland heb ik ervaren als een van de leukste en spannendste onderdelen van het onderzoek, mede dankzij de informatie die ik vooraf heb kunnen vergaren. Henk Menkhorst vertrouwde mij zijn collectie Griekse Clausiliidae toe ter determinatie, wat vele nieuwe gegevens opleverde. Willem Renema vertaalde de geologische kaart van Griekenland in voor biologen begrijpelijke termen (kalk- versus niet-kalkgebieden), waardoor een gerichte bemonstering van de Clausiliidae van Noord- en Midden-Griekenland mogelijk was. Robert Keizer, J. Heller, Wim Maassen, H.-J. Niederhöfer, H. Nordsieck en Thomas Reydon zorgden voor aanvullende monsters.

Met veel plezier kijk ik ook terug op de periode in het moleculaire lab. De omgang met de andere labgebruikers heb ik altijd als prettig en stimulerend ervaren. Zij hebben mij geholpen wegwijs te worden in het lab en zijn protocollen. René Glas en Marcel Eurlings vormden een constante basis in de voortdurend wisselende bezetting van het lab. Zij zijn in belangrijke mate bepalend geweest voor de goede werksfeer, en zorgden er voor dat alle voorwaarden voor het onbekommerd verrichten van moleculair onderzoek aanwezig waren. Met veel plezier heb ik in deze periode ook samengewerkt met Dennis Schneider. Zijn afstudeeronderzoek aan het tot dan toe genegeerde genus *Carinigera* heeft uiteindelijk een nieuwe richting gegeven aan dit proefschrift.

Tijdens de analyse- en de schrijffase kon ik met kleine en grotere vragen bij velen terecht, waarvan ik er hier slechts enkelen noem. Henk den Bakker attendeerde mij op de nieuwste versie van MrBayes en hielp mij het programma te doorgronden. Elizabeth van Ast-Gray hielp mij met het Engels en corrigeerde een deel van de tekst van het proefschrift. Adri ’t Hooft maakte vrijwel alle foto’s die het proefschrift sieren.

Mijn familie en vrienden toonden begrip voor de soms spaarzame en korte contacten die ik de afgelopen jaren met hen onderhield, en doorstonden bij die gelegenheden ook nog eens allerlei verhalen over slakken en DNA-analyse. Wolter, wat dat laatste betreft heb jij het zondermeer het zwaarst gehad. Ook jij hebt mijn vreemde “hobby” altijd gerespecteerd, ook al moest je daarvoor offers maken. De weekenden met jou waren de pijlers onder alle dagen die ik telkens van huis was.

CURRICULUM VITAE

Dennis René Uit de Weerd werd geboren op 31 oktober 1971 te Apeldoorn. In 1990 behaalde hij het Gymnasium β diploma aan het Veluws College te Apeldoorn, waarna werd begonnen met de opleiding biologie aan de Universiteit Utrecht. Het propedeuse diploma behaalde hij cum laude in 1991. Tijdens zijn verdere studie heeft hij zich laten leiden door zijn interesse in biologische evolutie. Zo specialiseerde hij zich in de richtingen evolutiebiologie, ecologie en palaeontologie. Naast het verplichte programma werden daarbij cursussen aan de Rijksuniversiteit Groningen en de Universiteit van Amsterdam gevolgd. Een eerste doctoraal onderzoek werd uitgevoerd aan het Instituut voor vakgroep Systematiek, Evolutie en Paleobiologie van Universiteit van Amsterdam en aan de vakgroep Biochemie van de Universiteit Nijmegen. Onder leiding van dr. G-J. Caspers, en onder supervisie van prof. dr. W. W. de Jong en dr. J. Wattel richtte hij zich op het ontrafelen van basale verwantschappen binnen de vogels op basis van αA-crystalline cDNA. Een tweede doctoraal onderwerp betrof onderzoek bij de werkgroep Populatiebiologie van Dieren (prof. dr. A. .J. van Noordwijk) van het Centrum voor Terrestrische Oecologie van het Nederlands Instituut voor Oecologisch Onderzoek te Heteren, onder leiding van dr. C. Both. Aan de hand van observaties in het veld, onderzocht hij de variatie in verenkleed tussen mannelijke koolmezen in relatie tot hun fitness. Na de opleiding Natuur- en Milieu-educatie aan de Universiteit Utrecht, werd op 26 augustus 1996 het doctoraal diploma aan deze universiteit behaald. Gedurende 1997 volgde hij de postdoctorale lerarenopleiding biologie van het IVLOS, Universiteit Utrecht. In november 1998 volgde een aanstelling als Assistent in Opleiding bij de sectie Theoretische Biologie en Fylogenetische Systematiek van het Instituut voor Evolutionaire en Ecologische Wetenschappen, Universiteit Leiden. Dit proefschrift is de weerslag van het onderzoek dat aldaar en in het Nationaal Natuurhistorisch Museum Naturalis werd uitgevoerd. In het kader van dit onderzoek heeft hij veldwerk verricht in Griekenland. De resultaten van het onderzoek zijn gepresenteerd op diverse (internationale) congressen. Daarnaast werd een deel van het onderwijs van de tweedejaars cursus Biodiversiteit en Patroonanalyse verzorgd. Vanaf december 2002 tot augustus 2003 was hij gastmedewerker aan het Instituut Biologie Leiden van de Universiteit Leiden. Sinds 1 augustus 2003 is hij binnen dit instituut aangesteld als docent en onderzoeker.