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Phylogenetic Relationships Inferred from the Sequence and Secondary Structure of ITS1 rRNA in *Albinaria* and Putative *Isabellaria* Species (Gastropoda, Pulmonata, Clausiliidae)[1]

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An analysis of the ITS1 sequence variation among five species of terrestrial pulmonate snails was performed to decide between two conflicting hypotheses concerning the phylogeny of these anatomically similar gastropods. It turned out that the so-called genus *Isabellaria* is a polyphyletic entity; the diagnostic, apomorphic structure of its clausilial apparatus, enabling a nearly complete obstruction of the shell aperture with the animal at rest, apparently evolved more than once from ancestors currently classified with the speciose genus *Albinaria*. The classification based on general shell shape and sculpture, and distributional patterns, turns out to be the natural one. This study also provides the first data on ITS1 sequences in gastropods. The recently published ITS1 sequence of another molluscan species, a bivalve, is quite different but similar in length to that of the snails. © 1995 Academic Press, Inc.

INTRODUCTION

The ribosomal DNA (rDNA) array of eukaryotes is a region with many properties of high value for the molecular systematist (Hillis and Moritz, 1990). First of all, it is well-known, with partial or complete sequences available for a wide variety of organisms. Second, it is usually present in a large number of (typically identical) copies, which facilitates its amplification. Its most valuable characteristic, however, is the fact that it contains both highly conserved and highly variable regions. In the past, systematic studies have focused on the more conserved regions, because these offer possibilities for resolving phylogenetic relationships among higher taxonomic categories. In recent years, however, the more variable regions, such as the internal transcribed spacers (ITS1 and ITS2), have gained attention from systematists working at lower taxonomic levels. A number of studies on various groups of organisms have shown the possibility of reconstructing phylogenies of closely related species on the basis of ITS sequence comparisons (e.g., Wesson *et al.*, 1992; Lee and Taylor, 1992; Saunders and Druehl, 1993; Smith and Klein, 1994). In molluscs, only a single ITS sequence has been published so far, *i.e.*, ITS1 for a species of bivalve, *Saccostrea glomerata* (Anderson and Adlard, 1994).

In the present paper, we investigate the possibilities of using ITS1 sequence variation for phylogenetic purposes in the pulmonate genus *Albinaria* (Clausiliidae). This genus forms an extremely speciose group of rock-dwelling land snails from Greece and surrounding regions. Its evolutionary history has been the focus of research for a decade now (see: Kemperman, 1992; Schilthuizen, 1994), but attempts to reconstruct the phylogeny of even a few species have invariably run into problems due to morphological character incompatibility (van Moorsel, unpublished MSc thesis) and uncertainties in homologizing allozyme variants (Schilthuizen and Gittenberger, in press).

As a pilot study, we have chosen to study ITS1 sequence variation in four Greek species, endemic to the Peloponnese. Two of these have traditionally been placed in a supposedly closely related genus *Isabellaria,* because of their apomorphic ("G-type": Nordsieck, 1978: 72) clausilial apparatus (a complicated system of lamellae, one of which developed into the clausilium, a door-like structure in the shell aperture of most species of the Clausiliidae). These two *Isabellaria* species, viz. *I. adriani* and *I. haessleini,* however, closely resemble the geographically neighboring *Albinaria hohorsti* and

[1] Sequence data from this article have been deposited with GenBank under Accession Nos. U19818–U19821.

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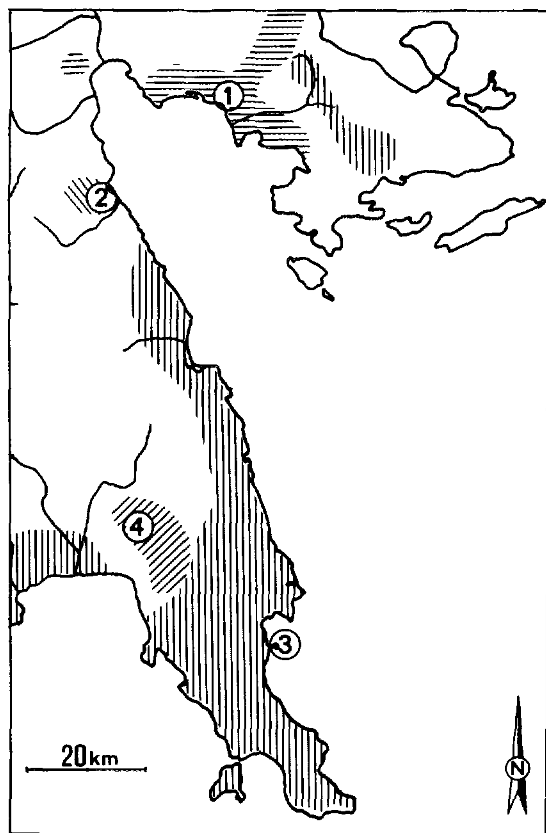

FIG. 1. The location of the collection sites in SE. Peloponnese, Greece, with different hatching indicating the ranges of the species (after samples in the collection of the Nationaal Natuurhistorisch Museum, Leiden, the Netherlands). 1, *Albinaria hohorsti* (disjunct); 2, *Isabellaria adriani;* 3, *Albinaria discolor* (disjunct); 4, *Isabellaria haessleini.*

A. discolor, respectively, in external shell traits. Clearly, the incongruence of these morphological characters points to convergence, either in the clausilial apparatus or in the external shell features (Gittenberger and Schilthuizen, in press). The present DNA analysis has been performed to investigate which set of characters is the phylogenetically informative one.

MATERIALS AND METHODS

Snail Specimens

Snails of the four ingroup species were collected by the second author (April 1994) at the following localities in Greece, Peloponnese: ancient Monemvasia (*A. discolor*), 1 km E. of Kandia (*A. hohorsti*), 3 km SE. of Astros (*I. adriani*), and 2 km SW. of Apidea (*I. haessleini*) (Fig. 1). Only *A. discolor* is widely distributed in the E. Peloponnese. A probably more distantly related species of the *Isabellaria* type from Macedonia, *I. praecipua,* was chosen as outgroup. A sample of this species was collected by the first two authors and C. van Moorsel near the village of Sérvia (April 1993). All samples were transported alive in plastic bags with tissue paper and frozen at −80°C upon arrival in the laboratory, until processing for DNA extraction.

Sample Preparation

Genomic DNA was isolated using a CTAB protocol based on Doyle and Doyle (1987). It involves digestion of pulverized tissue with CTAB and proteinase K, a phenol/chloroform extraction, treatment with RNase, and a second phenol/chloroform extraction. As recommended by Winnepenninckx *et al.* (1993), snails were dissected to produce separate organs. We did DNA extractions from foot, digestive gland, and genital tissue from single snails, but found that only foot tissue produced good quality, high-molecular-weight DNA. All sequences described in this paper, therefore, have been derived from DNA extracted from this type of tissue.

The target DNA was amplified by PCR, with primers from the conserved 18S and 5.8S regions, to produce fragments including the entire ITS1. The primers used were universal primers 5.8c ("Silkworm") and 18d ("Fruitfly") from Hillis and Dixon (1991). Amplification was performed with SuperTaq polymerase in 25µl total reaction volume (using, apart from standard reaction conditions, a final Mg^{2+} concentration of 3 mM). As target DNA, 5–10% of the amount extracted from a single foot was needed. Samples were amplified for 25 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1.5 min). PCR products were electrophoresed on a low-melting-point agarose gel containing ethidium bromide to verify band size and subsequently excised.

Direct sequencing was done by the dideoxy termination method (Sanger *et al.*, 1977; Lichtenauer-Kaligis *et al.*, 1993) using ^{35}S-radioactive labeling and manual gel reading. Partial sequences were confirmed by Fluore–dATP labeling and automated gel reading in a Pharmacia Automated Laser Fluorescent (A.L.F.) sequencer. The sample preparation for this latter approach involved PCR with a biotinylated 5.8S primer and the subsequent production of single-stranded DNA using Dynabead purification.

Sequence Analysis

Sequences were aligned manually. The positions of the 18S/ITS1 and ITS1/5.8S boundaries were determined by a comparison with the *Saccostrea* (see above) sequence. The 3′ end of the 18S ribosomal gene is well conserved and thus easily recognizable in the five gastropod species and quite different organisms, like *Saccostrea* (oyster; Anderson and Adlard, 1994), *Picea* (spruce; Smith and Klein, 1994), mouse, rat, *Xenopus,* and yeast (Goldman *et al.*, 1983). the 5′ end of the 5.8S ribosomal gene is less easy to identify because of a greater variation among various organisms. We aligned the sequence **TG**A**CA.A**CTCT.**AA**GT**G.GTGG** **A.T**C**A**CT.CGGC**T** in *Saccostrea* with **TG**G**CA.A.A**ACT

G.**AATCG.GTGGA.TTA**TA.ACTTT in the snails, noticing the similarity of especially the former one with the mouse sequence (bold type, underlined) **CG.ACTCT**.T **AGCG.GTGGA.TCACT.CGGCT**, apart from some missing nucleotides at the 5′ end. This fits in well with the local sequence variation in the 18S and 5.8S genes, as reported by Hillis and Dixon (1991).

Variable positions were identified and each (including deletions, which were coded as "fifth base") was used as a phylogenetic character in an exhaustive search for the optimal tree, using the computer program PAUP 3.1 (Swofford, 1993). We also included noninformative characters (such as autapomorphies) to obtain an impression of the amount of evolutionary change along each of the lineages in the phylogram.

Secondary structure analysis was performed with the STAR program (Abrahams *et al.,* 1990), using the "genetic algorithm" (van Batenburg *et al.,* 1995). The foldings yielded by the algorithm allowed speculations on alternative structures.

RESULTS AND DISCUSSION

Sequences were obtained from single individuals of each of the five species (Fig. 2). Due to incidental gel irregularities, a short (trinucleotide) stretch of the *A. hohorsti* and *A. discolor* sequences (269–271) and a longer (approximately 96 bp) stretch of the *I. praecipua* sequence (c. 284–379, corresponding with c. 260–355 of the ingroup species) could not be read reliably. Two partial sequences (approximately 200 bp from the 5′ end for *I. adriani* and *I. praecipua*) analyzed with the A.L.F. were identical to the manually derived ones. The flanking 18S and 5.8S regions were fully conserved across all five sequences. The ITS1, however, showed considerable variation across the five species studied, with substitutions, insertions, and deletions present. The outgroup was characterized by the presence of a large insertion of 22 bp. Among all variable positions, 32 were informative for resolving phylogenetic relations within the ingroup. The remaining variability concerned autapomorphies and total–ingroup synapomorphies. The length of ITS1 varied from 466 in *I. adriani* and *A. hohorsti* to approximately 486 in *I. praecipua.* In *Saccostrea* 438 bp was found (Anderson and Adlard, 1994). It was relatively G + C-rich: 59% in *I. haessleini,* 61% in *I. adriani,* the only two species for which complete sequences were available.

According to Goldman *et al.* (1983), "Two trends are apparent in the evolution of eucaryotic transcribed spacer regions" (p. 1498), which show an "increase in size and G + C content during evolution" (p. 1488). Regarding the former value, the following series emerges (data after Goldman *et al.,* 1983, unless indicated otherwise): yeast (363 bp), mosquito (419–426 bp) (Wesson *et al.,* 1992), oyster (438 bp) (Anderson and Adlard, 1994), snails (466–486 bp) (this paper), *Xenopus* (561 bp), mouse (998 bp), *Homo sapiens* (1067–1095 bp) (Gonzalez *et al.,* 1990). The latter value dictates (after the same authors): yeast (35.2%), mosquito (58%), snails (59–61%), mouse (70.1%), *Homo sapiens* (79–83%), *Xenopus* (83.9%). At both ends of the ITS1 5-nucleotide repeats are seen, i.e., CGCCC (8–12, 16–20) and GGCTC (436–440, 443–447), similar to what was found in other ITS1 RNAs (Goldman *et al.,* 1983; Wesson *et al.,* 1992). The comparison of the snail sequences with that of *Saccostrea,* thus comparing the two molluscan sequences, did not reveal apparent homologies, whereas their length is of the same order of magnitude.

Because of the incompleteness of some sequences, the secondary structure calculations for full-length ITS1 were carried out on *I. adriani* and *I. haessleini* sequences only. Mostly the predictions for these two RNAs were similar, deviating mainly in long-range pairings. The structure predicted for *I. adriani* is shown in Fig. 3. There are reasons to suggest that this prediction is very close to the actual folding.

First of all, the nucleotides in single-stranded regions were much more variable than the ones in stem regions. Among 272 nucleotides predicted to be paired in *I. adriani* ITS1, 28 positions are variable among the five sequences (10.3%). The variability in the loops is much higher, *i.e.,* 46 of 194 bases (23.7%). This suggests that at least some parts of the predicted secondary structure have some functional importance because they are conserved.

Furthermore, a lot of changes in the predicted stems were silent in terms of RNA structure, *i.e.,* not disturbing the pairing in question, or shortening a stem at the end by one base-pair.

The free energies of the predicted ITS1 structures for *I. adriani* and *I. haessleini* are about −130 kcal/mol (energies according to Jaeger *et al.,* 1989), which is very close to the value (−132.6 kcal/mol) reported for the mosquito ITS1, which is of comparable length (Wesson *et al.,* 1992). These values are also much lower than the mean values for energy minimum states of random sequences of comparable lengths (Fontana *et al.,* 1993).

The analysis of the other sequences shows that the structure of the *A. hohorsti* ITS1 could be very close to that of *I. adriani,* whereas the ITS1 folding of *A. discolor* could be similar to that of *I. haessleini.* Although about 100 nucleotides could not be sequenced in *I. praecipua,* the remaining part of the ITS1 clearly shows that this sequence may well serve as an outgroup. The large insertion of 22 nucleotides in *I. praecipua* may be folded separately in a relatively stable hairpin of 7 base-pairs (see the legend to Fig. 3), whereas its insertion point is located in a loop region of the structure that was calculated for the ingroup. Therefore, such an insertion would not distort the proposed folding.

In some ITS1 regions, alternative structures could be proposed. For example, in the region corresponding to the positions 25–83 in the *I. adriani* sequence, the clos-

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Isabellaria adriani         AAGAAGCTCG AACTCGATCG CTTGGAGAAA GTAAAAGTCG TAACAAGGTT  -30
Albinaria hohorsti          .......... .......... .......... .......... ..........  -30
Isabellaria haessleini      .......... .......... .......... .......... ..........  -30
Albinaria discolor          .......... .......... .......... .......... ..........  -30
Isabellaria praecipua       .......... .......... .......... .......... ..........  -30

                                              18S ][ ITS-1
Isabellaria adriani         TCCGTAGTGA ACCTGCGGAA GGATCATTAT CGGATTCGCC CACGCGCCCC   21
Albinaria hohorsti          .......... .......... .......... .......... ..........   21
Isabellaria haessleini      .......... .......... .......... .......... ..........   21
Albinaria discolor          .......... .......... .......... .......... ..........   21
Isabellaria praecipua       .......... .......... .......... .......... .........-   20

Isabellaria adriani         GAGCCGGCAC TGGCAAGAGG CAACGTAGGT TGAAGCTGAT GTCACGTCGT   71
Albinaria hohorsti          ......AG.. .C........ ..-G...... .......... ..........   70
Isabellaria haessleini      ......AG.. .C........ ..-G...... .......... ........AA   70
Albinaria discolor          ......AG.. .C........ ..-G...... .......... ........AA   70
Isabellaria praecipua       A.....TG.. ......A... ..-G...... .......... .AA.....AA   69

Isabellaria adriani         CATTCGTGCC GGACGGGGAC CGCATGAAGC GCCGCCCCGG CGGTTGAAAC  121
Albinaria hohorsti          .....T.... .......... .......... .......... ..........  120
Isabellaria haessleini      .TG.--.... .......... .......... .......... ..........  118
Albinaria discolor          .TG.--.... .......... .......... .......... ..........  118
Isabellaria praecipua       T--....... .......... ....A..... .......... ......GCG.  117

Isabellaria adriani         GTCCCCTTTT TC-GGGGTAC CTAGCTGGGC ----ACGACG ACCCACGGTG  166
Albinaria hohorsti          .......... ..G....... .......... .......... ..........  166
Isabellaria haessleini      .......... .......... ........A. .TTT...G.. ..........  166
Albinaria discolor          .......... .......... ........A. .TTT...G.. ..........  166
Isabellaria praecipua       T......... .......... ...C...CT. GATT.....C ....G.....  166

Isabellaria adriani         ACGGCTTGAG CAT-CTGGTG CTCGCCGGGT CGTC-GGTTC AAAGAGCGCT  214
Albinaria hohorsti          .......... ...T...T.. .......... .......... ..........  215
Isabellaria haessleini      .......... ..C.T..... .......... .......... ..........  214
Albinaria discolor          .......... ..C.T..... .......... ....A..... ..........  215
Isabellaria praecipua       .......... .......... .......... ....A..... ..........  215

Isabellaria adriani         GCAGTGACTG CTCGGCGTAG AATG------ ---------- ------AGCG  242
Albinaria hohorsti          .......T.. ........G. C......... .......... ..........  243
Isabellaria haessleini      .......T.. .......... .C........ .......... ..........  242
Albinaria discolor          .......T.. .......... .C........ .......... ..........  243
Isabellaria praecipua       ..C....T.. ......CC.. GC..GCGCTC ACTTTGAGTG AGCATG....  265

Isabellaria adriani         GTGCCGCCC- GGTAGGTTTA GAAGATCGGT GAGGTACCTA TGCATCCTGT  291
Albinaria hohorsti          .........C ...-..CC-. .......NNN .......... .........A  291
Isabellaria haessleini      .......... ........C. .........C .......... .........A  291
Albinaria discolor          .......... ........C. .......NNN .......... .........A  292
Isabellaria praecipua       .......... ........NN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN  315

Isabellaria adriani         CATGGGTGCG GCAGCTCCTT CTCAAACAAG GCGCGAGGCT TAAAGAGGTC  341
Albinaria hohorsti          .......... .T........ .......... .......... ..........  341
Isabellaria haessleini      T......... .......... .....G.... .......... ..........  341
Albinaria discolor          T......... .......... .....G.... .......... ..........  342
Isabellaria praecipua       NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN  365

Isabellaria adriani         GGCAAGCTCT CGGCAGACCC GCCCTCGGTC TTCTCTTGTC CATTTTTTTT  391
Albinaria hohorsti          .......... .......... .......... .......... ..AAC....C  391
Isabellaria haessleini      ...G...... .......... .......... ......C... ........-C  390
Albinaria discolor          ...G.....- .......... .......... ......C... .........C  391
Isabellaria praecipua       NNNNNNNNNN NNNN...... .......... .......... ..........  415

Isabellaria adriani         TTTTAAGACC GCTAATAAAC TTTTATTGTG GCT---T-GT GCAGGAGTGG  437
Albinaria hohorsti          .......... .......... .......... ..C....... ..........  437
Isabellaria haessleini      .....C.... .......... -........T ...TGT.T.. ........-.  438
Albinaria discolor          .....C.... ......G... -......... ...TGT.... ..........  439
Isabellaria praecipua       .....C.... ...T...... -A........ ...T..-... ........-.  459

Isabellaria adriani         CTCCTGGCTC GAGCGTCCC- GGCACTGAAA TGGCAAACTG AATCGGTGGA  466+20
Albinaria hohorsti          .......... .......... .......... .......... ..........  466+20
Isabellaria haessleini      .......... .........C ........T. .......... ..........  468+20
Albinaria discolor          .......... .......... ........C. .......... ..........  468+20
Isabellaria praecipua       .........T ..-......C .-...-.... .......... ..........  486+20
                                                     ITS1 ] [ 5.8S

Isabellaria adriani         TTATAACTTT GAGCGGT  466+37
Albinaria hohorsti          .......... .......  466+37
Isabellaria haessleini      .......... .......  468+37
Albinaria discolor          .......... .......  468+37
Isabellaria praecipua       .......... .......  486+37
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FIG. 2. Alignment of ribosomal DNA nucleotide sequences of the ITS1 and flanking portions of 18S and 5.8S regions for the five species that were studied. Indeterminable bases have been indicated with N. [Sequence data deposited with GenBank under Accession Nos. U19798 (*Isabellaria praecipua*), U19818 (*I. adriani*), U19819 (*I. haessleini*), U19820 (*Albinaria discolor*), and U19821 (*A. hohorsti*)].

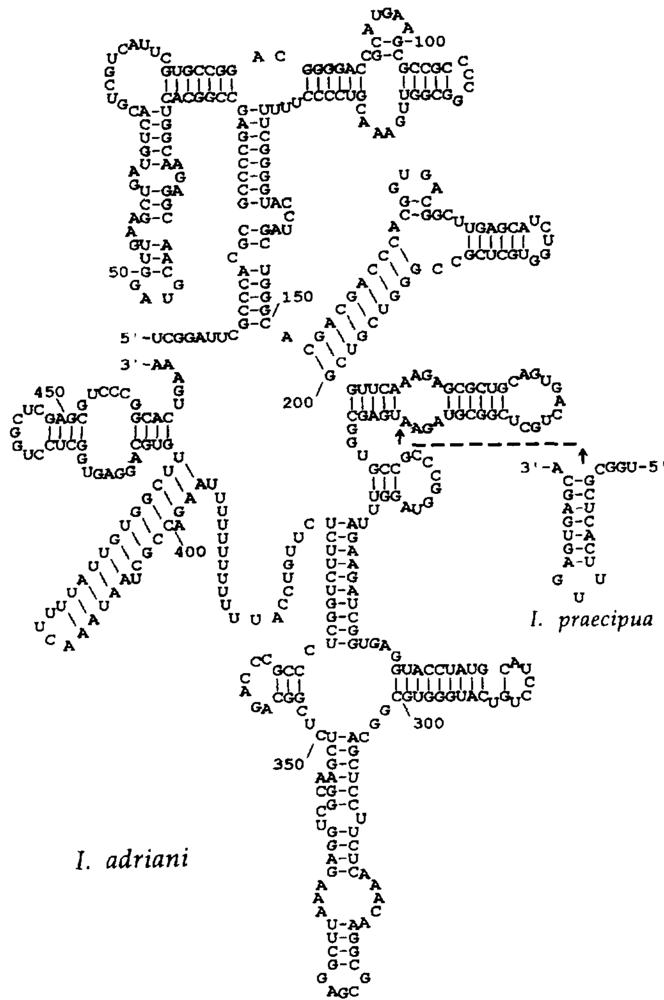

FIG. 3. The predicted structure for ITS1 of *Isabellaria adriani*. The site of the 22nt insertion in *Isabellaria praecipua* and the possible folding of the inserted nucleotides is also shown.

ing stem is folded in all four RNAs of the ingroup species (Fig. 4). However, internal stems may be folded in different ways. It is impossible to be certain about the exact actual folding in the absence of additional experimental or phylogenetic evidence.

Finally, the phylogenetic analysis, based on the primary RNA structure, resulted in a single optimal tree, with a rescaled consistency index (Farris, 1989) of 0.77 (Fig. 5). It shows the *Isabellaria* species as a polyphyletic group, which lends support to the hypothesis of Gittenberger and Schilthuizen (in press) that the G-type clausilial apparatus has frequently developed in parallel, and that external shell features are in fact better indicators of relatedness than the (traditionally highly valued) clausilial structures. The relatively clear phylogenetic picture emerging from this four-species sequence comparison, even with some parts of the sequence missing, offers good perspectives for using ITS1 sequence variation for resolving cladistic relationships among larger numbers of species from this and related genera. It should be stressed, however, that the present data were derived from single individuals representing each species. Both intrapopulation and intraspecific variation in rDNA sequence are known to be reduced because of concerted evolution (Hillis and Dixon, 1991). Nevertheless, further work should be carried out to reveal whether intraspecific variation is sufficiently limited to justify such a small sample size. Given the lack of reliable classifications in clausiliid snails, additional outgroups should be selected as well, although it is unlikely that this would overturn the conclusions in this particular case.

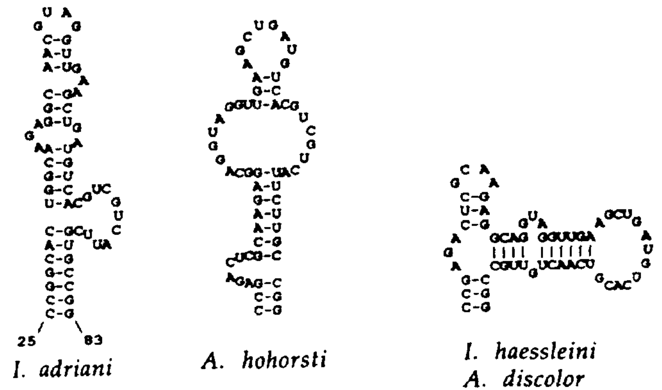

FIG. 4. Alternative foldings in the ITS1 region corresponding to the positions 25–83 in the *Isabellaria adriani* sequence.

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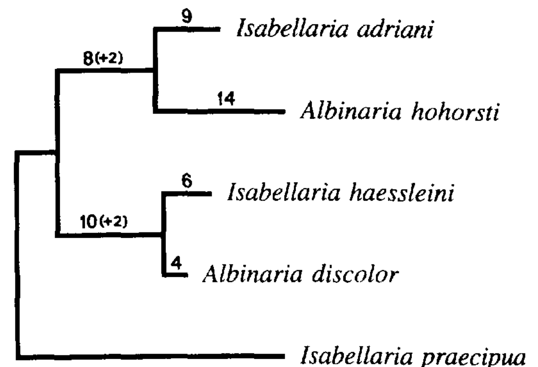

FIG. 5. Optimal tree for the five species studied. The numbers indicate the number of specific changes. Because the outgroup sequence is incompletely known, the status of two changes remains unclear.

lusc DNA extraction. Jenny Peters-van Rijn assisted in the lab and Dr. E. van Batenburg took part in our discussions on RNA secondary structure. Finally we thank two reviewers for critically commenting on the text.

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