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Evolution and Alignment of the Hypervariable Arm 1 of *Aeschynanthus* (Gesneriaceae) ITS2 Nuclear Ribosomal DNA

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Comparative ITS2 sequencing in the plant genus *Aeschynanthus* (Gesneriaceae) reveals an insertion/deletion (indel) hot spot in the ITS2 sequences that is difficult to align. Examination of other Gesneriaceae sequences shows that this is a widespread phenomenon in this plant family. Minimum free-energy secondary structure analyses localize the hot spot to the terminal part of arm 1. Arm 1 is twice as long in Gesneriaceae than in other asterids. In addition, the pattern of indels is consistent with this secondary structure model. The high variability of the extended terminal part of arm 1 in Gesneriaceae and the fact that it can be deleted altogether imply that it is functionally superfluous. In contrast, the base of arm 1 is relatively conserved and may function as an exonuclease recognition site. This study illustrates how comparative secondary structure analyses can be helpful in fine-scale alignment. Alignment based on secondary structure conflicts with our initial manual alignment and, to a lesser extent, with a CLUSTAL X alignment with default parameters. © 2001 Academic Press

***Key Words:* *Aeschynanthus;* alignment; Gesneriaceae; internal transcribed spacer; nuclear ribosomal DNA; secondary structure.**

INTRODUCTION

Internal transcribed spacers 1 and 2 (ITS1 and ITS2) of the nuclear ribosomal DNA (nrDNA) have become important nuclear regions for molecular systematic studies of flowering plants (reviewed by Baldwin *et al.*, 1995). These regions have fast rates of substitution, which are useful for evaluating relationships at generic and species levels. Sequences of the spacers can be obtained easily via polymerase chain reaction (PCR) amplification from conserved flanking regions in ribosomal genes. Furthermore, length conservation of ITS sequences greatly helps their sequence alignment and phylogenetic analysis (Baldwin, 1992).

Recent studies of ribosomal RNA (rRNA) processing in *Saccharomyces cerevisiae* (Lafontaine and Tollervey, 1995; Venema and Tollervey, 1995) show that rDNA transcription first produces a pre-rRNA which contains all three rRNAs found in the ribosome, i.e., 18S small subunit (SSU) rRNA, 25S large subunit (LSU) rRNA, and 5.8S rRNA. Cleavages of the pre-rRNA occur initially at the 5′ external transcribed spacer (ETS), followed by the 5′ end of the 18S rRNA and in the ITS1 to generate a mature SSU rRNA. Finally, cleavage occurs in the ITS2 and the 3′ ETS to generate mature 5.8S and LSU rRNAs. The pre-rRNA processing of plants appears to follow this pathway, but their rRNA processing sites have not yet been fully analyzed (Brown and Shaw, 1998).

The ITS2 region has been shown to be necessary for rRNA processing (van der Sande *et al.*, 1992; Hadjiolova *et al.*, 1994; van Nues *et al.*, 1995). It participates in guiding endonucleases to the correct rRNA processing sites. To assist the study of its function, secondary structure folding patterns of the ITS2 sequence have been examined in many organisms. To date, only yeast ITS2 structure has been unambiguously resolved, by a combination of minimum free-energy calculations, chemical and enzymatic probing, and mutation and insertion/deletion (indel) experiments (Yeh and Lee, 1990; van der Sande *et al.*, 1992; van Nues *et al.*, 1995). The yeast cross-like structure contains two long central stems and three short branches. The structure common to other organisms, i.e., *Drosophila* (Schlötterer *et al.*, 1994), trematodes (Michot *et al.*, 1993), monogeneans (Morgan and Blair, 1998), green algae (Mai and Coleman, 1997), and flowering plants (Hershkovitz and Zimmer, 1996; Mai and Coleman, 1997), appears to be based on a four-arm model, in which the third arm (sometimes called the central stem) is longest. The only significant difference between yeast ITS2 structure and those of other organisms is in this long central stem, which is branched in yeast with a conserved pairing region between the first and the second arms (branches) of the yeast structure. However, there are no clearly conserved ITS2 sequence motifs shared between yeast and either green algae or flowering plants (Hershkovitz and Lewis, 1996). It should also be noted

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that the 3′ sequence of ITS2 is conserved in yeast, whereas it is highly variable in angiosperms.

In the case of angiosperms, the ITS2 sequences have been found to be moderately conserved. Hershkovitz and Zimmer (1996) studied 75 angiosperm species and found that the region from the base (3′ side) of arm 1 to the loop end (5′ side) of arm 3, ca. 40% of the total ITS2 sequence of these species, was fairly well alignable except for two small variable regions. Among eudicots, about 47% of the total ITS2 sequence was found to be conserved. Most of the conserved regions are inherently GC rich, but a conserved region corresponding to the single-stranded core of the putative secondary structure is purine rich (Mai and Coleman, 1997). Moreover, secondary structure comparison of green algal, moss, and angiosperm ITS2 sequences reveals another two highly conserved characteristics, i.e., a pyrimidine mismatch loophole in arm 2 and a GGU triplet (UGGU in angiosperms) on the 5′ end of arm 3 (Liu and Schardl, 1994; Mai and Coleman, 1997).

Arm 1 has been found to be highly variable between taxa in both length and sequence. In angiosperms this arm shows a C- to G-rich transition from 5′ to 3′ and high length variability of ca. 26–54 bp (Hershkovitz and Zimmer, 1996). Even at the species level, sister taxa may show sequence and length variation in the arm 1 region. In two previous studies, 50 aligned base positions of the arm 1 region of ITS2 sequences of the plant genera *Streptocarpus* and *Saintpaulia* (Gesneriaceae) had to be excluded from the analysis because of alignment uncertainty (Möller and Cronk, 1997a,b). In our phylogenetic study of another genus, *Aeschynanthus* (Gesneriaceae), we also found that 45 aligned positions near the start of ITS2 had to be excluded from the analysis, as alignment in this region was ambiguous.

To study the nature of this variable area, we here analyze ITS2 arm 1 structures among *Aeschynanthus* species. We particularly wished to determine whether the structure of this region would offer any functional explanations for the hypervariability and whether structural analysis would help with the alignment of this otherwise intractable region.

MATERIALS AND METHODS

Plant materials. Fresh leaf material of one plant representing each species was taken from the living collection at the Royal Botanic Garden, Edinburgh. Voucher herbarium specimens of all accessions analyzed have been prepared (E). In a previous study on the phylogeny of *Aeschynanthus* (Denduangboripant and Cronk, 2000), we used the genera *Lysionotus* and *Cyrtandra* as the outgroups and 23 species of *Aeschynanthus* as the ingroups; 12 of these species were chosen for use in this study (Table 1). These 12 species represent both of the two main clades found in our *Aeschynanthus* phylogenetic trees.

TABLE 1

Accessions of *Aeschynanthus, Cyrtandra,* and *Lysionotus* Examined for ITS2 Sequence Variation

Taxon	Clade	RBGE Accession No.	GenBank Accession No.
Cyrtandra baileyi	—	Cronk T118[a]	AF349232
Lysionotus forrestii	—	19962269	AF349233
Aeschynanthus tricolor	II	19812968	AF349251
Aeschynanthus chrysanthus	II	19810535	AF349255
Aeschynanthus argentii	II	19801419	AF349261
Aeschynanthus guttatus	II	19841131	AF349287
Aeschynanthus pachyanthus	II	19623299	AF349267
Aeschynanthus albidus	I	19912436	AF349271
Aeschynanthus buxifolius	I	19970178	AF349279
Aeschynanthus ceylanicus	I	19850904	AF349281
Aeschynanthus hildebrandii	I	19850473	AF349285
Aeschynanthus hookeri	I	19892128	AF349289
Aeschynanthus mimetes	I	19900384	AF349293
Aeschynanthus sikkimensis	I	19611984	AF349295

[a] From material cultivated at Canberra Botanic Garden, Australia.

For comparison, GenBank ITS2 sequences from a range of families in the class Asteridae were examined, including two other members of the family Gesneriaceae, four members of Scrophulariaceae, three Solanaceae, and two Asteraceae. The scientific names and GenBank accession numbers of these plants are shown in Table 2.

Experimental strategy. Details of DNA extraction, the polymerase chain reaction amplification, the PCR cloning, and DNA sequencing strategies used are provided elsewhere (Möller and Cronk, 1997a,b; Denduangboripant and Cronk, 2000). Briefly, genomic DNA prepared by a modified CTAB procedure (Doyle and Doyle, 1987) was used as template for PCR amplification, yielding the complete internal transcribed spacer region (both ITS1 and ITS2 and 5.8S ribosomal DNA). The products were purified and ligated into plasmid vectors with the Prime PCR Cloner cloning system (5Prime–3Prime, Inc., Boulder, CO). The subcloned plasmids were extracted from transformants. Isolated plasmids were sequenced with the Amplitaq-FS dye terminator cycle-sequencing kit (Perkin–Elmer Biosystems, Inc., Warrington, UK) and analyzed on the ABI 377 prism DNA sequencer (Perkin–Elmer, Applied Biosystem, Inc., Foster city, CA). At least two transformant clones were sequenced. The Factura program version 1.2.Or6 (Perkin–Elmer, Applied Biosystem, Inc.) was used to handle the raw sequences. For the outgroups consensus sequences were obtained without cloning. All sequences listed in Table 1 have been submitted to the GenBank database.

TABLE 2

Other Representatives of the Order Asteridae Used for RNA Secondary Structure Analysis, Compared with Gesneriaceae, with Accession Nos. and Length of the Arm 1 Region

Taxon	Family	GenBank Accession No.	Base position of arm 1	Length of arm 1 (bp)
Other Asteridae				
Chionohebe densifolia	Scrophulariaceae	AF037375	6~47	42
Derwentia nivea	Scrophulariaceae	AF037382	6~47	42
Hebe tetragona	Scrophulariaceae	AF069467	6~47	42
Veronicastrum sibiricum	Scrophulariaceae	AF037398	6~46	41
Atropa belladonna	Solanaceae	AB019948	7~59	53
Lycopersicon esculentum	Solanaceae	X52265	3~48	46
Nicotiana tabacum	Solanaceae	AJ012367	7~60	54
Artemisia sublessingiana	Asteraceae	AF061394	7~52	46
Dahlia scapigeroides	Asteraceae	AF165845	2~54	52
Gesneriaceae				
Anna mollifolia	Gesneriaceae	AF055051	6~80	75
Chirita crassifolia	Gesneriaceae	AF055055	1~80	80
Cyrtandra baileyi	Gesneriaceae	AF349232	1~80	80
Lysionotus forrestii	Gesneriaceae	AF349233	6~79	74
Aeschynanthus tricolor	Gesneriaceae	AF349251	1~88	88
Aeschynanthus chrysanthus	Gesneriaceae	AF349255	1~87	87
Aeschynanthus argentii	Gesneriaceae	AF349261	1~46	46
Aeschynanthus guttatus	Gesneriaceae	AF349287	1~92	92
Aeschynanthus pachyanthus	Gesneriaceae	AF349267	1~89	89
Aeschynanthus albidus	Gesneriaceae	AF349271	8~86	83
Aeschynanthus buxifolius	Gesneriaceae	AF349279	1~78	78
Aeschynanthus ceylanicus	Gesneriaceae	AF349281	1~92	92
Aeschynanthus hildebrandii	Gesneriaceae	AF349285	4~94	91
Aeschynanthus hookeri	Gesneriaceae	AF349289	1~95	95
Aeschynanthus mimetes	Gesneriaceae	AF349293	1~99	99
Aeschynanthus sikkimensis	Gesneriaceae	AF349295	1~98	98

Note. Gesneriaceae have exceptionally large arm 1 lengths, except for *A. argentii,* which has a deleted top of arm 1.

Secondary structure analysis and sequence alignment. The ITS2 sequences were first aligned with the sequence alignment program CLUSTAL X (EMBL, Heidelberg, Germany), with "Do Complete Alignment" and "Slow/Accurate Pairwise Alignment" options, without changes in any of the default parameters. A manual alignment was also performed. CLUSTAL X is equivalent to the CLUSTAL W program of Thompson *et al.* (1994) but with a graphic user interface. RNA secondary structure analysis was introduced to optimize the initial alignment of the first ca. 100 aligned positions. First, the secondary structure study was performed for all ITS2 sequences by the RNA folding program RNAdraw version 1.1 (Matzura and Wennborg, 1996). RNAdraw uses the energy structure prediction algorithm MFOLD based on the work of Zuker and Stiegler (1981). The temperature parameter for folding was 20°C, as in the work of other flowering plants (Mai and Coleman, 1997), and yielded a minimum energy structure. All the complete ITS2 sequences were first examined for a common secondary structure, and then the analyses were repeated with partial sequences (arm1 only). The sequence alignment was then optimized with the secondary structure results. The optimized matrix was compared to the primary CLUSTAL and manual alignments.

Search for conserved and variable sites in ITS2. To understand the characteristics of the highly variable region of *Aeschynanthus* ITS2 sequences, the alignment was searched for conserved and variable sites along the sequences. The character conservation was determined by use of the Chart option of the program MacClade version 3.01 (Maddison and Maddison, 1992). Steps of character changes in each site were calculated over one of the most parsimonious trees, with the ambiguous area in the arm 1 still retained, and excluded outgroups from the analysis. The secondary structure of *A. mimetes,* which has the longest ITS2 sequence, was chosen to be the template onto which to plot the step change values. Indel events found in the *Aeschynanthus* matrix were also marked on the structure in three categories, i.e., insertions found in *A. mimetes,* insertions found in other species, and deletions found in other species. The details of the complete ITS2 alignment of 23 *Aeschynanthus* species (two

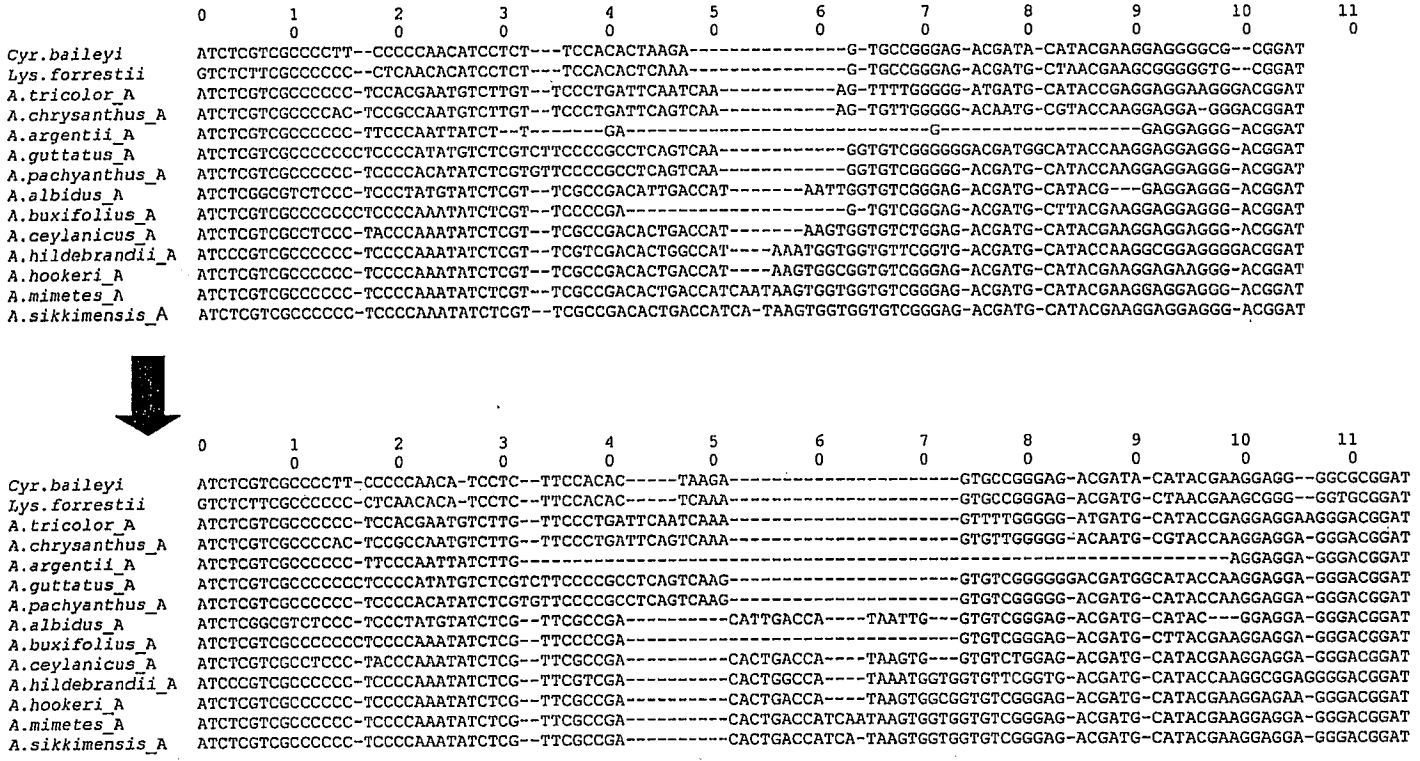

FIG. 1. Alignment of 12 *Aeschynanthus* taxa and two outgroups with CLUSTAL X with default parameters (top alignment), compared with the manual alignment (bottom alignment).

clones per species) and two outgroups can be obtained at the authors' website (http://www.icmb.ed.ac.uk/J_matrix.html).

RESULTS

Nature of hypervariable region in ITS2 sequence. An alignment of ITS2 sequences of 14 taxa analyzed revealed an ambiguous region that was difficult to align. This area ranges approximately from position 30 to position 90 in the primary alignment suggested by CLUSTAL (Fig. 1, top). The CLUSTAL alignment of this region clearly detected a very large deletion in the sequence of *A. argentii* and another single large deletion in that of *A. buxifolius.* The manual alignment (Fig. 1, bottom) clearly suggests two groups of species with different alignments between positions 40 and 73 (clade I species and clade II species). This area of 33 aligned positions contains almost the same proportion of purine and pyrimidine bases, whereas the whole 100-position aligned region shows a transition from C/T rich to A/G rich. The two groups of species are not unambiguously alignable, which might prompt this 70-position ambiguous region to be excluded for the purposes of phylogenetic analysis. For this reason, confirmation of the alignment with secondary structure was sought.

Secondary structure of ITS2 in Aeschynanthus. To help understand the nature of the 100-bp variable region of *Aeschynanthus* ITS2, RNA secondary structures were predicted. Minimum free-energy secondary structures of whole ITS2 sequences of the 14 taxa repeatably recovered a four-arm model, as shown in Fig. 2. The *A. mimetes* structure was used as a template onto which to map the hypervariable region. We found that this area formed the end part of the first stem helix of the ITS 2 standard structure (arm 1). Among the *Aeschynanthus* and outgroup taxa, arm 1 is characterized by a long stem with one end loop and one middle-stem loop (Fig. 3). Note that arm 1 of *A. argentii* is half as long as that in other species. Comparison of the models revealed a pattern of insertions/deletions, from 1 bp to several, that supported the predicted secondary structure. Arm 1 of clade II *Aeschynanthus* species lacks complementary sequence regions (found in that of the clade I species) near the stem end. The short *A. argentii* and *A. buxifolius* sequences result from the complete deletion of the previously extended stem (Fig. 3). Because the terminal half of the *A. argentii* arm 1 has been deleted, we suggest that this arm extension is superfluous, providing that the integrity of the basal half of the arm is maintained.

Arm 1 sequence realignment. Positioning of indels on a secondary structure was adopted to improve the sequence alignment. This allowed detection of the 5-bp complementary deletions, which cause the main difference between two *Aeschynanthus* groups (clade I and clade II). This in turn helps to combine these two sets

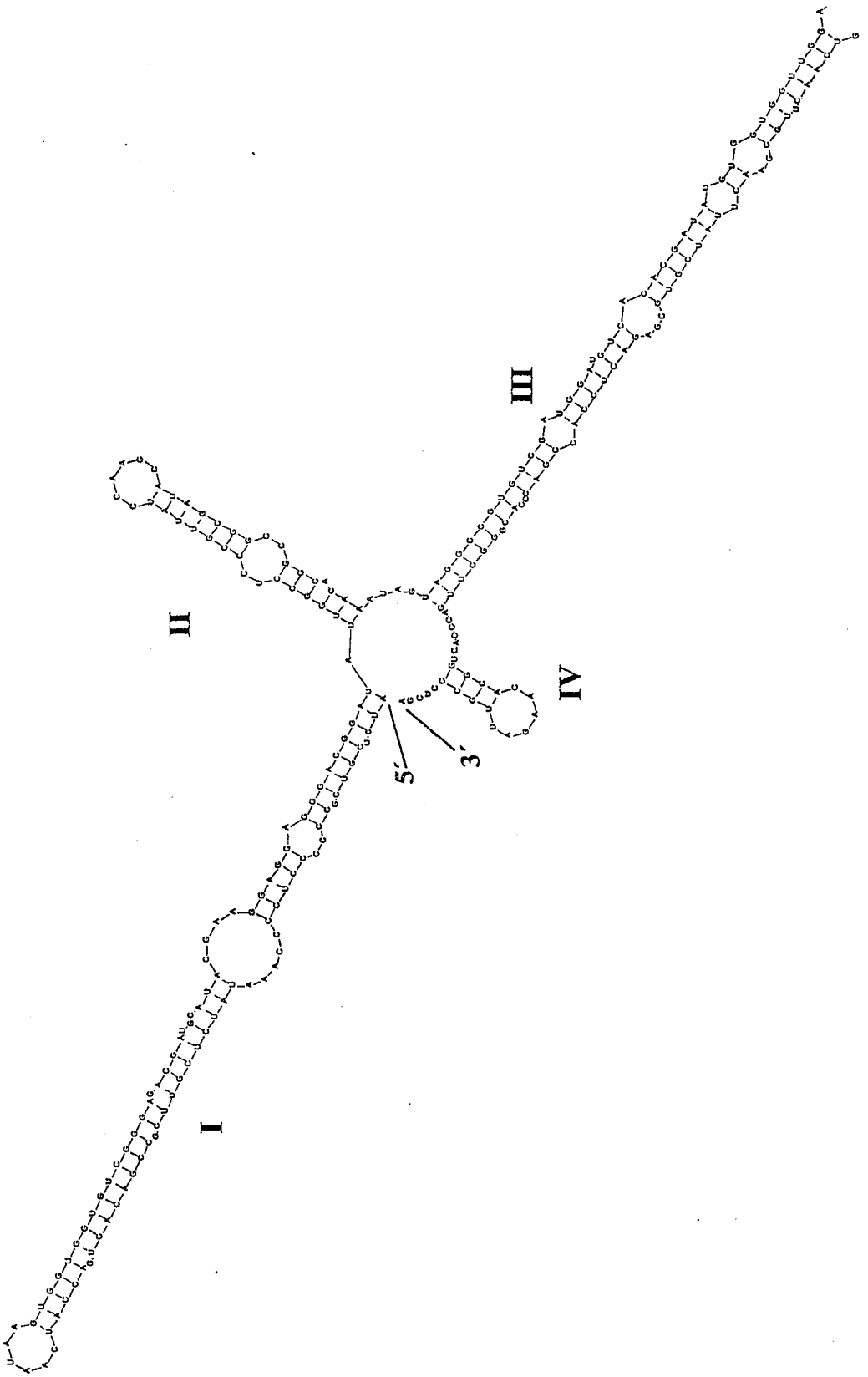

FIG. 2. RNA secondary structure of the ITS2 sequence of *A. mimetes* clone A (20°C, −117.89 kcal), showing a four-arm model.

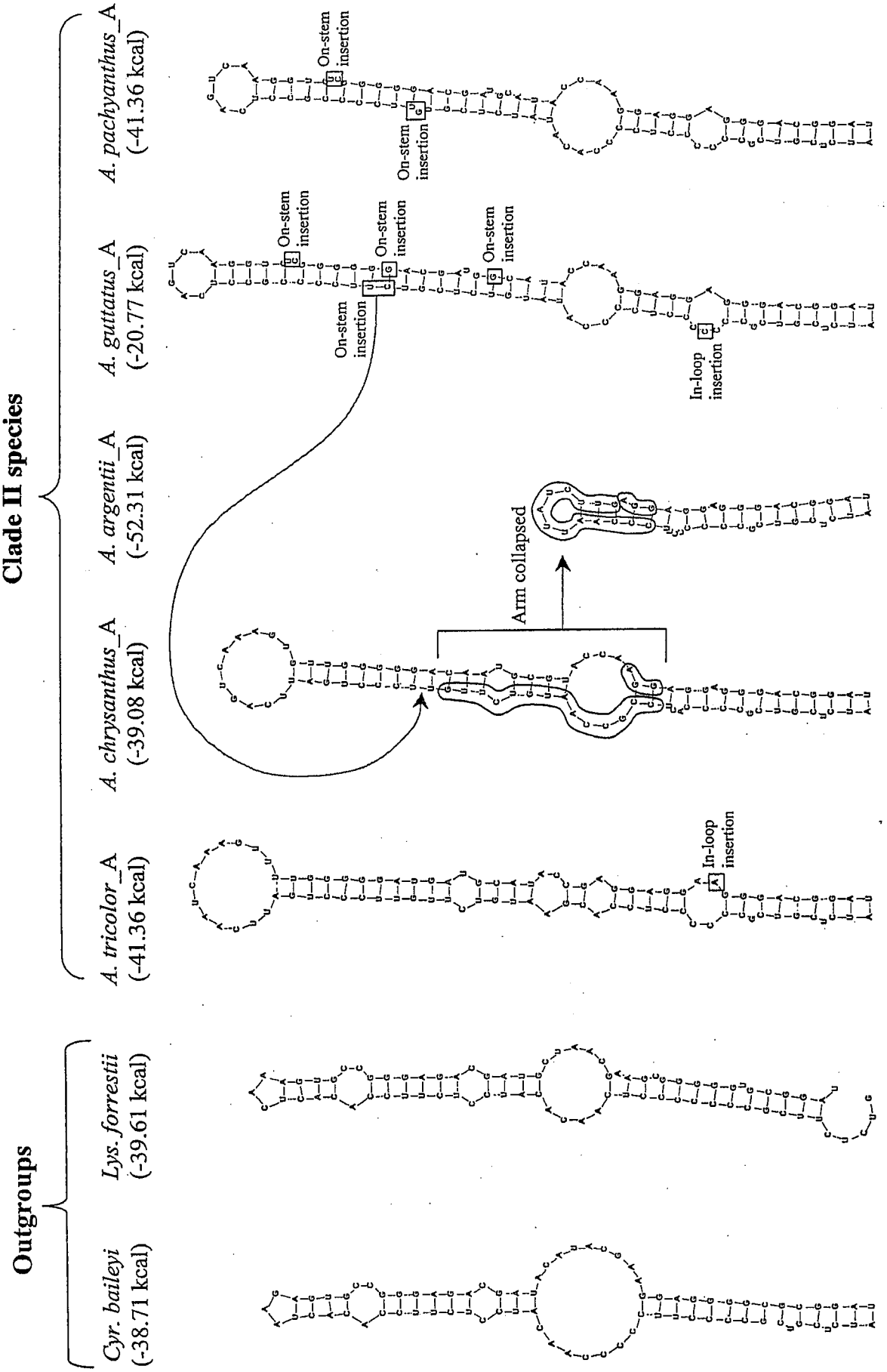

FIG. 3. RNA secondary structures of the arm 1 region for ITS2 sequences of 12 *Aeschynanthus* species and two outgroup taxa.

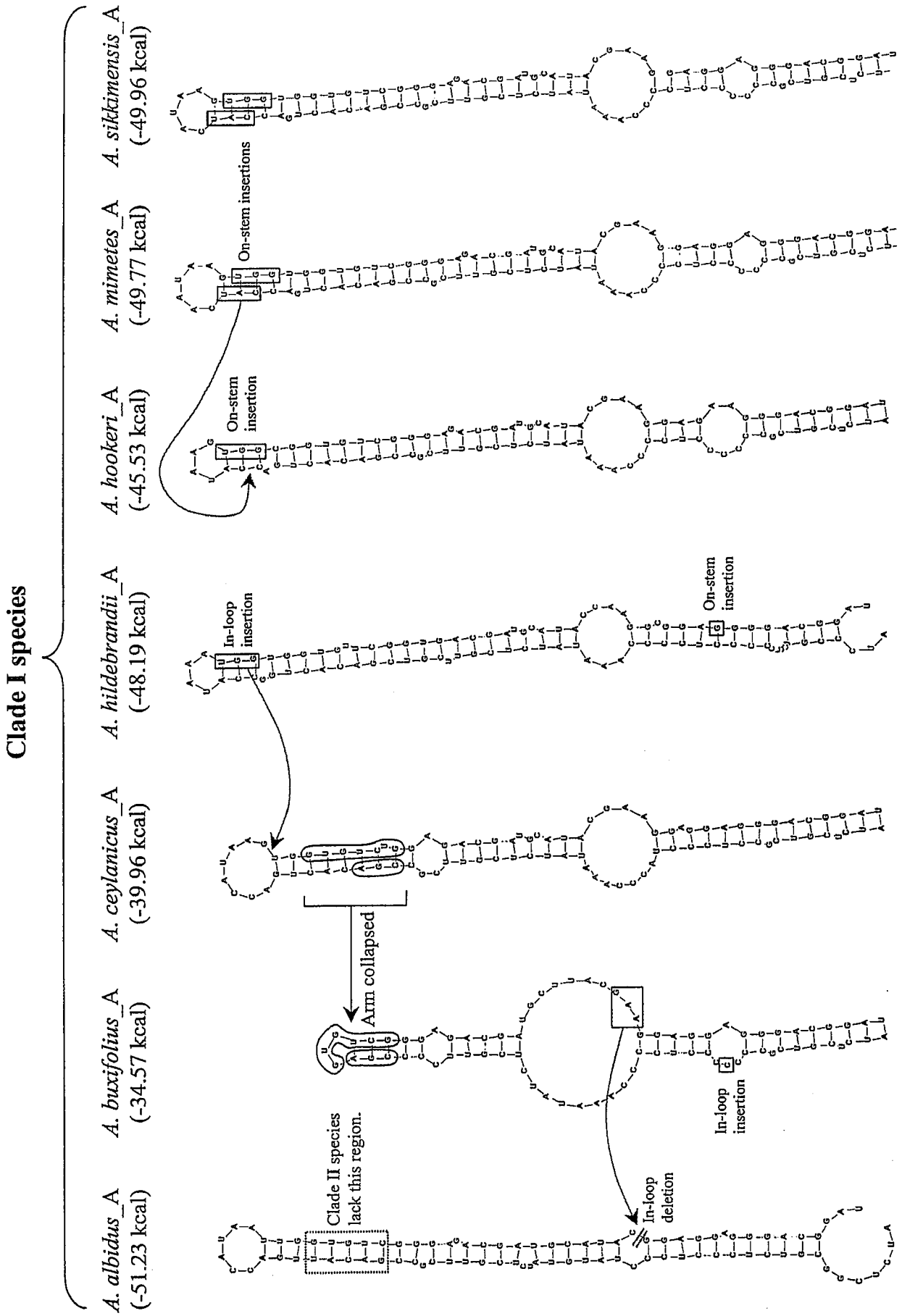

FIG. 3—*Continued*

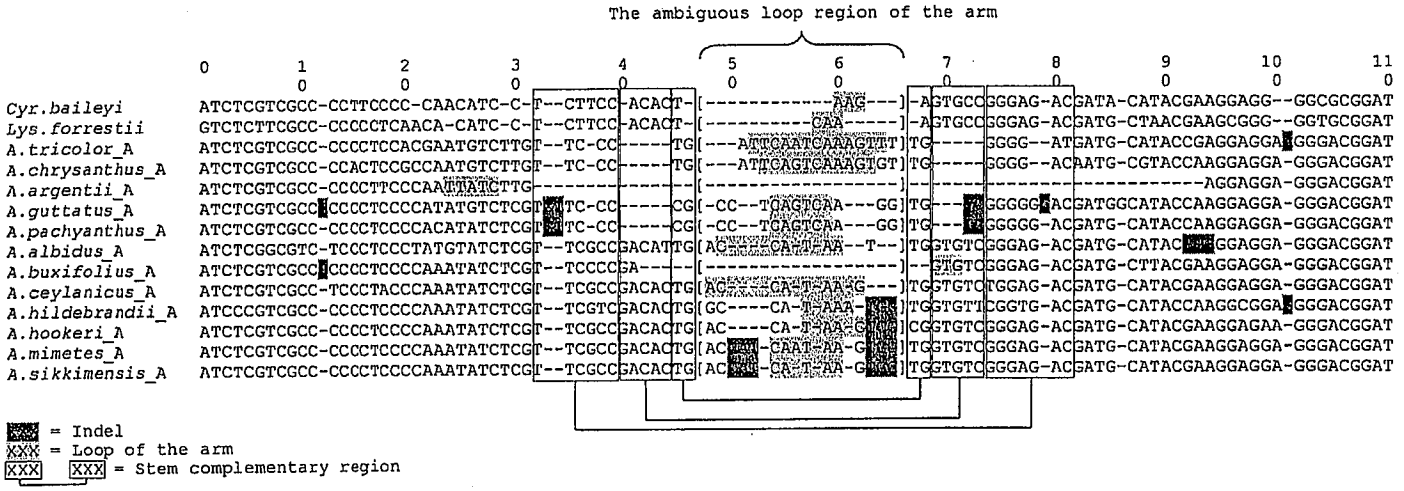

FIG. 4. New alignment suggested by secondary structure analysis.

of superficially dissimilar sequences. The new sequence alignment (Fig. 4) decreases the size of the ambiguous region from about 32 aligned positions to 18 aligned positions. This still ambiguous region is the loop-end region of the arm 1 structure, apparently composed of nonhomologous sequences in the two groups of species. The new alignment suggests a greater number of shorter indels than were predicted by the manual or CLUSTAL alignments. In addition to the 5-bp deletions, numerous shorter indels were found in this region: 1-bp insertions (one site in *A. tricolor, A. buxifolius, A. hildebrandii;* three sites in *A. guttatus*), 2-bp insertions (two sites in *A. gutttus* and *A. pachyanthus*), and 3-bp indels (one deletion site in *A. albidus;* one insertion site in *A. hildebrandii* and *A. hookeri;* two complementary insertions in *A. mimetes* and *A. sikkimensis*).

Superfluous arm 1 extension in Aeschynanthus. Nucleotide changes over the phylogenetic tree were calculated for each aligned position and mapped onto the secondary structure of *A. mimetes.* The position of indels was also mapped (Fig. 5). Most of the indels were located on the arm 1 extension. Some other indels were found along the arm 3 and the arm 4, and on the basal part of the arm 1, but no indel is located on arm 2. ITS2 sequences of *Aeschynanthus* are highly conserved on arm 2 and in the central core of the structure. To investigate whether the variable arm 1 extension is a unique character of the family Gesneriaceae, secondary structures were determined for some other representatives of the class Asteridae, which have ITS2 sequences available in GenBank. The extension was found only in the family Gesneriaceae. Six sampled genera of the family Scrophulariaceae (a family allied to Gesneriaceae) do not have the arm 1 extension. Examples from the Solanaceae (three genera) and Asteraceae (two genera) also revealed the same lack of arm 1 extension as in Scrophulariaceae. Table 2 shows the comparison of the arm 1 length from each of the taxa analyzed. Gesneriaceae arm 1 sequences are almost two times longer than those of other plants.

DISCUSSION

Functional significance of conserved sites versus the superfluous Gesneriaceae arm extension. We suggest that approximately 50 bp at the terminal part of arm 1 in the *Aeschynanthus* ITS2 sequence is superfluous as it is an indel hot spot and has been completely lost in *A. argentii.* The arm extension cannot therefore be important in ribosomal RNA processing, which is a main role of the ITS2 region (Hadjiolova *et al.,* 1994; van Nues *et al.,* 1995). Conversely, the conservation of arm 2 and the central core suggest possible functional significance. *Aeschynanthus* ITS2 (as in Fig. 5) shows a pyrimidine mismatch loophole in arm 2 and a UGGU triplet at the 5′ end of arm 3, reported as characteristic of flowering plants (Liu and Schardl, 1994; Mai and Coleman, 1997). We therefore suggest that ITS2 processing in angiosperms may occur in a way analogous to that of yeast exonuclease ITS2 processing (Mitchell *et al.,* 1996). To remove the ITS2 sequence from 5.8S–26S premature RNA, exonuclease enzymes in plant cells will bind to the UGGU binding site on the arm 3 of the ITS2 sequence and have 3′ → 5′ activity to digest the sequence. By analogy with yeast, the exoribonuclease activity probably degrades RNA in two steps: initial rapid 3′ → 5′exonuclease processing from the end of the arm to the central core and then slower trimming activity. It would then stop the processing when it reaches the recognition sites at the basal parts of arm 1 and arm 2. A similar mechanism has also been suggested for mammalian processing by Michot *et al.* (1999).

It is interesting to consider why only the family Gesneriaceae has this superfluous arm 1 extension. It

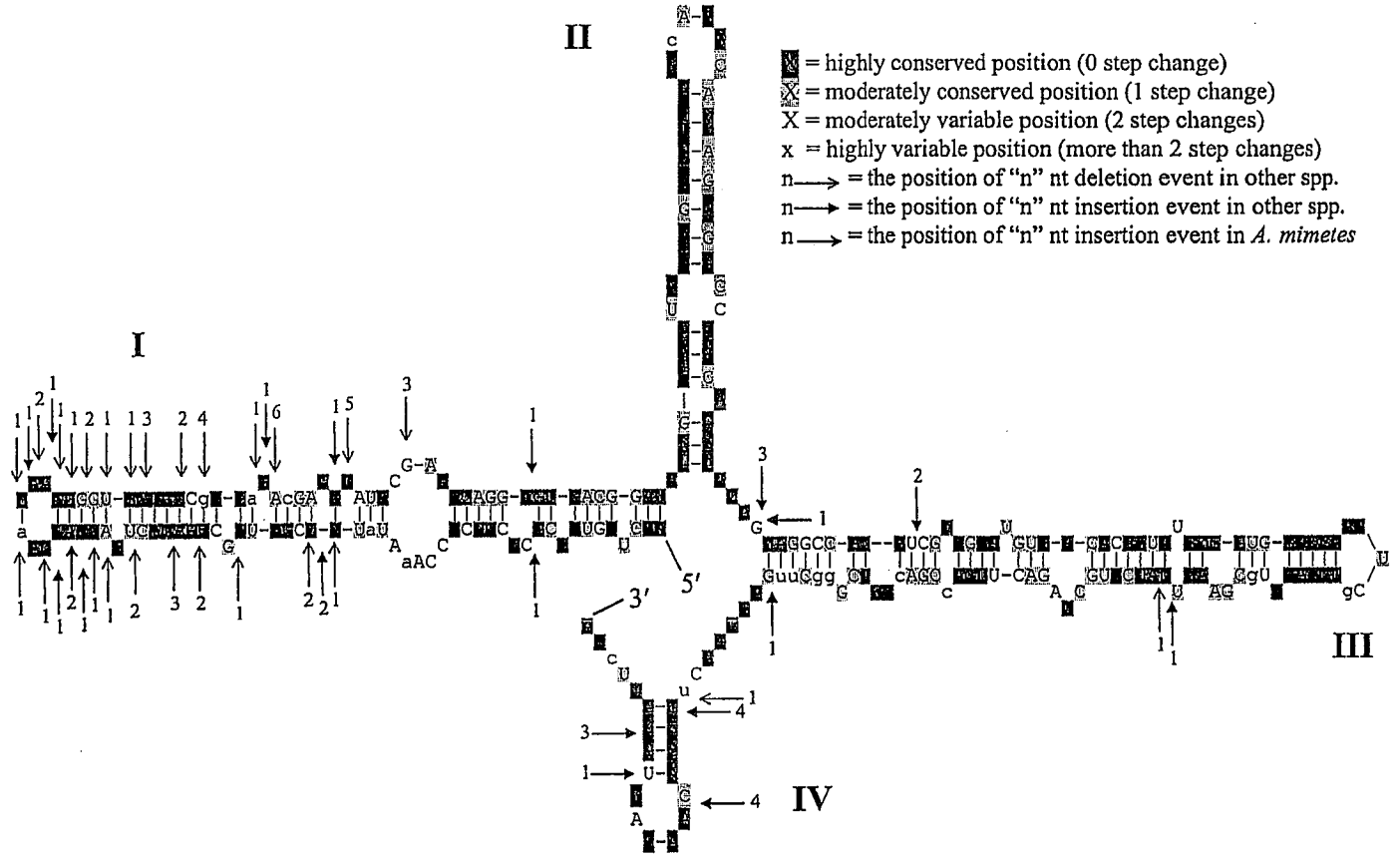

FIG. 5. Conserved and variable sites and indels among *Aeschynanthus* ITS2 sequences, mapped on the predicted secondary structure of the ITS2 sequence of *A. mimetes*. Arm 1 is an indel hotspot but not a substitution hotspot.

is most likely that a Gesneriaceae ancestor either gradually extended the terminal region by complementary insertions or duplicated a long stretch of sequence which by chance could pair to form an arm twice as long. As this extension did not interfere with the ITS2 RNA processing, it has been retained. However, in some species the extension has been diminished by deletions, particularly by the large deletions in *A. argentii* and *A. buxifolius*.

Use of RNA secondary structure for DNA sequence alignment. Our study of *Aeschynanthus* ITS2 sequence alignment shows that CLUSTAL X with default parameters is fairly reliable, but does not detect the complementary indels suggested by the secondary structure alignment. In cases in which primary homology assessment based only on sequence similarity is ambiguous, it is reasonable to take secondary structure as an alternative guide. Our secondary structure realigned matrix is advantageous in allowing the unambiguous combination of two very different sequence types (clade I and clade II), thereby reducing the size of the ambiguous region, which might otherwise be excluded from phylogenetic analysis. This allows further hypotheses of homology to be tested by congruence with other characters.

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REFERENCES

Baldwin, B. G. (1992). Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: An example from the Compositae. *Mol. Phylogenet. Evol.* **1:** 3–16.

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S., and Donoghue, M. J. (1995). The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. *Ann. Mo. Bot. Gard.* **82:** 247–277.

Brown, J. W. S., and Shaw, P. J. (1998). Small nucleolar RNAs and pre-rRNA processing in plants. *Plant Cell* **10:** 649–657.

Denduangboripant, J., and Cronk, Q. C. B. (2000). High intra-individual variation in ITS sequences in *Aeschynanthus* (Gesneriaceae): Implications for phylogenetics. *Proc. R. Soc. London B* **267:** 1407–1415.

Doyle, J. J., and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19:** 11–15.

Hadjiolova, K. V., Normann, A., Cavaillę J., Soúpene, E., Mazan, S.,

Hadjolov, A. A., and Bachellerie, J.-P. (1994). Processing of truncated mouse or human rRNA transcribed from ribosomal minigenes transfected into mouse cells *Mol. Cell. Biol.* **14:** 4044–4056.

Hershkovitz, M. A., and Lewis, L. A. (1996). Deep-level diagnostic value of the rDNA-ITS region. *Mol. Biol. Evol.* **13:** 1276–1295.

Hershkovitz, M. A., and Zimmer, E. A. (1996). Conservation patterns in angiosperm rDNA ITS2 sequences. *Nucleic Acids Res.* **24:** 2857–2867.

Lafontaine, D., and Tollervey, D. (1995). Trans-acting factors in yeast pre-rDNA and pre-snoRNA processing. *Biochem. Cell Biol.* **73:** 803–812.

Liu, J.-S., and Schardl, C. L. (1994). A conserved sequence in internal transcribed spacer 1 of plant nuclear rRNA genes *Plant Mol. Biol.* **26:** 775–778.

Maddison, W. P., and Maddison, D. R. (1992). "MacClade, v. 3.01", Sinauer, Sunderland, MA.

Mai, J. C., and Coleman, A. W. (1997). The internal transcribed spacer 2 exhibits a common secondary structure in green algae and flowering plants. *J. Mol. Evol.* **44:** 258–271.

Matzura, O., and Wennborg, A. (1996). RNAdraw: An integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. *CABIOS* **12:** 247–249.

Michot, B., Desprès, L., Bonhomme, F., and Bachellerie, J.-P. (1993). Conserved secondary structures in the ITS2 of trematode pre-rRNA. *FEBS Lett.* **316:** 247–252.

Michot, B., Joseph, N., Mazan, S., and Bachellerie, J. P. (1999). Evolutionarily conserved structural features in the ITS2 of mammalian pre-rRNAs and potential interactions with the snoRNA U8 detected by comparative analysis of new mouse sequences *Nucleic Acids Res.* **27:** 2271–2282.

Mitchell, P., Petfalski, E., and Tollervey, D. (1996). The 3 end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism. *Gene Dev.* **10:** 502–513.

Möller, M., and Cronk, Q. C. B. (1997a). Origin and relationships of *Saintpaulia* (Gesneriaceae) based on ribosomal DNA internal transcribed spacer (ITS) spacers. *Am. J. Bot.* **84:** 950–965.

Möller, M., and Cronk, Q. C. B. (1997b). Phylogeny and disjunct distribution: Evolution of *Saintpaulia* (Gesneriaceae). *Proc. R. Soc. London B* **264:** 1827–1836.

Morgan, J. A. T., and Blair, D. (1998). Trematode and monogenean rRNA ITS2 secondary structure support a four-domain model *J. Mol. Evol.* **47:** 406–419.

Sambrook, J., Fritch, E. F., and Maniatis, T. (1989). "Molecular Cloning," Cold Spring Harbor Laboratory Press, Plainview, NY.

Schlötterer, C., Hauser, M.-T., von Haeseler, A., and Tautz, D. (1994). Comparative evolutionary analysis of rDNA ITS regions in *Drosophila. Mol. Biol. Evol.* **11:** 513–522.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL-W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.

van der Sande, C. A. F. M., Kwa, M. R., van Nues, W., van Heerikhuizen, H., Raué, H. A., and Planta, R. J. (1992). Functional analysis of internal transcribed spacer 2 of *Saccharomyces cerevisiae* ribosomal DNA. *J. Mol. Biol.* **223:** 899–910.

van Nues, R. W., Rientjes, J. M. J., Morré, S. A., Mollee, E., Planta, R. J., Venema, J., and Raué, H. A. (1995). Evolutionarily conserved structural elements are critical for processing of internal transcribed spacer 2 from *Saccharomyces cerevisiae* precursor ribosomal RNA. *J. Mol. Biol.* **250:** 24–36.

Venema, J., and Tollervey, D. (1995). Processing of pre-ribosomal RNA in *Saccharomyces cerevisiae. Yeast* **11:** 1629–1650.

Yeh, L.-C. C., and Lee, J. C. (1990). Structural analysis of the internal transcribed spacer 2 of the precursor ribosomal RNA of *Sacchoromyces cerevisiae. J. Mol. Biol.* **211:** 699–712.

Zuker, M., and Stiegler, P. (1981). Optimal computer folding of larger RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* **9:** 133–148.