J Plant Res (2002) 115:71–76

ORIGINAL ARTICLE

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Phylogeny of Saururaceae based on mitochondrial *matR* gene sequence data

Received: January 19, 2001 / Accepted: October 22, 2001

Abstract DNA sequences of *matR* gene from three species of Saururaceae and the selected outgroups, *Chloranthus holostegius* and *Zippelia begoniaefolia*, are reported. All DNA sequences of six species in four genera of Saururaceae and the two outgroups are analyzed on PAUP 4.0 8b to reconstruct the phylogeny. A single *matR* gene tree is generated from parsimony, distance, and likelihood analyses, respectively. The three trees with the same topology are slightly different in bootstrapping support for some clades. The result indicates that Saururaceae is monophyletic. *Anemopsis* is sister to *Houttuynia*, and the two genera form the first diverging lineage of the family. The sister group relationship between *Saururus* and *Gymnotheca* is also supported by a relatively high bootstrap value. The result is different from all the former phylogenetic opinions on Saururaceae based on morphology, but it is supported by the evolution of flower-bract stalk in Saururaceae. In addition, some characteristics of the *matR* gene are analyzed. The *MatR* gene is a relatively better tool to reconstruct the molecular clock because the base substitution bias greatly decreases in the gene.

Key words *MatR* sequences · Phylogeny · Saururaceae

Introduction

Saururaceae, a member of paleoherbs, are an ancient and relic family with six species in four genera, i.e., *Anemopsis*, *Gymnotheca*, *Houttuynia*, and *Saururus* (Liang 1995). They are perennial herbs with simple flowers. The flowers have bracts and no perianths. Saururaceae are typically East Asian–North American disjuncted, with *Anemopsis* and *Saururus cernuus* in North America, and *Gymnotheca*, *Houttuynia*, and *Saururus chinensis* in East Asia.

Because of its interesting geographical distribution pattern and discordance between speculated phylogenetic relationships based on non-molecular data, Saururaceae has been an interesting family to study, although it includes only a few species. However, the viewpoints on the phylogeny of Saururaceae are very different based on morphology. Wu and Wang (1957) posed a systematic tree of Saururaceae based mainly on the gross morphology and geography. They included *Anemopsis*, *Circaeocarpus*, *Gymnotheca*, *Houttuynia*, and *Saururus* in Saururaceae. Later, however, Wu and Wang (1958) detected that the new genus, *Circaeocarpus*, was in fact a member of Piperaceae, and *Circaeocarpus saururoides* C.Y. Wu and *Zippelia begoniaefolia* Blume were conspecific. Moreover, also according to the gross morphology and geograpic distribution, Wu (1984) thought that *Anemopsis* was sister to *Houttuynia*. Basic chromosome numbers are different among genera of Saururaceae. For example, *Saururus*, $x = 11$; *Houttuynia*, $x = 12$; *Anemopsis*, $x = 22$ (Okada 1986); and *Gymnotheca*, $x = 9$ (Lei et al. 1991). Okada (1986) then suggested that *Saururus* was cytologically the most primitive in the family, and Lei et al. (1991) supported the view of Okada (1986). On the basis of a cladistic analysis for some conventional morphological a1nd ontogenetic characters, Tucker et al. (1993) concluded that *Saururus* was the first to diverge from the ancestral family stock, followed by *Gymnotheca*, with *Houttuynia* and *Anemopsis* being sister taxa. Combining the data from gross morphology, anatomy, embryology, palynology, cytology, and flower development (Liang and Tucker 1990; Liang 1991, 1992, 1994; Meng and Liang 1997), Liang (1995) proposed that the ancestral stock of Saururaceae had diverged into two branches early on to give the *Gymnotheca–Anemopsis* branch, and the *Saururus–Houttuynia* branch.

MatR is a mitochondrial functional gene that encodes a maturase-related protein, and has been conserved during plant evolution (Wahleithner et al. 1990). Generally, it is

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Table 1. List of Saururaceae and the designed outgroups

Species	Origin	Collection number	GenBank accession number
Chloranthaceae:			
Chloranthus holostegius (Hand.-Mazz.) Pei et Shan	Xingyi, Guizhou	*Meng 99708*	AF332104
Piperaceae:			
Zippelia begoniaefolia Blume	Menglun, Yunnan	*Meng 99715*	AF332107
Saururaceae:			
Anemopsis californica (Nutt.) Hook.	From GenBank		AF197747*
Gymnotheca chinensis Decne.	Xuanwei, Yunnan	*Meng 99003*	AF332103
Gymnotheca involucrata Pei	Ermei, Sichuan	*Liang 97015*	AF332105
Houttuynia cordata Thunb.	From GenBank		AF197749*
Saururus chinensis (Lour.) Baill.	Menglun, Yunnan	*Meng 99525*	AF332106
Saururus cernuus Linn.	From GenBank		AF197748*

* AF197747, AF197748, and AF197749 are cited from GenBank

used to reconstruct higher-level phylogeny, such as the relationships of orders, families, or distant genera (Qiu et al. 1999). We selected the *matR* gene to reconstruct the phylogeny of the relict family of Saururaceae. Overall research on the molecular systematics of Saururaceae is very much needed, even though some genera have been represented in recent studies as having higher-order relationships within the angiosperms (Chase et al. 1993; Soltis et al. 1997, 2000; Qiu et al. 1999). This study presents an assessment of the phylogeny of the Saururaceae.

Materials and methods

Plant materials

Five species, *Chloranthus holostegius* (Chloranthaceae), *Zippelia begoniaefolia* (Piperaceae), *Gymnotheca chinensis*, *Gymnotheca involucrata*, and *Saururus chinensis*, were collected from natural populations or cultivated plants. Vouchers are deposited in the herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences, Heilongtan, Kunming, Yunnan, People's Republic of China (KUN). The sequences of the other three species are cited from the GenBank (Table 1). *Chloranthus holostegius* (Chloranthaceae) and *Z. begoniaefolia* (Piperaceae) are designated as outgroups in this study because Piperaceae is the sister group of Saururaceae and Chloranthales is close to, and more basal than, Piperales (Soltis et al. 2000).

DNA extraction, PCR amplification and sequencing

Total DNA was isolated using the CTAB method (Doyle and Doyle 1987). The materials for extracting DNA were silica-gel-dried or fresh leaves. The DNA was precipitated using iso-Propyl alcohol for half an hour at −20°C, then washed with 70% ethanol two or three times and with 100% ethanol one time. The concentrated DNA was then stored in TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0) at −20°C.

Double-stranded DNA was directly amplified by PCR. The primers *matR* 26F (5′ GACCGCTNACAGTAGTTCT 3′) and *matR* 1858R (5′ TGCTTGTGGGCYRGGGTG AA 3′) were used for PCR amplification. Reaction volumes were 20 μl and contained 1.5 U Ampli*Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), Replitherm TM buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTP, 0.1 μM primer, 25–60 ng sample DNA. PCR reactions were performed in GeneAmp 9600 (Perkin-Elmer, Applied Biosystems, Norwalk, CT, USA) and consisted of initial denaturation at 94°C (4 min), followed by 35 cycles of 94°C denaturation (1 min), 55°C annealing (1 min), and 72°C extension (90 s), with a final extension for 7 min at 72°C. PCR products were separated with 1.5% agarose TAE gel and purified using Wizard PCR preps DNA purification system (Promega, Madison, WI, USA).

Purified PCR products were sequenced using the Dideoxy Chain Termination method and an ABI PRISM Bigdye Terminator Cycle Sequencing Ready Reaction Kit with Ampli*Taq* DNA polymerase FS (Applied Biosystems). The four primers 26F (5′ GACCGCTNACAGTA GTTCT 3′), 1858R (5′ TGCTTGTG GGCYRGGGTGAA 3′), 879F (5′ ACTAGTTATCAGGTCAGAGA 3′), and 1002R (5′ CACCCACGATTCCCAGTAG T 3′) were used for sequencing. Primers were designed by Mark Chase's Molecular Systematic Lab at the Royal Botanic Gardens (Kew, UK). All protocols of DNA sequencing followed the recommendations of the manufacturers (Applied Biosystems), with slight modification in some cases. Sequencing was performed using an ABI 377 automated sequencer.

Alignment and analysis of the sequence data

Chloranthus holostegius and *Zippelia begoniaefolia* were designated as outgroups in this study. Sequences were assembled using the program Seq^{Ed} (PE Applied Biosystems). The boundaries of the *matR* gene were determined by comparison with the sequences of AF197747, AF197748, and AF197749 from the GenBank. All *matR* sequences were aligned using the software Clustal X (Thompson et al. 1997) and Mega2b3 (Kumar et al. 2000). The basic sequence sta-

tistics, including nucleotide frequencies, ns:nv ratio, and variability in different regions of the sequences were computed by Mega2b3 and PAUP 4.0 8b (Swofford 2001).

The aligned sequences were analyzed through the program PAUP 4.0 8b. Phylogenetic analyses were performed using maximum parsimony, distance, and maximum likelihood (ML). In the maximum parsimony analysis, branch-and-bound search, furthest addition sequence, and ACCTRAN character-state optimization were used. Gaps were treated as missing data. Un-rooted trees were rooted using the outgroup method. In distance analysis, neighbor-joining search and uncorrected ("P") distance measure were used. The objective function was minimum evolution. In the maximum likelihood analysis, branch-and-bound search, as-is addition sequence, the HKY (1985) base substitution model, statistical base frequencies, equal distribution of rates at variable sites, and enforced molecular clock (parameterization for clock optimization was Rambaut), or not were used. Starting branch lengths were obtained by using the Rogers-Swofford approximation method, and the transition/transversion ratio was also used statistical value. Support for topologies obtained with all three analyses was estimated with the bootstrap option in PAUP 4.0 8b using 1000 replicates, and the other settings were the same as the previous analyses.

Results

After alignment and deletion of some sites from each end, there are 1716 bp in the matrix; 100 are variable and 18 are parsimony informative. The percentage of variable sites was 5.83%, and the percentage of phylogenetically informative sites was 1.05%. Uncorrected sequence divergence ranged from 0% to 1.70% among species of Saururaceae and from 1.36% to 4.11% between outgroups and the ingroup (Table 2).

Characteristics of the *matR* gene

The 1716 bp of the *matR* gene sequenced from Saururaceae and the outgroups represented about 83% of the gene and mainly covered the 5′ end. The first base of the sequence in the study is not the first base of the *matR* gene sequence because the primer 26F (5′ GACCGCTNACAGTAGT TCT 3′) begins from the 26th base of the *matR* gene sequence. However, the first base of the sequence in the study is the first position of a certain triplet because the whole sequence can be translated into amino acids by the software EditSeq (DNASTAR, Madison, WI, USA). However, there are many terminators in the amino acid sequence if the nucleotide sequence is translated after one or two bases at the 5′ end have been deleted. Moreover, there were a few gaps in the matrix, three gaps of 6 bp in *Zippelia*, one gap of 9 bp in *Chloranthus*, and one gap of 3 bp in *Houttuynia*.

The average base frequencies within Saururaceae were as follows: A, 26.2; C, 27.0; G, 25.7; T, 21.1, and those among the Saururaceae and outgroups: A, 26.1; C, 27.1; G, 25.7; T, 21.1. These values were almost identical with the empirical base frequency (A = 0.26123, C = 0.27108, G = 0.25706, T = 0.21063). The G + C content of this section was as follows: *Chloranthus* 53.2%, *Zippelia* 53.1%, *Anemopsis* 52.6%, *Houttuynia* 52.5%, and *Gymnotheca* and *Saururus* 52.8%, with an average of 52.7% in Saururaceae and an average of 52.8% in Saururaceae and the outgroups. The G + C content of the part in Saururaceae was a little higher than that for the whole gene as calculated from broad bean (51.2%; Wahleithner et al. 1990).

In order to evaluate the functional constraints on *matR* gene, the DNA sequences were translated to amino acids to calculate codon position substitution. For *Anemopsis californica*, *Gymnotheca chinensis*, *G. involucrata*, *Saururus chinensis*, and *S. cernuus*, the 1716 bp were translated into 572 amino acids. However, for *Houttuynia cordata*, the 1713 bp were translated into 571 amino acids, for *Zippelia begoniaefolia*, the 1698 bp were translated into 566 amino acids, and for *Chloranthus holostegius*, the 1707 bp were translated into 569 amino acids. In the matrix of 572 amino acids, 69 (12%) sites were variable and 8 (1.4%) sites were phylogenetically informative in Saururaceae and outgroups.

The distribution of the number of variable sites was calculated by dividing the sequences into sectors of 99 bp. For the most part, even distribution of variable sites was observed. The average number of variable sites was 5.8 bp per 99-bp sector (Fig. 1).

Among Saururaceae and the outgroup, the substitution rates of codon positions were as follows: the first position of

Table 2. Pairwise distances between taxa of Saururaceae and outgroups

	1	2	3	4	5	6	7	8
1 CHLO	–	0.03612	0.03574	0.03515	0.03515	0.04110	0.03574	0.03456
2 ZIPP	61	–	0.01355	0.01355	0.01355	0.01889	0.01531	0.01413
3 ANEM	61	23	–	0.00874	0.00874	0.01227	0.01049	0.00932
4 GCHI	60	23	15	–	0.00000	0.01519	0.00641	0.00641
5 GINV	60	23	15	0	–	0.01519	0.00641	0.00641
6 HOUT	70	32	21	26	26	–	0.01694	0.01577
7 SCHI	61	26	18	11	11	29	–	0.00233
8 SCER	59	24	16	11	11	27	4	–

Total character differences are indicated below diagonal, and mean character ones above
CHLO, *Chloranthus holostegius*; ZIPP, *Zippelia begoniaefolia*; ANEM, *Anemopsis californica*; GCHI, *Gymnotheca chinensis*; GINV, *Gymnotheca involucrata*; HOUT, *Houttuynia cordata*; SCHI, *Saururus chinensis*; SCER, *Saururus cernuus*

codon, 6.29% (36 sites); the second, 4.89% (28 sites); and the third, 6.29% (36 sites). The distribution of parsimony informative sites was as follows: the first position of codon, 0.52% (3 sites); the second, 0.87% (5 sites); and the third, 1.75% (10 sites).

The ratios of transition–transversion for pairs of sequences are shown in Table 3. Within Saururaceae, the average ns:nv ratio was 0.5; the ns:nv ratio in the first position of codon was 0.8, and in the second or the third position, 0.5. Among Saururaceae and outgroups, the average ns:nv ratio was 0.7; the ns:nv ratio in the first or second positions of the codon was 0.8, and in the third position, 0.5.

Sequence analysis through the maximum parsimony method

A single most parsimonious tree of 106 steps was obtained (Fig. 2; RC = 0.86, RI = 0.88). Saururaceae are supported as a monophyletic group, with a bootstrap value of 92%. *Anemopsis* is sister to *Houttuynia* with a bootstrap value of 71%, and they constitute the first diverging lineage of Saururaceae. *Saururus* is sister to *Gymnotheca* with a bootstrap value of 92%. There is also good support for the monophyly of the two genera, *Gymnotheca* and *Saururus*, with bootstrap values of 99% and 95%, respectively.

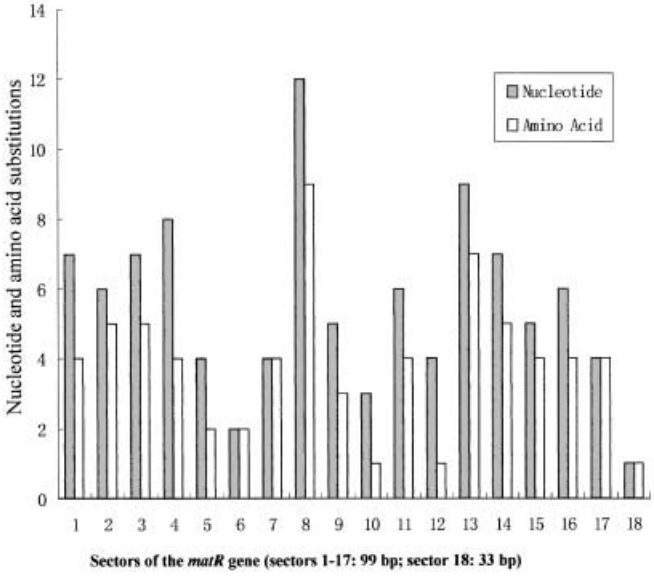

Fig. 1. Variability of the *matR* gene among Saururaceae and outgroup: *x-axis* shows the sectors of the *matR* gene, and the *y-axis* is the number of nucleotide and amino acid substitutions. *Gray columns* express nucleotide, and *white columns* amino acids

Sequence analysis through the distance method

Distance analysis yielded a tree of identical topology to Fig. 2 (ME-score = 0.06081). Again, there was strong support for the monophyly of the Saururaceae (91%, bootstrap value, same thereafter), *Saururus* (96%) and *Gymnotheca* (100%), and for the sister relationship between *Anemopsis* and *Houttuynia* (88%), and between *Saururus* and *Gymnotheca* (93%).

Sequence analysis through the maximum likelihood (ML) method

When the molecular clock was not enforced, ML generated the tree of topology shown in Fig. 2 (-Ln likelihood = 3072.44465). Again, there were strong supports for the monophyly of Saururaceae (88%), *Saururus* (95%) and *Gymnotheca* (98%), and for the sister relationship between *Anemopsis* and *Houttuynia* (73%), and between *Saururus* and *Gymnotheca* (89%). When the molecular clock was enforced, ML generated a tree identical with that in Fig. 2 (-Ln likelihood = 3076.27074). Moreover, there were also strong supports for the monophyly of Saururaceae (92%), *Saururus* (99%), and *Gymnotheca* (100%), and for the sister relationship between *Zippelia* and Saururaceae (100%), between *Anemopsis* and *Houttuynia* (70%), and between *Saururus* and *Gymnotheca* (97%).

Discussion

Characteristics of the *matR* gene

All of the gaps in the matrix are multiples of 3 bp so they do not result in the reading frame shift. Furthermore, none of the gaps are informative. The rate of substitution in amino acids is higher than that of the nucleotide, implying relatively less functional constraints on the the *matR* gene.

Theoretically, the first, second, and third positions of the codon are translated to 4%, 0%, and 69% synonymous

Table 3. The ratios of transition–transversion among Saururaceae and outgroups

	1	2	3	4	5	6	7	8
1 CHLO	–							
2 ZIPP	0.7949	–						
3 ANEM	0.8458	0.2785	–					
4 GCHI	1.0697	0.4378	0.5000	–				
5 GINV	1.0697	0.4378	0.5000	Undefined	–			
6 HOUT	0.9453	0.5242	0.9111	0.7343	0.7343	–		
7 SCHI	0.9676	0.3007	0.2857	0.3750	0.3750	0.5276	–	
8 SCER	0.9668	0.2637	0.2308	0.5714	0.5714	0.5011	0.3333	–

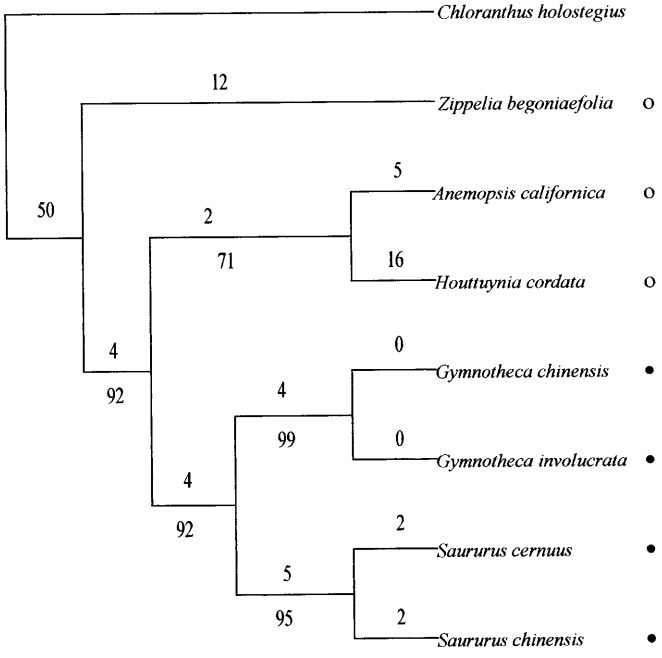

Fig. 2. The single most parsimonious tree of Saururaceae based on *matR* sequences. Length = 106, CI (Consistency index) = 0.9717, RI (Retention index) = 0.8846, RC (Rescaled consistency index) = 0.8596. Number of bootstrap replicates = 1000. Base substitutions are indicated *above branches*, and bootstrap values (%) *below branches*. Species without flower-bract stalk (○), and species with the stalk (●)

substitutions, respectively (Li and Grauer 1991). However, for the *matR* gene, the substitution rate in the first position of the codon is 6.29%, 4.89% in the second, and 6.29% in the third. The relatively high percentages of substitution in the first and third positions of the codon explain the high variability in amino acids in the *matR* gene. Moreover, the almost identical substitution percentages in the first, second, and third positions of the codon indicate that there is no substitution bias in positions of the codon in the *matR* gene. On the other hand, according to the result of variable site distributions in different sectors, most of the sectors in the *matR* gene have a similar substitution rate (Fig. 1). These characteristics also indicate that *matR* is a relatively fine tool to construct a molecular clock.

Holmquist (1983) reported that the commonly observed ns:nv ratio was 2:1, as DNA sequences were more likely to undergo transition than transversion. However, the average ns:nv ratio of the *matR* gene within Saururaceae is 0.5, i.e., 1:2. Transversion is considered a more reliable type of mutation in constructing phylogenies (Quicke 1993). In addition, the low frequency of transversion compared with transition implies that the accumulation of transversion would accrue over a longer time span (Liang and Hilu 1996). Therefore taxa with lower ns:nv ratios would be older, diverging earlier in the evolution history of the group. Thus, the ns:nv values between the designated outgroup *Chloranthus* and Saururaceae coincide with the topology of Saururaceae (Table 3). However, the ns:nv values between the outgroup *Zippelia* and Saururaceae are not supported by the cladistic analysis.

According to the analyses, we know that in *matR* gene, all indels are multiples of 3 bp and the base substitution bias greatly decreases. It is very interesting that these characteristics were also observed in the chloroplast-encoded *matK* gene (Liang and Hilu 1996; Hilu and Liang 1997).

The phylogenetic value of *matR* gene sequences

There were 100 variable sites and 18 informative ones when gaps were treated as missing, and uncorrected sequence divergence ranged from 0% to 1.69% among Saururaceae species, and from 1.36% to 4.11% between the outgroups and the ingroup (Table 2). Thus, we could easily distinguish genera and even species of Saururaceae through the analyses of *matR* gene sequences (Fig. 2). Moreover, the bootstrap supports are very high. For example, in Fig. 2, the monophyly of Saururaceae (92%), *Gymnotheca* (99%) and *Saururus* (95%) are strongly supported, and the sister group relationships between *Anemopsis* and *Houttuynia* (71%), and *Saururus* and *Gymnotheca* (92%) are also well supported. Therefore *matR* DNA sequence data should be valuable for the phylogenetic reconstruction among genera of some ancient and relic families such as Saururaceae.

The phylogeny of Saururaceae

According to parsimony analysis (Fig. 2), the monophyly of Saururaceae (92%), *Gymnotheca* (99%), or *Saururus* (95%) is strongly supported. The sister group relationships between *Anemopsis* and *Houttuynia* (71%), and between *Gymnotheca* and *Saururus* (92%), also are well supported. Moreover, *Anemopsis* and *Houttuynia* constitute the first diverging lineage of Saururaceae. This result is also supported by distance and ML analyses. The result is in agreement with Wu (1984) and partly agrees with Tucker et al. (1993), but disagrees with the opinions of Wu and Wang (1957, 1958), Okada (1986), Lei et al. (1991), and Liang (1995).

The topology in Fig. 2 shows that the *Anemopsis* – *Houttuynia* clade is the first diverging lineage in Saururaceae. However, many researchers using the morphological data of Saururaceae thought that *Saururus* was the most primitive in Saururaceae because they paid more attention to characteristics such as the position of ovaries or stamina, and the number of stamina, but neglected the great variation of flower organs. Tucker (1975) observed the high variation of the number of stamina and carpels in *S. cernuus*, as did Liang (1994) in *Gymnotheca*. The flower-bract stalks are absent in *Zippelia*, *Anemopsis*, and *Houttuynia*, but present in *Saururus* and *Gymnotheca*. According to the principle of comparison with outgroups, the *matR* gene tree is clearly supported by the evolution of flower-bract stalks in Saururaceae (Fig. 2).

Acknowledgments We express our hearty thanks to Ms. Cha-Cha Huang, Ms. Ying-Xue Sun, Mr. Ya-Ping Hong, Ms. Bao-Hua Song, and Mr. Jun-Bo Yang for their assistance during the experiments, to Prof. Shirley C. Tucker and Dr. Andrew W. Douglas for helpful suggestions,

and to Profs. Xiao-Quan Wang and Song Ge for useful discussions. This research was supported by a Special Grant of the National Natural Science Foundation of China (39725001 to De-Zhu Li), the National Natural Science Foundation of China 39930020, and the Hundreds of Talents Program of the Chinese Academy of Science CAS [1998] 0054 (to De-Zhu Li).

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