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# Molecular phylogenetic analysis of mangroves: independent evolutionary origins of vivipary and salt secretion

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**Abstract**

The most remarkable morphological specializations of mangroves are vivipary, salt secretion, and aerial roots. There has been a long debate on whether the complex traits vivipary and secreters have a single origin, the answer to which has profound implications for the mechanism of evolution in mangroves. We took a large and representative sample across mangroves and sequenced the 18S rRNA, *rbc*L, and *mat*R genes. Together with the outgroups, our data yielded a high resolution phylogeny which allowed us to gain much needed insight into the distributions of the two characters and address their evolutionary origins. For each character, its ancestral state in the phylogeny was estimated by the maximum likelihood method. Overall evidence is in favor of a multiple origin for both vivipary and salt secretion in mangroves.


*Keywords:* Vivipary; Salt secretion; Mangrove; Character evolution; Ancestral state

## 1. Introduction

Mangroves, which include all the tropical trees restricted to intertidal and adjacent communities, share an interesting mixture of attributes (Tomlinson, 1986), of which vivipary, salt exclusion, and aerial roots are the three most important morphological and physiological traits. Although these characters are widely thought to have facilitated the adaptation of mangroves to harsh coastal environments, no single structural feature uniquely characterizes mangroves (Tomlinson, 1986). Vivipary is a condition found in some seed plants in which the sexually produced embryo of the seeds, while still attached to the parent plant, continues its development without dormancy. Vivipary can be divided into two major different forms, known as "true vivipary" and "cryptovivipary," representing the two situations in which the embryo grows to break through the fruit wall, and the seed coat, respectively (Tomlinson and Cox, 2000).

Among seed plants, vivipary is most well developed in mangroves (Tomlinson, 1986). True vivipary occurs occasionally in some seagrasses, such as *Amphibolis*. (A third kind of vivapary, the pseudovivipary, is clearly unrelated to the true vivapary (Elmqvist and Cox, 1996; Tomlinson, 1986), and is hence excluded from this analysis.) Since vivipary is such a remarkable character, the mechanism, including its evolutionary origin, is of great interest to many plant biologists. In particular, whether

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vivipary resulted from single or multiple origins has been debated and analyses based on the viviparous structure alone have not been conclusive (Farnsworth, 2000; Guppy, 1906; Van der Pijl, 1983).

Another important character enabling mangroves to establish along the sea coast is their ability to exclude salt in seawater. According to the mechanism of salt secretion, mangroves can be divided into two groups: secreters and non-secreters. Secreters control their salt balance by excreting the absorbed salt metabolically via salt glands (Fahn, 1979; Fahn and Shimony, 1977; Tomlinson, 1986). In comparison non-secreters selectively absorb only certain ions from the solution they come into contact with by the process of ultrafiltration (Morgany et al., 1999; Tomlinson, 1986). The structure of salt glands in salt-secreting mangroves is surprisingly similar in view of the fairly remote systematic affinity of the several families involved (Atkinson et al., 1967; Tomlinson, 1986). Again, whether salt secretion among mangroves evolved once or mutiple times cannot be resolved on the sole basis of anatomy.

A third feature of more highly specialized mangroves is that some parts of the root system become exposed to the atmosphere (Gill and Tomlinson, 1975; Tomlinson, 1986). Tomlinson (1986) defined several types of aerial roots in mangroves, including stilt roots, pneumatophores, root knees, and plank roots. The mangrove root system plays three distinct roles—the aerating, anchoring/absorbing, and cable system. These morphological components apparently have different origins in different species (Tomlinson, 1986; Troll and Dragendorff, 1931).

How and why do not all mangroves share their chief attributes—vivipary, salt secretion, and aerial roots? Are the special and complex traits vivipary and salt secretion of single or multiple origin? Many paleobotanists have argued that the mangrove habitat is an ancient one and many seed plants share some primitive characters of mangroves. For example, vivipary was suggested to be the rule under uniform climatic conditions of early geological periods (Cridland, 1964; Guppy, 1906; Raymond and Phillips, 1983). Retallack and Dilcher (1981) went even further, in light of fossil evidence, to suggest that angiosperms may have all radiated from coastal environments. The mainstream view, however, appears to be that most of those important adaptive attributes of mangroves were derived in specialized habitats rather than lost in general habitats. This view of independent and multiple evolutionary origins through convergent evolution is largely based on the occurrence of traits in different unrelated angiosperm families (Cox and Humphries, 1993; Ellison and Farnsworth, 2001; Farnsworth, 2000). While the scattered distribution of any adaptive trait in a phylogenetic framework may seem to be the prima facie evidence for its multiple origin, the statistical support for such intuitions can often be flimsy (Mooers and Schluter, 1999; Oakley and Cunningham, 2002; 2002; Pagel, 1999). This is especially true when the loss of a character is more likely than the gain which we believe are the case for vivipary and salt secretion.

The purpose of this paper is to report our conclusions about the evolutionary origin of vivipary and salt secretion based on a combination of phylogeny reconstruction of most extensive set of data to date and statistical analyses. Taking advantage of molecular systematics and the recent methodological advances in analyzing character evolution in a given phylogeny. Mangroves are found in about 20 families, 27 genera, and 69 species (Duke, 1992). They provide an impressive instance of trait evolution and a combination of diverse morphological and physiological adaptations. We mapped vivipary and secreter traits onto the phylogeny of a large sample of major mangroves with the outgroup and then reconstructed their evolutionary paths, from which the hypotheses about their evolutionary origin were evaluated. Since both vivipary and salt secretion appear much more frequently in mangroves than in any of their close relatives, our sampling scheme is thus, deliberately in favor of the single origin hypothesis. Therefore, when the conclusion of multiple origins is reached, the evidence is thus, much stronger than that from a more balanced sample of plants including many non-mangroves.

## 2. Materials and methods

### 2.1. Taxon sampling

We sampled 26 representative genera of major groups (17 families) of mangroves and mangrove associates, of which eight genera (five families) germinate viviparously and four genera (four families) control their salt balance by secreting sodium chloride (1, see Fig 1). *Nypa* is the only palm that has a viviparous fruits. We sequenced all samples of 18S rDNA and *mat*R mtDNA genes, and about 73% samples of *rbc*L cpDNA gene (19/26). Taxa sampled in this study are listed in Table 1, along with voucher information, literature citations, and GenBank accession numbers for the three data sets. The presence or absence of viviparous propagules and salt secreting leaves in mangroves are referenced primarily in Tomlinson's summary (Tomlinson, 1986).

We chose *Amborella* and *Nymphaea* as outgroups because they are successive sister taxa to the rest angiosperms (Qiu et al., 1999). *Amborella* was used to root the phylogeny, but not used for ancestral state analysis. The root node was treated the same as other nodes as suggested by Mooers and Schluter (1999). *Nymphaea*, however, was used as a root for reconstructing ancestral states.

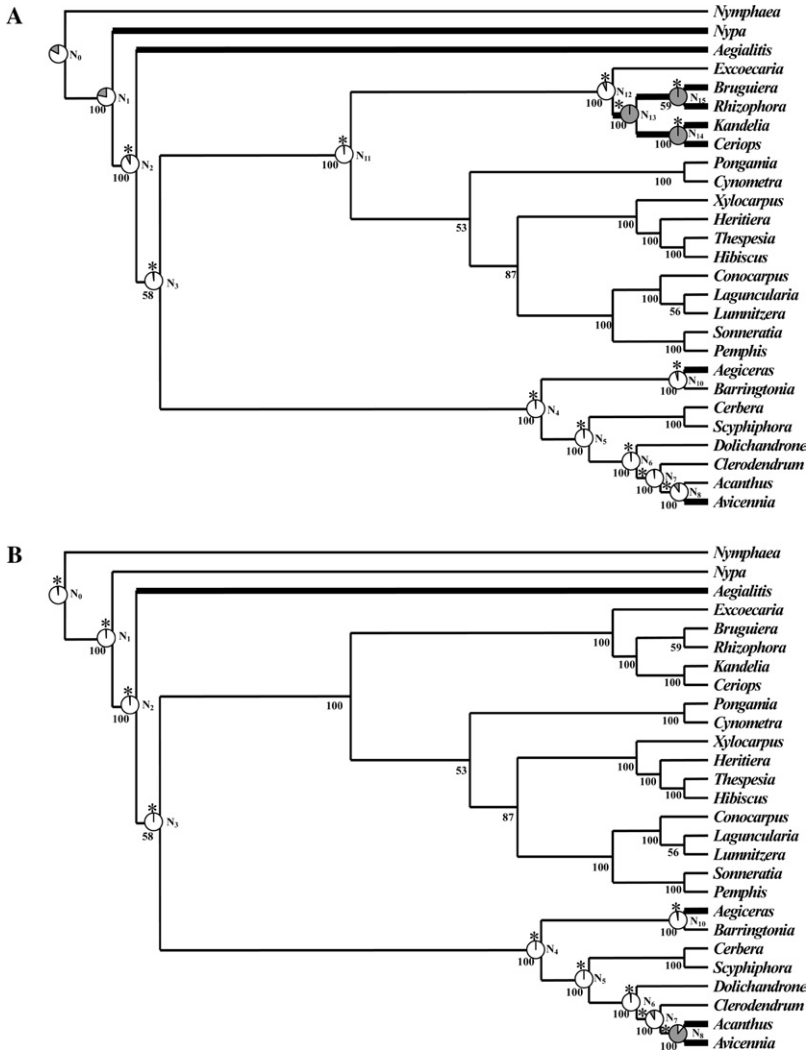


Fig. 1. Phylogenies of mangroves constructed by using Bayesian inference (BI) with the combined data set from *rbc*L, 18S, and *mat*R genes and maximum likelihood (ML) character mapping of vivipary and salt secretion, respectively. Clade credibility values for the phylogeny are noted below branches. (A) Pie charts represent relative ML supports at ancestral nodes for presence (black) and absence (white) of vivipary. (B) Pie charts represent relative ML supports at ancestral nodes for presence (black) and absence (white) of secreter. Asterisks indicate significant results (ln likelihood difference >2) using the same phylogeny and equal rates models of character evolution. The nodes ($N_0$, $N_1$,...) were labeled to the right.

### 2.2. Sequences and phylogenetic analysis

Total DNAs were extracted using the CTAB method of Doyle and Doyle (1987). Double-stranded copies of all regions {encoding 18S rRNA (nrDNA), *rbc*L (cpDNA), and *mat*R (mtDNA)}, were amplified using standard polymerase chain reaction (PCR) in 25–50 μl reactions. The primers of *rbc*L, 18S, and *mat*R followed Huang and Shi (2002), Whiting et al. (1997), and Meng et al. (2002), respectively. The PCR products of all samples were purified by using the QIAquick PCR Purification Kit (CN 28104, QIAGEN), and were sequenced in both directions by using an ABI 377 Genetic Analyzer (Applied Biosystems, CA). All sequences have been deposited in GenBank (for accession numbers see Table 1).

We aligned sequences with CLUSTALX (Thompson et al., 1997). We assessed the data congruence using the partition homogeneity test (Farris et al., 1994, implemented with PAUP*4.0b5) before combining the data sets (18S plus *mat*R: $P = 0.489$; 18S plus *rbc*L: $P = 0.097$; and *mat*R plus *rbc*L: $P = 0.565$; each with 1000 replicates using heuristic search conducted with 100 random-taxon-addition replicates with TBR branch swapping and MulTrees selected).

We used Metropolis-coupled Markov chain Monte Carlo (or MCMCMC) algorithm within a Bayesian framework to estimate the posterior probability of phylogenetic trees based on the combined data set (Lutzoni et al., 2001). Bayesian inference was carried out using MrBayes v2.01 (Huelsenbeck and Ronquist, 2001). We determined the best-fit model of molecular evolution

Table 1
Accessions of mangrove species used in this study

| Family | Species | Voucher | Source | GenBank Accession No. | | |
|---|---|---|---|---|---|---|
| | | | | 18S | *mat*R | *rbc*L |
| Acanthaceae | *Acanthus ebracteatus* | *X. Z. Qiu 171 (SYS)* | Futian, Shenzhen, Guangdong, China | AY289642 | AY289667 | AY289682 |
| | *Avicennia marina* | *Q. J. Zhan 99112201 (SYS)* | Futian, Shenzhen, Guangdong, China | AY289641 | AY289666 | AY289681 |
| Apocynaceae | *Cerbera manghas* | *S. G. Jian 0461(SYS)* | Qinglangang, Hainnan, China | AY289645 | AY289670 | AY289685 |
| Arecaceae | *Nypa fruticans* | *Z. H. Zhang 0744(SYS)* | Dongzhaigang, Hainan, China | AY289649 | AY289674 | AY289688 |
| Bignoniaceae | *Dolichandrone spathacea* | *S. G. Jian 0465(SYS)* | Qinglangang, Hainnan, China | AY289643 | AY289668 | AY289683 |
| Combretaceae | *Conocarpus erectus* | *X. J. Ge 1014 (SYS)* | Cult. Qinglangang, Hainan, China | AY289636 | AY289662 | AF281477[a] |
| | *Laguncularia racemosa* | *X. J. Ge 1011 (SYS)* | Cult. Qinglangang, Hainnan, China | AY289635 | AY289661 | AF425715[a] |
| | *Lumnitzera littorea* | *S. C. Chen 480 (SYS)* | Dongzhaigang, Hainan, China | AY289637 | AY289663 | AF425718[a] |
| Euphorbiaceae | *Excoecaria agallocha* | *Z. H. Zhang 0459 (SYS)* | Qinglangang, Hainnan, China | AY289628 | AY289654 | AY289675 |
| Fabaceae | *Cynometra iripa* | *S. H. Shi. 0522 (SYS)* | Daintree River, Cairns, Queensland, Australia | AY289630 | AY289656 | AY289677 |
| | *Pongamia pinnatas* | *S. G. Jian 0397(SYS)* | Qi'ao Island, Zhuhai, Guangdong, China | AY289629 | AY289655 | AY289676 |
| Lecythidaceae | *Barringtonia racemosa* | *C. P. Zhang 488 (SYS)* | Dongzhaigang, Hainan, China | AY289647 | AY289672 | AF088853[b] |
| Lythraceae | *Pemphis acidula* | *C. C. Liao 1150 (A)* | Deposited in A, collected from Lanyu Island, Taiwan, China | AY289639 | N/A | AY036138[c] |
| | *Sonneratia ovata* | *H. T. Chang 9711912(SYS)* | Dongzhaigang, Hainan, China | AY289638 | AY289664 | AY036143[c] |
| Malvaceae | *Heritiera littoralis*e | *S. G. Jian 0168(SYS)* | Qinglangang, Hainnan, China | AY289633 | AY289659 | AY289679 |
| | *Hibiscus tiliaceus* | *S. G. Jian 0247(SYS)* | Touyuan, Wenchang, Hainan, China | AY289631 | AY289657 | AY289678 |
| | *Thespesia populnea* | *S. H. Shi. 640(SYS)* | Futian, Shenzhen, Guangdong, China | AY289632 | AY289658 | L01961[d] |
| Meliaceae | *Xylocarpus granatum* | *S. G. Jian 0458(SYS)* | Qinglangang, Hainnan, China | AY289634 | AY289660 | AY289680 |
| Myrsinaceae | *Aegiceras corniculatum* | *S. H. Shi 0519(SYS)* | Daintree River, Cairns, Queensland, Australia | AY289648 | AY289673 | AY289687 |
| Plumbaginaceae | *Aegialitis annulata* | *Z. H. Zhang 0753(SYS)* | Dongzhaigang, Hainan, China | AY289640 | AY289665 | AJ312252[e] |
| Rhizophoraceae | *Bruguiera sexangula* | *S. H. Shi 2000-01001 (SYS)* | Dongzhaigang, Hainan, China | AY289626 | AY289652 | AF127691[f] |
| | *Ceriops tagal* | *H. T. Chang 9711902 (SYS)* | Dongzhaigang, Hainan, China | AY289624 | AY289650 | AF127684[f] |
| | *Kandelia candel* | *H. T. Chang 9711905 (SYS)* | Dongzhaigang, Hainan, China | AY289625 | AY289651 | AF127682[f] |
| | *Rhizophora stylosa* | *X. Z. Qiu 974306 (SYS)* | Futian, Shenzhen, Guangdong, China | AY289627 | AY289653 | AF127686[f] |
| Rubiaceae | *Scyphiphora hydrophyllaceae* | *Z. H. Zhang 0750(SYS)* | Dongzhaigang, Hainan, China | AY289646 | AY289671 | AY289686 |
| Lamiaceae | *Clerodendrum inerme* | *S. G. Jian 0396(SYS)* | Qi'ao Island, Zhuhai, Guangdong, China | AY289644 | AY289669 | AY289684 |

SYS, Sunyatsen (Zhongshan) University; A, Harvard University, Arnold Arboretum. Classification follows APG (2003).

[a] Tan et al. (2002).
[b] Tsou et al. (Direct submission to GenBank in 1998).
[c] Huang and Shi (2001).
[d] Albert et al. (1992).
[e] Lledo et al. (2001).
[f] Schwarzbach and Ricklefs (2000).

to be general-time-reversible model with invariant sites and gamma distributed rates for variable sites (GTR + I + $\Gamma$) by using MrModeltest (Posada and Crandall, 1998, written by Johon A.A. Nylander). We used the equivalent model in MrBayes (basefrequence, estimate; nst, 6; rates, invgamma; gamma shape, estimate) to initiate the maximum likelihood (ML) search. The Markov chains were run for one million generations sampling every 10 generations for a total of 100,000 samples each run to assure that successive samples were independent. We then removed the first 10,000 samples from each run to avoid including any trees that might have been sampled before convergence of the Markov chain. The remaining samples were combined into a single file and analyzed using the 'sumt' command in MrBayes. We calculated the posterior probability of ancestral nodes based on the consensus tree with mean branch lengths and clade probabilities.

### 2.3. Ancestral state reconstruction

We applied the continuous-time Markov model and ML estimate (Pagel, 1994, 1999) to reconstruct ancestral states of vivipary and salt secretion in mangroves using DISCRETE 4.0. We performed likelihood ratio tests (model test) to determine whether the two-rate model fits the data significantly better. We also used DISCRETE to calculate the ML estimate of $\alpha$ (forward rate) and $\beta$ (backward rate) of each trait under the selected model in model test. We preferred the "global" method to the "local" method because the "global" method is simpler and it enabled us to evaluate the relative ML support [a difference between ln (likelihood) of the two states] at each node (Mooers and Schluter, 1999).

### 2.4. Likelihood sensitive analysis

We used the likelihood sensitive analysis (LSA) developed by Oakley and Cunningham (Oakley and Cunningham, 2002) to evaluate whether a trait is of single- or multiple-origin. We applied this method to both vivipary and secretors. The calculation was done in Mathematica 4.0 (Wolfram) by using the 'pruning' algorithm (Felsenstein and Churchill, 1996). Our implementation extends slightly Oakley and Cunningham's original method. Taking vivipary as an example, in Oakley and Cunningham's method, the nodes $N_j$ ($j = 0, 1, \ldots, 8, 10, 11, 12$) are set to 1 (having the specific trait) under the homology hypothesis, and set to 0 (not having the trait) under the multiple-origin hypothesis. In our implementation, however, we further assign definite values to several nodes, i.e., setting $N_k = 1$ ($k = 13, 14, 15$) under both hypotheses. This implementation would make our method more conservative.

## 3. Results

### 3.1. Phylogeny

The phylogeny of mangroves constructed by using Bayesian inference (BI) from the combined data of 18S rRNA (nrDNA), *rbc*L (cpDNA), and *mat*R (mtDNA) (Fig. 1) shows a similar, but more highly resolved phylogenetic pattern for mangroves than recognized by the Angiosperm Phylogeny Group (APG, 2003). All groups with vivipary (Fig. 1A), and salt secretion (Fig. 1B) are polyphyletic and nested within other mangrove species that lack the traits.

### 3.2. Ancestral state reconstruction

The results of ancestral state reconstruction are presented in Fig. 1. Vivipary and salt secretion significantly favor multiple origins. For vivipary, the five top nodes all favor the absence of the character and the likelihood ratio test is significant for three of them (Fig. 1A). For salt secretion, all top four nodes significantly favor the absence of the character (Fig. 1B).

In the analysis of either character, the one-rate model, in which the rate of character-loss equals that of char-

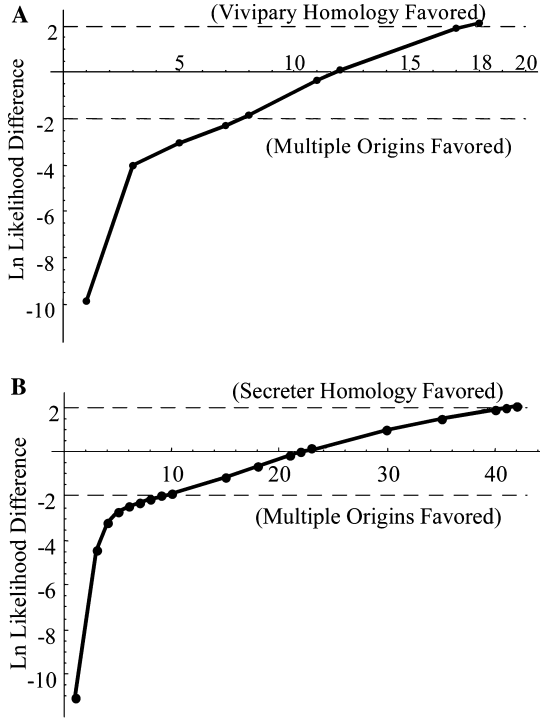


Fig. 2. The sensitivity analyses comparing likelihood models with differing amounts of asymmetry in rates of the evolutions of vivipary (A) and salt secretion (B) in mangroves. The *y*-axis in both diagrams represents the difference between the ln likelihood of homology hypothesis and ln likelihood of multiple origins hypothesis. The *x*-axis values refer to the ratio of backward rate ($\beta$) to forward rate ($\alpha$). A 10 on the *x*-axis is a model, where $\beta = 10 \times \alpha$.

acter-gain, is favored over the two-rate model. The rates are $\alpha$ (forward rate) $= \beta$ (backward rate) $= 11.09$ for vivipary and $\alpha = \beta = 6.19$ for salt secretion. Mooers and Schluter (1999) studied the effects of using one- vs. two-rate models and concluded that a one-rate model of discrete trait evolution worked better in most situations, which is consistent with our results. Although we do not have independent evidence for the relative rate of character gain vs. loss, a simple parsimony analysis suggests that the equal rate assumption is not farfetched. In Fig. 1A for vivipary, the minimal number of gain or loss to account for the observations is 5 or 7, respectively. In Fig. 1B for secretor, the minimal gain:loss numbers are 3:5.

*3.2.1. Likelihood sensitive test*

In the likelihood sensitive test, a multiple origin hypothesis of vivipary holds when the ratio of loss to gain is less than 8 (Fig. 2A). The homology hypothesis of vivipary would not be favored before the assumed evolution model is skewed to more than 12:1 and would not be statistically significant before 18:1 (upper critical value). Similar results have been derived from secreters (Fig. 2B) with three different critical values, 10, 23:1, and 42:1.

## 4. Discussion

In the statistical analysis, we did not (in fact, could not) determine a priori if any of the two traits is truly homologous across taxa. We reasoned that, if these traits are not homologous, then the statistical test will reveal their multiple origins. Indeed, we were able to conclude that both vivipary and salt secretion are most likely of multiple origins in mangroves. In the phylogenetic framework of Figs. 1A and B, the multiple origin hypothesis of vivipary is supported even when the chance of the loss of vivipary is higher than the gain (Fig. 2). The single origin hypothesis is only supported when the ratio of the rate of loss to gain is 12:1 or larger, and must be 18:1 or larger for the result is significant (Fig. 2). Similar conclusions hold for the secretion. Specifically, the ratio of loss to gain must be larger than 23:1 to favor the single origin hypothesis, and the result will be significant only when the ration is larger than 42:1 (Fig. 2). Therefore, the evidence for the conclusion of multiple origins for salt secretion is stronger than that for vivipary. We mentioned in Section 1 that our sampling scheme is in favor of the hypothesis of single origin. This is because both traits are most frequent in mangroves than any of their relatives, thus, it will require the least number of loss to explain the data than it would have been were a more balanced sample used, including some non-mangroves. For this reason, we conclude that the rate of loss must be at least 18 times and 42 times higher than gain to favor the hypothesis of single origin for vivipary and salt secretion, respectively.

The likelihood sensitive analysis we implemented is more flexible than the original proposed by Oakley and Cunningham (2002) because it allows assigning definite status to both external nodes (leaves) and internal nodes. We deem that fixing the status of some external nodes when justifiable will lead to more powerful analysis in general. In vivipary, for example, under the hypothesis of multiple origins, we set $N_i = 0$ ($i = 0, 1, \ldots, 8, 10, \ldots, 11, 12$). We further set $N_j = 1$ ($j = 13, 14, 15$). Doing so resulted in slightly stronger evidence supporting the hypothesis of multiple origins for both vivipary and salt secretion.

Although we are in favor of the multiple origin hypothesis for both vivipary and salt secretion based on our analysis, we realize the final conclusion will depend much on the knowledge about the rate of loss and gain of these characters, which can not be resolved by the sequence data presented in this study. Unfortunately such information is scarce either from molecular biology or plant physiology. To date, only vivipary morphology and physiology have been well examined previously in mangroves and these data are consistent with the salient adaptive values and vivipary homology (Farnsworth and Farrant, 1998; Tomlinson and Cox, 2000; Van der Pijl, 1983). Why vivipary should be so common in many unrelated mangrove taxa is a topic that has fostered considerable discussion but not generally accepted explanation (Tomlinson, 1986). Farnsworth and Farrant (1998) compared the multiple independent origins of vivipary within a phylogenetic context to address how physiological changes occurred during evolution of this trait. They investigated physiological mechanisms behind the convergent evolutionary loss of seed dormancy in mangrove lineages. Chapin et al. (1993) indicated that it was reasonable to conjecture relatively simple evolutionary changes in hormonal control may alter many plant behaviors simultaneously because phytohormones critically control multiple aspects of plant life histories. Present studies provided molecular phylogenetic data and character evolution analysis to reconstruct vivipary's ancestral state. Our analyses under maximum likelihood strongly support the multiple origins of vivipary in mangroves. Some equivocations, however, still exist based on the MP ancestral state reconstructions of this special trait.

The mechanism of salt secretion is even less understood than vivipary. Although the structure of salt glands in different salt-secreting mangroves is fairly similar which is evidence for the evolutionary convergence (Fahn, 1979; Tomlinson, 1986). Our results from the ML ancestral state reconstruction relatively strongly favor its multiple origin in mangroves.

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