RAPID SPECIATION AND THE EVOLUTION OF HUMMINGBIRD POLLINATION IN NEOTROPICAL COSTUS SUBGENUS COSTUS (COSTACEAE): EVIDENCE FROM nR DNA ITS AND ETS SEQUENCES

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We estimate phylogenetic relationships and the biogeographic and pollination history of Costus subgenus Costus (Costaceae) using sequence data from the internal and external transcribed spacer (ITS and ETS) regions of 18S–26S nuclear ribosomal DNA. The African members of the subgenus form a series of lineages basal to a monophyletic neotropical species radiation. The neotropical species have large, showy flowers visited by either euglossine bees or hummingbirds. The hummingbird pollination syndrome is supported as a derived character state from the bee pollination syndrome, and we estimate that it has evolved independently seven or more times in the neotropics. A molecular clock approach suggests that diversification of the neotropical clade has been recent and rapid and that it coincides with dramatic climatic and geologic changes, Andean orogeny, and the closing of the Panama isthmus that occurred in the Pliocene and Pleistocene epochs. We propose a scenario for the diversification of Costus, in which rapid floral adaptation in geographic isolation and range shifts in response to environmental changes contribute to reproductive isolation among close relatives. We suggest that these processes may be common in other recently diversified plant lineages centered in Central America or the Northern Andean phytogeographic region.

Key words: Costaceae; ETS; euglossine bees; hummingbirds; ITS; neotropics; pollination syndromes; speciation.

Neotropical forests harbor the highest plant diversity of any tropical region (Raven, 1976; Gentry, 1982), yet the evolutionary history of most resident plant lineages is poorly understood. Dynamic climatic and geological changes, especially in recent epochs, have been proposed as a causative agent of allopatric speciation (Haffer, 1969; Bush, 1994; Graham, 1997; Haffer and Prance, 2001). Some have suggested that speciation may occur at a faster rate in the tropics (Fischer, 1960; Gentry, 1989; Schemske, 2002) and that strong biotic interactions may contribute to higher rates of diversification (Dobzhansky, 1950; Corner, 1954; Ashton, 1969; Schemske, 2002). In particular, plant–pollinator interactions have been suggested to be more specialized in tropical regions (Johnson and Steiner, 2000; but see Ollerton and Cranmer, 2002), and specialized pollination systems may drive adaptive divergence in floral morphology and contribute to reproductive isolation between close relatives (Stebbins, 1970, 1974; Grant, 1981). In contrast, others have stressed the relative antiquity of neotropical plant lineages (Dick et al., 2003) and the long-term stability of high neotropical plant diversity (Colinvaux and De Oliveira, 2001; Wilf et al., 2003) and have criticized hypotheses for rapid and recent diversification, such as Pleistocene refugia (Haberle and Maslin, 1999; Colinvaux et al., 2001). Nevertheless, there has been little empirical evidence regarding the biogeographic pattern and timing of diversification of neotropical plant lineages, and few studies have examined the evolutionary history of changes in pollination systems that may contribute mechanistically to speciation.

The debate over the diversification of the neotropical flora may be partly resolved by recognizing that different lineages have different histories and responses to environmental change (Bush, 1994; Morley, 2000). The flora is thought to consist primarily of lineages of Gondwanan origin that dominate the vast lowland forests, along with some of Laurasian origin that are relegated mainly to high montane temperate-like habitats (Raven and Axelrod, 1974). Within the groups categorized as Gondwanan, Gentry (1982) recognized two main categories by their phytogeographic region of highest diversity and endemism: woody canopy trees and lianas with a center of diversity in the Amazon and epiphytes, understory shrubs, and understory monocots with a center of diversity at the base and along the lower slopes of the Northern Andes or in Southern Central America. The plants in this second category constitute over half of the neotropical species diversity and include orchids, bromeliads, gingers, gesneriads, acanths, Inga, and Psychotria, among others.

Gentry suggests that contrary to the gradual diversification of Amazon-centered trees, these lineages have undergone "ex-...
plosive speciation and adaptive radiation” (Gentry, 1982, p. 587) in response to dramatic changes in geology and climate. The entire neotropics experienced climatic oscillations during the Pleistocene, but volcanism in southern Central America, the closing of the isthmus of Panama approximately 3.5 million years ago (Ma), and the uplift of the Northern Andes from about 5 Ma (Gentry, 1982, Graham, 1997) would have affected these lineages, in particular. Gentry also notes that specialized pollination, particularly by hummingbirds, is a prominent feature of these groups and that shifts in specific pollinators appear to be a common mode of speciation. The center of hummingbird diversity is in the northern Andes (Bleiweiss, 1998), and this wide array of potential pollinators may have further promoted speciation in plant lineages already experiencing dramatic spatial and temporal environmental heterogeneity.

These ideas for the diversification of northern Andean-centered herb, shrub, small tree, and epiphyte lineages require evidence from phylogenetic studies. Support for rapid and recent speciation has been found for the Andean-centered small tree genus Inga (Richardson et al., 2001). Several other neotropical lineages have been examined for specialized pollination systems, some in a phylogenetic context, including such diverse groups as Aphelandra (Madden, 1984, 1992), Heliconia (Stiles, 1975), tribe Sinningieae (Gesneriaceae; Perret et al., 2001, 2003), Erythrina (Bruneau, 1997), Dalechampia (Armbuster, 1993, 1994), and Costus (Kay and Schemske, 2003). Studies are needed, however, that combine phylogenetic analysis of the biogeographic history and timing of diversification along with an examination of the evolution of ecological features, such as pollination systems, that may contribute mechanistically to speciation. Understanding the diversification of these lineages will contribute fundamentally to resolving the mystery of the extraordinary species richness of the neotropical flora.

Costus subgenus Costus provides an opportunity to examine the diversification of species and pollination systems of a northern Andes/Central America-centered plant group. The family Costaceae, commonly known as the spiral gingers, has its origin in Africa (Specht et al., 2001) and contains four genera, of which Costus is by far the most species rich. Costus subgenus Costus, with approximately 51 species in the neotropics and several species in Africa, comprises most of the diversity in the genus (Maas, 1972, 1977; Maas and Maas, 1990; Garcia-Mendoza, 1991; Maas and Maas-van de Kamer, 1997). Its primary center of diversity is in Costa Rica, Panama, western Colombia, and Ecuador, although it ranges from Mexico to Brazil. Although there are a few widespread species, many are locally restricted, with several known only from their type locality. All species are herbaceous, and they occur in a variety of habitats, including streamsides, treefall gaps, and limestone outcrops. Species are found from low to mid elevation, but are most common in wet, low-elevation sites.

The neotropical Costus species are remarkable for their floral biology. The characteristic spiraling stems support terminal inflorescences that generally produce a single large showy flower each day of an extended flowering season. Each species can be classified as having either a bee- or hummingbird-pollination syndrome based on distinct suites of floral characters. In his monograph for Flora Neotropica, Maas (1977) divided the subgenus into sections Costus and Ornithophilus according to pollination syndromes. Flowers of bee-pollinated Costus have a broad, pale labellar tube with a distinct white or yellow limb, the lateral lobes of which are often striped with red or purple, and the floral bracts are green. Hummingbird-pollinated species have flowers with a narrow, tubular labellum without an attached limb, and the labellum and floral bracts are yellow, orange, or red. Flowers in both pollination categories are odorless and diurnal, and they produce relatively large quantities of nectar. In a study of 11 species, Kay and Schemske (2003) found that these syndromes accurately predicted the type of pollinators, with species in sections Costus and Ornithophilus visited almost exclusively by orchid bees ( Euglossini: Apidae) and hummingbirds, respectively.

Here we use rDNA internal and external transcribed spacer (ITS and ETS) sequence data to examine the hypothesis of explosive recent speciation and pollination specialization within in Costus subgenus Costus using a phylogenetic approach. We estimate species-level relationships using Bayesian inference and parsimony, and we reconstruct ancestral states for pollination syndromes and biogeographic distributions. We attempt to determine the biogeographic origin of subgenus Costus, whether it has undergone rapid and recent diversification in the neotropics, and whether the diversification is associated with repeated evolutionary shifts in the pollination system. Because there are no fossils known from the Costaceae, estimates of divergence times and diversification rates are made with a molecular clock approach from published rates of ITS evolution and from a biogeographic calibration.

**MATERIALS AND METHODS**

**Taxon sampling**—We sampled leaf tissue from 41 species of Costus, 38 of which are classified in subgenus Costus. When possible, we attempted to include individuals from different subspecies, varieties, or geographically dispersed populations within each species. Overall, we sampled 63 individuals. Leaf tissue was collected on silica gel in the field or fresh from the collection in the Michigan State University greenhouse. Additional silica-dried samples were contributed by botanical gardens and a private collection. Taxonomic determinations were made by K.M.K., D.W.S., or the contributing garden or collection. Voucher and collection information is summarized in the Appendix. Costus laterifolius, C. leteusti, and C. talbotii were selected as outgroups based on a previous phylogenetic analysis of the Costaceae (Specht et al., 2001).

The taxonomy of the paleotropical Costus is poorly understood (reviewed in Kay, 2004). Much conflict exists in the subgeneric assignment of species (Schumann, 1904; Koechlin, 1964; Maas, 1979; Specht et al., 2001), and many named species are effectively indistinguishable and have been put into synonymy (Koechlin, 1964; Hutchinson and Dalziel, 1968; Maas, 1979; Lock, 1985). Therefore, we sampled the three most geographically widespread and frequently collected African species that likely belong to subgenus Costus sensu Specht et al. (2001) as representative of paleotropical molecular diversity within the subgenus.

**Molecular methods**—Total genomic DNA was extracted from fresh or silica-dried tissue using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) or the FastDNA Kit (Bio 101, Carlsbad, California, USA). The ITS region (ITS1, 5.8S, and ITS2) was amplified using the ITS4 and ITS5 primers (Baldwin, 1992). To design a Costus-specific primer for ITS, the entire ITS region was amplified from a subset of taxa by a long polymerase chain reaction (PCR) using the primers 18S-IGS and 26S-IGS (Baldwin and Markos, 1998). The 3’ region of the ITS was sequenced and a 5’ primer (5’- CTTTGTTGTGCTCGGCGGAGTTC-3’) was designed. The 3’ end of the ITS was amplified from all taxa using this primer and 18S-IGS. Long PCR conditions were as follows: 94°C for 75 s, 30 cycles of 94°C for 15 s, and 68°C for 8 min, with a final extension of 72°C for 10 min. All other PCR reactions consisted of 2.5 µL Promega (Madison, Wisconsin, USA) 10X reaction buffer, 2.5 µL 25 mmol/L MgCl2, 1.25 µL 40 mmol/L dNTPs,
Analyses—The ITS and ETS sequences were aligned manually using the program Se-Al version 1.0a1 (A. Rambaut, University of Oxford, Oxford, UK). Unambiguous gaps were scored as missing data and then recorded as binary characters in a separate matrix following the procedure of Simmons and Ochoterena (2000).

A preliminary parsimony analysis including all sequenced clones and PCR products was conducted using PAUP* version 4.0b10 (Swofford, 2002) separately for ITS and ETS. A heuristic search strategy was used to construct a strict consensus tree, with 1000 random sequence addition replicates and 10 best trees held per iteration. If all the clones from one individual were supported as monophyletic or as components of the same polytomy, a representative tree was selected from each PCR product for sequencing. Sequences were deposited in GenBank (AY972877–AY973004).

Bayesian phylogenetic analysis was performed using MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001) with five data partitions: ETS, ITS1, 5.8S, ITS2, and gaps. The first four partitions employed the general time reversible (GTR) model of DNA substitution with gamma-distributed rate variation across sites. For the gap partition, rate variation was modeled using a gamma distribution but with a correction for scoring only variable sites ("coding = variable"). All default priors were used except that rates were allowed to vary across partitions ("ratep = variable") and for the gap partition, the dirichlet prior was fixed for equal character state frequencies ("symdirhyperp = fixedinfinity"), as recommended in the MrBayes manual for data in which states have different meanings across characters. All priors were unlinked except branch lengths were allowed to vary proportionally across partitions. Four replicates of the Markov chain Monte Carlo (MCMC) search were run with four chains and 5,000,000 generations each, and trees were sampled every 100 generations. A burn-in of 1,000,000 generations for each replicate was determined by stabilization of the likelihood scores, and these trees were removed from the analysis. A majority rule consensus tree of the remaining trees from each replicate provided posterior probabilities for each node.

We also conducted parsimony analysis for the combined data set. Running PAUP* version 4.0b10 on a computer cluster (Reeves et al., 2005), we conducted a single heuristic parsimony search using tree bisection-reconnection (TBR) branch swapping with MULTREES = ON, followed by 360 200 further replicate searches with the above settings, except that only 100 trees were saved per replicate. A strict consensus of the resulting trees was computed. Clade support was estimated using bootstrap values (Felsenstein, 1985), which were calculated using 1000 bootstrap replicates of 10 random additions each and 10 trees saved per replicate (DeBry and Olmstead, 2000).

To explore the evolutionary history of pollination syndromes, we attempted to determine transformation rates between pollination syndromes as well as estimate the total number of shifts that have occurred in the neotropics. Each species was scored as either bee or hummingbird pollinated based on pollinator observations or pollination syndrome. To avoid overestimating the number of shifts in pollination syndrome, trees were first pruned so that each species or variety was represented only once. Because different samples of C. wilsonii and C. varzeaum were found in disparate parts of the tree and likely represent distinct lineages, both accessions of each were retained in the pollination analyses. In neither case did this affect the estimates of the number of shifts in syndrome. Local maximum likelihood estimators of transformation rates were determined using Multistate (Pagel, 1994, 1999) on the Bayesian majority rule consensus tree with branch lengths averaged over all trees from the posterior distribution and branch length scaling set to its maximum likelihood value. Polytomies in the consensus tree were resolved randomly with branch lengths of 0.001 for the maximum likelihood analysis. Asymmetries in transformation rates were tested with a likelihood ratio test.

To estimate conservatively the minimum number of shifts between syndromes while accounting for uncertainty in tree topology, ancestral states were reconstructed using parsimony in MacClade version 4.06 (Maddison and Maddison, 2001) across the sample of the posterior distribution of trees from the Bayesian analysis. This is a sample of the trees found by the MCMC search after stabilization of the likelihood scores (burn-in) and is thought to represent the optimal neighborhood of tree space. For each of the 160000 trees, we recorded the number of unambiguous shifts between pollination syndromes, which ignores any shifts occurring at unresolved nodes and is thus likely an underestimate. These values were then used to calculate a mean number of shifts and a 95% confidence interval. To determine further the support for multiple transformations to hummingbird pollination, we also conducted a parsimony search on the combined data set with all hummingbird-pollinated taxa constrained to be monophyletic (1000 heuristic search replicates, 100 trees saved per replicate). The resulting strict consensus tree was compared to the strict consensus tree from the unconstrained parsimony search with a one-tailed Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) with resampling estimated log-likelihood (RELL) optimization and 1000 bootstrap replicates.

To reconstruct the biogeographic history of subgenus Costus, trees were pruned so that each species or variety was represented only once. Species were coded according to the following phytogeographic regions or combinations thereof: Africa, Central America, northern Andes, southern Andes, the greater Amazon basin, and the Caribbean islands. Ancestral states for phytogeographic region were reconstructed using parsimony in MacClade. We were particularly interested in determining the continent of origin of the entire subgenus and the specific biogeographic origin of the neotropical radiation. Therefore, we examined the sensitivity of the reconstruction of these nodes to both uncertainty in tree topology and the addition of unsampled species that clearly belong in subgenus Costus. The inferred character states of the basal nodes of the subgenus and of the neotropical radiation were examined across the posterior distribution of trees from the Bayesian analysis. We also coded 16 unsampled neotropical species for phytogeographic region and attached them at random to neotropical branches of the Bayesian consensus tree using the TreeFarm module of Mesquite (Maddison and Maddison, 2004). With 1000 replications of this tree augmentation, we were able to examine possible phylogenetic scenarios that would change our inference for the biogeographic origin of the neotropical species. A test for evolutionary rate constancy across taxa for ITS1 and ITS2 was conducted by using a tree-wide likelihood ratio test for differences between clock-constrained and clock-unconstrained trees (Felsenstein, 1988). Likelihood scores were obtained using a K80 + G model of sequence evolution (as chosen by MODELTEST for ITS 1 and ITS 2) for the majority rule consensus tree identified through Bayesian analysis of the full data set. The like-
Results

The total aligned length of the ITS region was 708 base pairs (bp) (ITS1 = 286 bp, 5.8S = 164 bp, ITS2 = 258 bp), and the aligned ETS region was 579 bp. Twenty-two gaps were scored, nine from ITS and 13 from ETS. The overall data set included 1309 characters, 223 of which were informative for parsimony. The ITS and ETS substitutions yielded 71 and 130 informative substitutions, respectively; all the gaps scored were informative. Excluding the outgroups reduced the number of parsimony-informative characters within subgenus Costus to 128.

The ILD test showed that the ITS and ETS partitions of the data were not significantly incongruent (P = 0.66). Strict consensus trees based on separate parsimony analysis of ITS and ETS were examined for topological conflicts, and none was found. All further analyses were conducted on the total data set.

Bayesian MCMC analyses of the partitioned data set resulted in stabilization of likelihood scores well before the generations discarded as burn-in, and consensus trees were consistent across the four replicate analyses. Many nodes were resolved with high posterior probability (Fig. 1). The neotropical species within subgenus Costus were supported as monophyletic, while the African taxa formed a series of lineages basal to this radiation. Multiple populations sampled for a given species were often not monophyletic and were typically part of a multi-species polynomy.

A single heuristic parsimony search using TBR branch swapping with MULTREES = ON produced 156609 equally parsimonious trees of length 557. Sixty-three additional unique trees of length 557 were recovered after performing 360200 replicate searches. Trees had a consistency index (CI) of 0.835, retention index (RI) of 0.891, and rescaled consistency index of 0.744. A strict consensus of the 156672 most parsimonious (MP) trees was computed. There were no differences between the consensus trees from the Bayesian and parsimony analyses except for a few clades reconstructed in the former but not the latter (Fig. 1).

Pollinator shifts—Hummingbird pollination has evolved multiple times since subgenus Costus colonized the neotropics (Fig. 2). The hummingbird-pollination syndrome is supported as derived, relative to bee pollination, with no strong evidence for reversals. Across the posterior distribution of trees from the Bayesian analysis, parsimony analysis finds a mean of 6.5 unambiguous shifts from bee to hummingbird pollination (median = 7; 95% CI = 3–9; Fig. 3) and a mean of 0.1 unambiguous shifts from hummingbird to bee pollination (median = 0; 95% CI = 0–1; Fig. 3). Parsimony analysis conducted with the constraint that all hummingbird-pollinated taxa are monophyletic yields most parsimonious trees that are 36 steps longer than the best trees from the unconstrained analysis. An SH test of the strict consensus trees from the constrained and unconstrained analyses shows that the data better fit the unconstrained tree (P < 0.001), supporting multiple origins of hummingbird pollination. Maximum likelihood estimates of transformation rates using mean branch lengths are α = 1.61 (bee to hummingbird pollination) and β < 0.00 (hummingbird to bee), with an optimal branch length scaling parameter of 0.70. The asymmetrical model of unequal transformation rates between syndromes is significantly better than a model in which rates are constrained to be equal (χ² = 4.33, df = 1, P = 0.04).

Biogeography—Reconstructing the biogeographic history of the subgenus suggests that Central America was colonized from Africa, with subsequent dispersal to the Andes, the Amazon, and the Caribbean (Fig. 4). Across all trees from the posterior distribution, the African taxa are resolved as basal to the neotropical clade, and the base of the neotropical clade is resolved as Central American. This second result is robust to most topological arrangements of unsampled species on the strict consensus tree. The unsampled species included only two from the Amazonian phytogeographic region; the other 14 were either Central American or Andean. Out of 1000 tree augmentation replicates, the origin is resolved unequivocally as Central American 939 times. In 61 replicates, the origin is equivocal, but always includes Central America as one of the possible states, along with the northern Andes (31 times), the southern Andes (22 times), and/or the Amazon (12 times).

Diversification rate—Rates of evolution of ITS 1 and 2 within Costus are consistent with a molecular clock (χ² = 52.78, df = 63, P = 0.82). Maximum likelihood branch lengths show 0.0122 substitutions per site in ITS since the basal divergence between Africa and the neotropics. This suggests an approximate date for that node of 1.5–7.1 Ma using the range of published ITS substitution rates for herbaceous plants (Richardson et al., 2001; Zhang et al., 2001) or 4.6 Ma using the closing of the Panama isthmus as a calibration for Costus. Assuming that all c. 51 neotropical species of subgenus Costus are monophyletic, including the 16 known but unsampled species, a diversification rate of 0.6–2.6 species per million years is estimated from the date for the basal split from the African subgenus Costus using the range of published ITS rates or 0.9 species per million years using the closing of the isthmus as a calibration. The earliest dispersal to South America is similarly estimated at 1.1–5.4 Ma using the range of published rates.
DISCUSSION

Phylogenetic analysis and taxonomic considerations—The ITS and ETS rDNA sequences proved to be useful tools for estimating phylogenetic relationships within Costus subgenus Costus. The results of this study are entirely consistent with the broader phylogenetic analysis of Specht et al. (2001), but provide much more detail for the neotropical species radiation. As expected with a rapid diversification, not all nodes were resolved even with these quickly evolving loci. New World Costus subgenus Costus was found to be monophyletic, with the African lineages resolved as basal. The result was not surprising, given that there are no species that naturally occur on both continents and that the frequency of intercontinental dispersal is likely to be low. Our study does not clarify the sister group of the neotropical radiation because of incomplete sampling of African species and low support for basal relationships. In a post hoc parsimony analysis (1000 heuristic search
replicates, 100 trees saved per replicate), we found that constraining the African subgenus *Costus* taxa to be monophyletic resulted in MP trees of a single additional step. An SH test of the strict consensus trees from constrained and unconstrained analyses (1000 bootstrap replicates and RELL optimization) shows that the data do not reject the hypothesis of reciprocal monophyly between the African and neotropical members of the subgenus ($P = 0.167$). Additional sequence and more thorough taxon sampling will be necessary to address that question. Within the neotropical clade, Maas’ (1972, 1977) sections *Ornithophilous* and *Costus*, based on pollination syndromes, clearly are not supported as natural groups.

For the species in the study, we often found divergent sequences when more than one accession was sampled. In some cases, accessions from distinct geographic areas were distantly related, and we suggest that they may represent independent evolutionary lineages that have converged on a similar phenotype. The varieties of *C. guanaiensis*, for example, deserve further evaluation for elevation to species status, as does the *C. aff. wilsonii* from the Monteverde region. For both of these...
cases, multiple individuals were sequenced, and sequences were found to be identical within a given site (results not presented). In other cases, we found that different populations of a species yielded divergent sequences that were not monophyletic but that stemmed from the same polytomy. For example, our extensive sampling of the geographically widespread Costus woodsonii but that stemmed from the same polytomy. For example, a species yielded divergent sequences that were not monophyletic (represented). In other cases, we found that different populations of species were nested. C. pulverulentus and C. scaber resulted in a large polytomy, within which were nested C. spicatus, C. woodsonii, and the Montevede accession of C. aff. wilsonii. A cautious interpretation is that these five species are very recently diverged, and for such closely related species, the ITS/ETS gene tree has not yet attained reciprocal monophyly for the constituent species. A better estimate of the evolutionary history of such a recent diversification would require multiple unlinked loci and more thorough population sampling.

We sometimes observed divergent ITS or ETS alleles within a single individual. It is possible that these were pseudogenes, but they did not have mutations in the conserved 5.8S region. Their presence may also be explained by incomplete concerted evolution, minor replication errors propagated during PCR, or gene flow between divergent populations.

Pollination systems—Reconstruction of ancestral pollination states suggests that changes in pollination biology have been a prominent feature in the neotropical radiation of Costus subgenus Costus. Although there is almost no information on the pollination systems of African subgenus Costus, their floral traits are characteristic of bee pollination, all possessing white tubular flowers with pink and/or yellow nectar guides and green floral bracts. In the neotropical clade, the majority of the species have a hummingbird pollination syndrome, and the bee-pollinated species for which there are pollinator observations have further specialized on orchid bees (Kay and Schemske, 2003). Neither hummingbirds nor orchid bees occur in Africa, so all neotropical species likely have derived pollination systems, although the characters involved in hummingbird pollination are especially distinct, including bright reds and yellows, tubular curved corollas, and a reduction in the labellum that bee pollinators use as a landing platform. Our parsimony reconstructions suggest that hummingbird pollination has evolved multiple times in the neotropics, with a conservative estimate of seven unambiguous shifts from bee to hummingbird pollination, and no support for reversals. This direction of change in pollination is consistent with the wide floral phenotypic diversity among the hummingbird-pollinated species, but relative consistency in floral traits among the bee-pollinated species (Kay and Schemske, 2003). All but three of the 16 unsampled neotropical species have a hummingbird-pollination syndrome, and thus our results are likely to underestimate the number of shifts to hummingbird pollination.

It is possible to calculate a per speciation “hummingbird pollination shift rate” as the number of independent origins of hummingbird pollination divided by the number of speciation events. Ignoring extinction and assuming a bifurcating phylogeny, the number of speciation events is the current number of species in the clade minus one. For Costus subgenus Costus, with approximately seven independent origins of hummingbird pollination across 38 sampled species or varieties, the rate is 0.18. In other words, approximately one-fifth of the speciation events have involved a shift to hummingbird pollination. This is a high rate compared to other plant lineages that have been studied. For comparison, we calculated hummingbird pollination shift rates in some other groups that have multiple transformations reported. We found a rate of only 0.03 for the western North American Mimulus (Beardsley and Olmstead, 2002; Beardsley et al., 2003, 2004), 0.05 for Penstemon s.l. (Castellanos, et al., 2004; Wilson et al., 2004), 0.11 for Erythrina (Bruneau, 1997), and 0.03 for tribe Sinningieae (Perret et al., 2003).

In Costus, shifts in pollination syndrome are concentrated in the species of Central American or Andean distribution, suggesting that proximity to montane environments is associated with the evolution of hummingbird pollination. Hummingbird diversity is centered in the northern Andes and encompasses hundreds of species. The major hummingbird lineages diversified in the Miocene and now comprise over 300 species (Bleiweiss, 1998). Thus, there were many potential hummingbird pollinators at the time subgenus Costus arrived in the neotropics.

Biogeography and diversification rates—Our dating based on a molecular clock suggests that the rapid speciation in Cost-
Fig. 4. Biogeographic distribution of *Costus* species depicted on the consensus tree from the Bayesian analysis, pruned so that each taxon is represented once. Branch lengths are averaged across all trees containing a particular branch in the Bayesian posterior distribution of trees.
Costus subgenus Costus coincided with important climatic and geological events in Central America and northern South America, notably the uplift of the northern Andes from about 5 Ma, the closing of the Panama isthmus at about 3.5 Ma, and the dramatic climatic oscillations from warm and wet to cool and dry that were frequent during the Pleistocene. Although fossil evidence is lacking for Costaceae, we used a molecular clock approach to estimate the arrival date of Costus in the neotropics at 1.5 to 7.1 Ma and a calibration from the closing of the Panama isthmus to estimate it at 4.6 Ma. Even the oldest estimate dates the basal divergence between Africa and the neotropics as no earlier than the beginning of the Pliocene and suggests long-distance intercontinental dispersal instead of ancient vicariance (>95 Ma; Morley, 2000) or even Miocene dispersal across tropical Laurasian habitat (Davis et al., 2002).

The parsimony reconstruction of ancestral biogeography supports Central America as the original site of the neotropical diversification. The earliest estimated date of spread to South America was 1.1 to 5.4 Ma (using published rates), consistent with dispersal across the closing Panama isthmus at approximately 3.5 Ma. The hypothesis of a Central American origin and subsequent dispersal to South America runs contrary to the pattern hypothesized for the majority of neotropical plant groups (Raven and Axelrod, 1974). It is possible that more complete taxon sampling could change the estimate of Central America as the site of origin. Our exploration of randomly attaching unsampled species to branches of the tree found no arrangements out of 1000 replicates that unequivocally supported either an Amazonian or an Andean origin of the neotropical radiation. However, we only included extant described species in this simulation, and it is possible that the Central American Costus have experienced lower rates of extinction and/or have been more thoroughly collected and described than the South American Costus, either of which could lead to an error in our estimate of the neotropical origin. More thorough sampling and a more resolved phylogeny are needed to support our result.

Although all dates should be considered rough estimates because of the lack of an independent fossil calibration within Costus and possible error in our branch length estimates, rates of ITS evolution in Costus would have to be as slow as the slowest known rates from all plants, in the woody Winteraceae (Suh et al., 1993) and Hamamelis (Wen and Shi, 1999), to alter our conclusion that neotropical Costus subgenus Costus is a recent radiation. Recency of divergence is further supported by the ease with which fertile hybrids can be made between many of the neotropical species in greenhouse crosses (D.W. Schemske and K.M. Kay, unpublished data). Our diversification rates of 0.6-2.6 species per lineage per million years estimated from the published ITS rates or 0.9 species per lineage per million years estimate from the ishmus calibration suggest that Costus subgenus Costus has diversified at a rate comparable to the fastest known plant radiations for which diversification rates have been estimated. For example, the Hawaiian silversword alliance is estimated at 0.56 ± 0.17 species per lineage per million years (Baldwin and Sanderson, 1998), the paleotropical shrub genus Gaertnera (Rubiaceae) at 0.717-0.832 (Malcomber, 2002), the South African ice plants (Aizoaceae) at 0.77-1.75 (Klak et al., 2004), and neotropical Inga at approximately 0.5 (calculated from data in Richardson et al., 2001).

Diversification in the neotropics—Our results suggest a recent and rapid radiation in the neotropics that has gone hand in hand with changes in specialized pollination systems, a key part of Gentry’s (1982) hypothesis for the diversification of the Central America and Northern Andes-centered neotropical flora. Questions remain about how these striking shifts in floral traits have occurred and how they may have contributed mechanistically to speciation. We propose the following scenario for the diversification of Costus. African species in subgenus Costus have large flowers with a bee pollination syndrome, and the ancestor of the neotropical radiation quickly adopted orchid bees for pollination. Orchid bees are prominent and species rich in lowland neotropical rainforest, relatively large in body size, and are known to travel between isolated nectar-rich plants on their long-distance foraging routes (Cameron, 2004). This would be a similar phenomenon to that hypothesized for Dalechampia, in which migration to a different continent, in this case from America to Africa, involves utilizing different pollinating species, but maintaining the same general pollination syndrome (Armbuster, 1994).

Climatic fluctuations and geological uplift caused range shifts and occasional isolation of Costus populations along the slopes and valleys of the Central American cordillera and northern Andes. In the tropics, even relatively minor topological features have been suggested to be effective dispersal barriers (Janzen, 1967). Isolated populations at higher elevations adapted to the local hummingbird fauna for pollination, because orchid bees are less active in the cool, wet weather (Armbuster and McCormick, 1990; Armbuster and Berg, 1994) that is common at higher elevations in the tropics and are rarely found above 2000 m a.s.l. (Dressler, 1982). In contrast, hummingbirds are common and remain highly active at higher elevations (Cruden, 1972; Altschuler et al., 2004). Intermediate floral forms are missing in Costus, suggesting that the shifts to hummingbird pollination occur relatively quickly or involve a limited number of genetic changes. The shifts involved changes in shape and color, but the volume and concentration of the nectar reward appears to have remained relatively constant for both orchid bee and hummingbird attraction (Schemske, 1981; K. M. Kay and D. W. Schemske, unpublished data).

In times of climatic cooling, these newly hummingbird-adapted lineages could have migrated to lower and warmer areas, but would have been substantially reproductively isolated from their bee-pollinated relatives. Shifts in specialized pollination systems are well known to contribute to reproductive isolation (Grant, 1994; Fulton and Hodges, 1999; Schemske and Bradshaw, 1999), and in Costus, pollinator specialization is sufficient to preclude most pollen flow between sympatric species that differ in syndrome (Kay and Schemske, 2003). Climatic oscillations occurred repeatedly as the Andes rose, creating many opportunities for allopatric speciation and secondary range expansions (Graham, 1997). Not all speciation events in Costus have been accompanied by a dramatic shift in pollination syndrome, but with such a young radiation, it is likely that strong prezygotic isolation maintains the distinctiveness of sympatric species, whether by floral specialization within a pollination syndrome or other ecological differences acquired in an environmentally heterogeneous landscape.

Putative sister species differing in pollination syndrome suggest that this process may be continually occurring. For example, the low-elevation C. bracteatus is strongly supported.
as sister to the cloud forest *C. lasius* (Fig. 1), and pollinator observations show that the former is visited exclusively by euglossine bees, whereas hummingbirds are the only visitors to the latter. Similarly, *C. wilsonii* is a rare species, restricted to a single region of cloud forest in the Central Cordillera of southern Costa Rica, and we find its nrDNA alleles nested within the alleles of geographically widespread lowland *C. laevis* (Fig. 1). *Costus laevis* is primarily pollinated by euglossine bees, with occasional hummingbird visits, whereas only hummingbirds have been seen at *C. wilsonii* (Kay and Schemske, 2003).

Although this scenario for the neotropical *Costus* radiation is speculative, it integrates much of what is known about pollination biology and the climatic and geologic history of the region with the results of our phylogenetic analysis. Furthermore, it provides a general hypothesis for the diversification of many herbaceous, epiphytic, or shrubby lineages in the region—lineages that altogether make up a substantial portion of neotropical floristic diversity. Perhaps many of these lineages of relatively short-lived plants have been able to diverge quickly, especially in response to the local biotic assembly, when isolated by rapidly changing topographic and climatic barriers, while many longer-lived trees and lianas diversify gradually across the vast and comparatively uniform Amazon basin. Many more time-referenced, species-level phylogenies are needed from a diversity of taxa, along with information on pollination systems, but this possibility suggests that the tropics may be both a museum of plant diversity and a cradle for recent and “explosive” speciation.

**LITERATURE CITED**


HABERLE, S. G., AND M. A. MASLIN. 1999. Late Quaternary vegetation and
APPENDIX. Taxa used in this study, GenBank accession numbers for the two regions studied (ITS followed by ETS), voucher specimen/tissue source information, and geographic var. Sources for plant material are as follows: University Botanic Gardens, PO. Box 80,162, NL-3508 TD Utrecht, The Netherlands (Utrecht); Jardin Botanique National de Belgique, Nationale Plantentuin van België, Domaine van Bouchout, B-1860 Meise, Belgium; Tom Wood living collection, Archer, Florida; and the University of Washington Botany Greenhouse living collection, Department of Biology, Box 351800, Seattle, Washington 98195-1800 (UW). Field collections were made by K.M.K. or D.W.S. Voucher specimens are deposited in the herbarium at Michigan State University (MSC).

**Taxon:** ITS, ETS; voucher/source; geographic origin.

- **C. afer** Maas; AY972877, AY972941; (Loes.) Macbride ssp. Maas; AY972881, AY972944; C. arabicus Linn.; AY972880, AY972945; C. asplundii Linn.; AY972891, AY972946; C. afer Maas; AY972887, AY972947; Depto. La Paz, Bolivia.
- **C. africeps** Baker; AY972938, AY973002; T. Wood living collection; El Valle de Anton, Panama.
- **C. albiviridis** Baker; AY972937, AY973001; T. Wood living collection; Panama.
- **C. alpinus** Maas; AY972897, AY972955; T. Wood living collection; Barro Colorado Island, Panama.
- **C. alpinus** Maas; AY972892, AY972943; T. Wood living collection; Barro Colorado Island, Panama.
- **C. americana** Maas; AY972892, AY972943; T. Wood living collection; Barro Colorado Island, Panama.
- **C. americana** Maas; AY972892, AY972943; T. Wood living collection; Barro Colorado Island, Panama.