American Journal of Botany

American Journal of Botany 100(3): 000–000. 2013.

Variation in the strength of association among pollination systems and floral traits: Evolutionary changes in the floral traits of Bornean gingers (Zingiberaceae)[1]

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- *Premise of the study:* Diversification of floral traits in angiosperms is often attributed to have been driven by adaptations to pollinators. Nevertheless, phylogenetic studies on the relationships among evolutionary changes in floral traits and pollination systems are still limited. We examined the relationships between floral trait changes and pollinator shifts in Bornean gingers (Zingiberaceae). These plants have strongly zygomorphic flowers pollinated by spiderhunter birds, bees of the genus *Amegilla*, and halictid bees.
- *Methods:* We identified pollination systems through field observations and recorded petal color, quantity of floral rewards, and seven measures of flower morphology in 28 ginger species. Phylogenetic trees were constructed from nucleotide sequences of the *matK* and ITS regions. We examined the correlations between the evolution of pollination systems and floral traits using phylogenetically independent contrasts.
- *Key Results:* Significant association was found between pink color and spiderhunter pollination, orange and *Amegilla* pollination, and yellow and white and halictid pollination. Sugar production was higher in spiderhunter-pollinated species and lower in halictid-pollinated. Meanwhile, there was a significant association only for a subset of the floral morphological characters measured. Floral tube length, which is often thought to evolve to match the lengths of pollinator probing apparatuses, did not show any correlation.
- *Conclusions:* There is considerable variation in the strength of association among pollination systems and floral traits. Lack of significant correlation in some traits could partly be explained by floral functions other than pollination, such as adaptations to prevent herbivore damage to the ovules. Further studies on these factors may improve understanding of plant–pollinator interactions.

Key words: Borneo; floral morphology; flower color; Lambir; phylogenetically independent contrast; pollination; rewards for pollinators; Zingiberaceae.

Biologists are fascinated by the huge diversity of colors, morphologies, and complexities of flowers. This diversity is understood primarily as the evolutionary outcome of selection for successful reproduction, especially pollination by different groups of animals (e.g., Faegri and van der Pjil, 1978; Fenster et al., 2004). Flowers visually attract pollinators from a distance with vivid colors and prominent displays (Klinkhamer et al., 1989; Melendez-Ackerman et al., 1997). Most animal-pollinated flowers offer reward (usually nectar) to pollinators to ensure repeated visits to conspecific plants (Simpson and Neff, 1981). In addition to attracting desirable pollinators, some floral traits may have evolved to deter unsuitable visitors (Johnson and Steiner, 2000); for example, many plants have long floral tubes, which limit access to nectar by visitors with unsuitable mouth parts (Heinrich, 1979; Laverty, 1980). Other morphological characters, such as anther and stigma position, are important to achieve successful deposition of pollen grains from the anther onto the pollinator body and subsequent deposition on the stigma (Murcia, 1990; Kudo, 2003). Determining pollinator-mediated evolution of floral traits to better understand the evolution and diversification of flowers has long been a central topic of study in plant biology.

In studies of the factors associated with trait variation among species, those of phylogenetic approaches have become popular recently. Phylogenetic information is extremely useful for determining phylogenetic homology and convergence. To date, various statistical methods for incorporating phylogenetic information have been developed (e.g., Felsenstein, 1985; Pagel, 1994; Martins and Hansen, 1997; Martins 2004; Pagel et al., 2004; Maddison et al., 2007; Webb et al., 2008). Earlier studies of pollination using phylogenetic information investigated patterns of evolutionary change in pollination systems, which were identified by floral characteristics thought to be linked to particular pollinator groups, or more rarely, by field observation (e.g., Armbruster, 1993, 1997; Johnson et al., 1998). These works demonstrated that pollination systems are evolutionarily labile but that shifts in pollination systems do not occur randomly. Indeed, shifts may repeatedly occur only between particular pairs

[1]Manuscript received 16 July 2012; revision accepted 15 January 2013.

The authors thank A. A. Hamid, Lucy Chong, and other staff at the Sarawak Forestry Department and Sarawak Forestry Corporation for permission and support, and also W. Scott Armbruster, Tetsukazu Yahara, and Makoto Kato for valuable advice, information, and comments on the study. This work was supported by the Research Institute for Humanity and Nature Project (D-04) and JSPS KAKENHI Grant Numbers 1645006 and 20405009.

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doi:10.3732/ajb.1200359

of pollination systems (e.g., Whittall and Hodges, 2007; Wilson et al., 2007) or can be directional (e.g., Kay et al., 2005; Wilson et al., 2007; Tripp and Manos, 2008). However, few studies to date have statistically examined the association between changes in floral traits and pollination systems (Smith, 2010). Of those that have, most focused on single traits of particular relevance (e.g., spur length), and only a few (Smith et al., 2008, Martén-Rodríguez et al., 2010) compared multiple floral traits in relation to pollination systems from a phylogenetic perspective. Smith et al. (2008, 2009) examined the role of pollinator shifts in the evolutionary changes in floral traits. They did not find a significant correlation among pollination systems and floral color and morphology (corolla tube length), which are usually considered to be the most important traits with regard to pollinators. Instead they found correlations among pollination systems and the amount of reward and display size, as also suggested by an experimental work using hybrids in *Penstemon* (Plantaginaceae) (Wilson and Jordan, 2009). Martén-Rodríguez et al. (2010) studied the evolution of floral traits in the tribe Gesnerieae (Gesneriaceae) and found that all four traits examined, i.e., floral color, corolla shape, timing of anther dehiscence, and nectar production, were correlated to an extent with the functional groups of their pollinators. In these studies, floral morphology was described based on a single categorical variable, and relative importance of different functions of floral morphology in pollination was not investigated.

The ginger family Zingiberaceae includes strongly zygomorphic, animal-pollinated flowers. It is the largest of eight families in the order Zingiberales, which includes more than 1200 species (Kress, 1990; Kress et al., 2002). The family has a pantropical distribution, with a center of diversity in the Malesian biogeographic region. In particular, about 170 species have been reported from Borneo, where more than 40 species may coexist in a single forest stand (Sakai and Nagamasu, 2006). The considerable diversity of ginger flowers has stimulated a large number of pollination studies in the family (Ippolito and Armstrong, 1993; Kato et al., 1993; Kato 1996; Sakai et al., 1999; Li et al., 2002; Gao et al., 2004; Takano et al., 2005; Zhang and Li, 2008; Zhang et al., 2011). These works have demonstrated that each ginger species has a specialized pollination system involving bees or birds as important pollinators. Sakai et al. (1999) reported on the pollination systems of 27 ginger species co-occurring in the Lambir Hills, Borneo. The species are pollinated by three pollinator groups (Fig. 1):

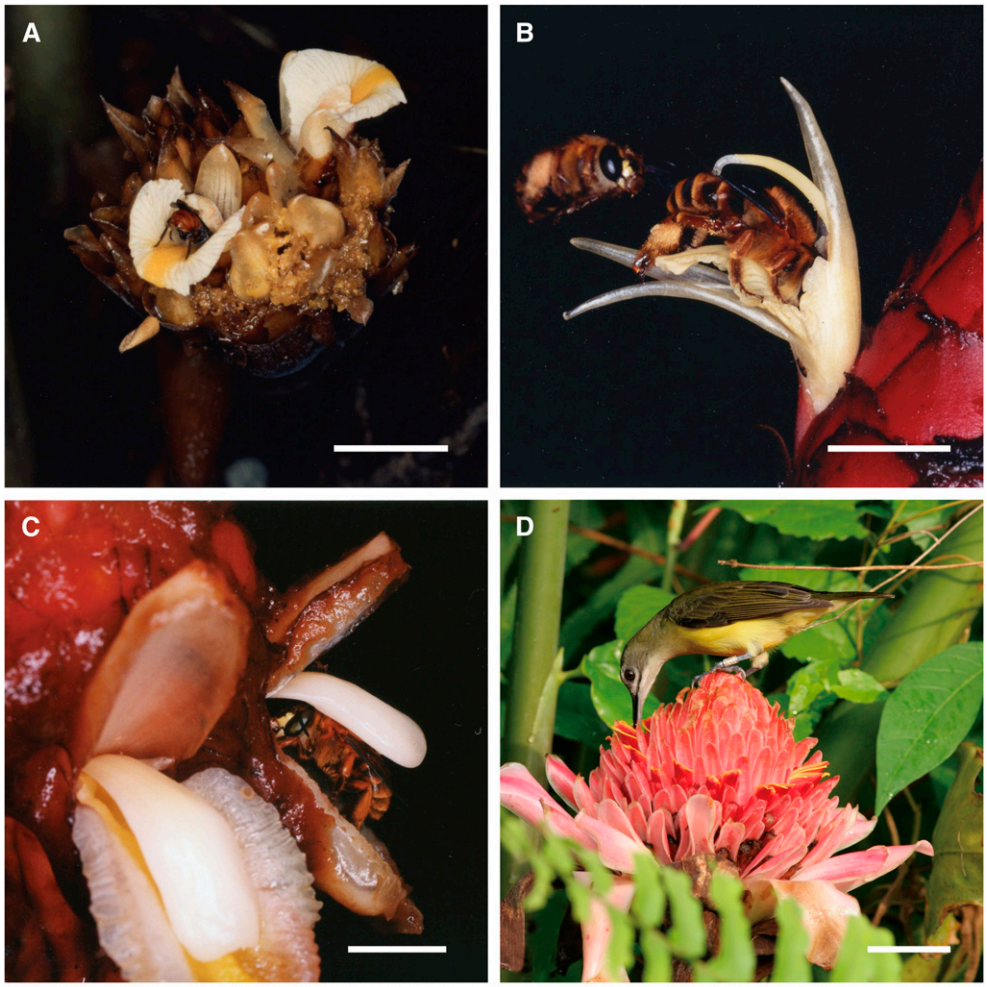

Fig. 1. Three pollinator groups of Bornean gingers: halictid-bee (A); bee of the genus *Amegilla* (B, C); and spiderhunter-bird (D). (A) Flower of *Amomum durum* visited by *Nomia* sp. (scale = 1 cm). (B) *Zingiber longipedunculatum* visited by *Amegilla pendleburyi* (scale = 1 cm). (C) *Plagiostachys crocydocalyx* visited by *A. pendleburyi* (scale = 1 cm). (D) *Etlingera elatior* visited by the small spiderhunter (*Arachnothera longirostra*) (scale = 5 cm).

spiderhunter birds (*Arachnothera longirostra* (Latham) and rarely *A. robusta* Müller & Schlegel, Nectariniidae), medium-sized bees of the genus *Amegilla* (*A. pendleburyi* Cockerell and *A. insularis* Smith, Apidae), and small halictid bees (*Nomia* spp. and *Thrincostoma afasciatum* Michener, Halictidae). A correlation was found between pollination system and floral traits, including morphology, petal color, and quantity of floral rewards. However, whether the correlation reflects an underlying evolutionary trend remained unclear due to lack of phylogenetic information.

In the current study, we investigate associations among changes of floral traits and pollinator shifts by constructing a molecular phylogeny of Bornean gingers. We use the phylogenetic information to assess correlations between the evolution of various floral traits and pollination systems. Based on the results, we discuss how evolutionary diversification of ginger flowers can be better understood with the aid of phylogenetic information.

MATERIALS AND METHODS

Sampling—We sampled 32 of the 41 ginger species that we had examined in a previous pollinator-assessment and morphometric analysis (Sakai et al., 1999). In addition, we collected two additional ginger species (*Burbidgea pauciflora* Val. and an undescribed species of *Alpinia*) from the same field site and included them in the molecular analysis. Our sampling was concentrated in a particular geographic location (Lambir Hills, Borneo, see Roubik et al., 2005 for detailed information) and therefore was not designed for broad taxonomic interpretations within the family. Hence, we did not aim to reconstruct an overall pattern of the evolution of the pollination systems in Zingiberaceae, but instead used the phylogenetic information to correct for statistical nonindependence of species (resulting from shared evolutionary history) in our assessment of correlation between pollinator types and floral characters.

For outgroups, we sampled six species of Costaceae, Marantaceae, and Lowiaceae, which are closely related to the Zingiberaceae within the order Zingiberales. A list of species sampled and GenBank accession numbers is provided in Appendix 1.

DNA extraction, molecular markers, and sequencing—Total DNA was extracted from leaf samples that had been frozen at −80°C or dried with silica gel using the modified CTAB DNA extraction method (Doyle and Doyle, 1987). We examined the chloroplast DNA *matK* gene in all samples. We also examined the internal transcribed spacer (ITS) region of nuclear ribosomal DNA from Zingiberaceae species. We failed to obtain ITS sequences for *Boesenbergia ischnosiphon* S.Sakai & Nagam. because it contained two or more sequences of different lengths; thus, it was excluded from the analyses.

Double-stranded DNA was amplified using the polymerase chain reaction (PCR) with 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Santa Clarita, California, USA) and were immediately sequenced using a DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer, Foster City, California, USA). Sequences were obtained with an ABI automated DNA sequencer (model 373A, Applied Biosystems, Carlsbad, California, USA) according to the manufacturer's instructions. We used the primers listed in Appendix 2 for the PCR and sequencing.

Phylogenetic analysis—Sequences obtained were aligned with ClustalX (Thompson et al., 1997), and obvious misalignments were adjusted by eye. Using PAUP* version 4.0b10 software (Swofford, 2002), we first performed a partition-homogeneity test with 999 replications to determine whether the *matK* and/or ITS regions contained conflicting phylogenetic signals. Because the test indicated no significant conflict ($P = 0.75$), the two regions were analyzed simultaneously in all the following phylogenetic reconstructions.

TABLE 1. The proportions of visits to inflorescences by spiderhunters (*Arachnothera* spp.), *Amegilla* bees, halictid bees and others. In addition to the flower visitor data presented in Sakai et al. (1999), additional data for *Etlingera elatior* and *Alpinia ligulata* were collected in 2004 at the same field site. The plant species code follows Sakai et al. (1999).

Plant species name	Plant species code	Percentage of visit				Total no. of visits	Observation period (Hrs)
		Spiderhunter	*Amegilla*	Halictid	Others		
Alpinia glabra Ridley	A1	0	100	0	0	7	2
Alpinia ligulata K. Schum.	A2	0	0	100	0	30	18
Boesenbergia lambirensis S. Sakai & Nagam.	B1	0	0	100	0	5	5
Boesenbergia hirta (Ridley) Merr.	B6	0	0	98	2	42	10
Elettaria longituba (Ridley) Holt.	D1	0	0	98	2	44	9
Etlingera elatior (Jack) R.M. Smith	E1	100	0	0	0	32	12
Etlingera velutina (Ridley) R.M. Smith	E2	82	0	0	18	338	30
Etlingera punicea (Roxb.) R.M. Smith	E3	100	0	0	0	106	4
Etlingera inundata S. Sakai & Nagam.	E4	100	0	0	0	2	7
Globba brachyanthera K. Schum.	G1	0	100	0	0	11	11
Hornstedtia reticulata (K. Schum.) K. Schum.	H1	100	0	0	0	3	1
Hornstedtia leonurus (J. König) Retz.	H2	100	0	0	0	3	9
Hornstedtia minor (Bl.) K. Schum.	H3	97	0	0	3	67	18
Amomum calyptratum Nagam. & S. Sakai	M1	0	100	0	0	15	5
Amomum somniculosum S. Sakai & Nagam.	M10	0	0	100	0	340	15
Amomum coriaceum R.M. Smith	M2	0	4	75	21	24	8
Amomum durum S. Sakai & Nagam.	M3	0	17	83	0	18	3
Amomum gyrolophos R.M. Smith	M4	0	100	0	0	47	11
Amomum oliganthum K. Schum.	M6	0	100	0	0	43	4
Amomum dimorphum M.F. Newman	M7	18	0	82	0	66	22
Amomum angustipetalum S. Sakai & Nagam.	M8	0	50	0	50	2	14
Amomum roseisquamosum Nagam. & S. Sakai	M9	100	0	0	0	2	6
Tamijia flagellaris S. Sakai & Nagam.	O2	0	100	0	0	10	6
Elettariopsis aff. *kerbyi* R.M. Smith	O3	0	0	100	0	30	9
Plagiostachys crocydocalyx (K. Schum.) B.L Burtt & R.M. Smith	P1	0	95	0	5	19	13
Plagiostachys glandulosum S. Sakai & Nagam.	P2	9	91	0	0	102	33
Plagiostachys strobilifera (Bak.) Ridley	P3	99	0	0	1	2816	33
Zingiber longipedunculatum Ridley	Z1	0	97	0	3	67	12

We obtained phylogenetic trees using maximum-parsimony (MP), maximum-likelihood (ML), and Bayesian methods. We conducted MP heuristic searches using PAUP* software with 100 random addition analyses and tree bisection–reconnection (TBR) branch swapping; the robustness of the results was validated through bootstrap analysis with 1000 replications. Prior to the ML analysis, we used the Modeltest 3.0 package (Posada and Crandall, 1998) to select best fitting models of base substitution (GTR + Γ + I) and to estimate model parameters. We performed ML heuristic searches using PAUP*, with 10 random addition analyses and TBR branch swapping. Because ML bootstrap analysis was too computer intensive to be done in PAUP*, we performed ML bootstrap analysis using the PhyML software (Guindon and Gascuel, 2003), with 1000 replications. For the Bayesian analysis, we used MrModeltest 2.2 software (Nylander, 2004) to infer appropriate models of base substitution. Since the following Bayesian analysis allows separate models to be assigned to each gene partition, we chose the best-fit model separately for each gene (*matK* and ITS). Using the selected models (GTR + Γ for both partitions), we performed Bayesian analyses with MrBayes 3.1.2 software (Ronquist and Huelsenbeck, 2003). The unlink command was enforced to estimate substitution parameters separately for each partition. Analyses comprised runs of four simultaneous chains for 2×10^6 generations, sampling trees every 1000 generations, for a total of 2001 trees. We plotted ln-likelihood of the sampled trees against generation time to identify the region of the analysis in which the parameter estimates were stable. Then we discarded the burn-in region (trees and parameters estimated obtained before equilibrium, i.e., the initial 1001 trees), and the remaining 1000 samples were used to estimate tree topology, branch lengths, and substitution parameters. To ensure that analyses were not trapped at local optima, we performed three separate runs and compared three topologies and parameter estimates for consistency.

***Characterization of pollination system*—**We previously identified pollinators of 28 ginger species based on the observation of flower visitors (Sakai et al., 1999). Pollination data for *Etlingera elatior* (Jack) R.M. Smith and *Alpinia ligulata* K. Schum. were collected in 2004 at the same field site. All of the observed plant and flower visitors were native species to the study site. To attempt to distinguish between nonpollinating floral visitors and animals that have the ability to transfer pollen, we carefully observed visitor behavior and checked whether they contacted the reproductive organs. We also collected bees to examine pollen on the body, and observed pollen attachment to the bill of some spiderhunter species using a video camera (Sakai et al., 1999). We defined the visitors that made contact with the reproductive organs as effective visitors and counted their total visits. Nonpollinating visitors, while they are very rare, were excluded from analysis. We recognize that visit frequency is only an approximation of total contribution to pollination and that it would be ideal to include other measures such as pollen grain deposition onto stigmas by each pollinator group (Reynolds and Fenster, 2008). However, due to the logistical difficulties of obtaining pollen removal and deposition data for a large number of species, we believe that the approach we adopted provided a better characterization of the pollination system than a simple list of floral visitors.

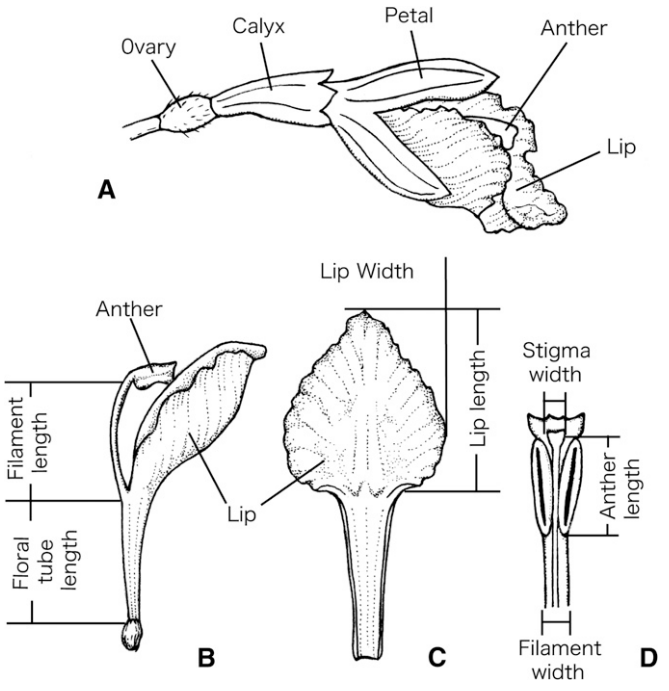

Fig. 2. Floral structure of a ginger flower and seven morphological characters measured (lip length, lip width, floral tube length, filament length, filament width, anther length, and stigma width). (A) Ginger flower, with bract and bracteole removed. (B) Lateral view of corolla with petals removed. (C) Dissected corolla. (D) Pistil and stamen. (Modified from Sakai et al., 1999.)

***Correlated evolution of pollination systems and floral traits*—**We tested for correlations between pollinator shifts and floral trait changes using a phylogenetically independent contrast analysis (Felsenstein, 1985), in which pollination systems were response variables, and floral traits were explanatory variables. Pollination systems were treated as a quantitative trait because more than a third of the species were visited by animals other than the main pollinator groups (Table 1), and because the method assume continuous values as response variables. Three separate variables were used to represent the pollination system, corresponding to the relative importance of the spiderhunter bird, *Amegilla* bee, or halictid bee. The importance of each was calculated as the number of visits by each pollinator group as a proportion of the total number of visits recorded for each species (Sakai et al., 1999).

As explanatory variables, three classes of floral traits were considered. i.e., floral morphology, reward, and color. Floral morphology was based on measurements reported in Sakai et al. (1999), which included floral tube length (FL), lip length (LL), lip width (LW), anther length (AL), stamen length (anther + filament length, SL), filament width (FW), and stigma width (SW) (Fig. 2). These seven variables were subjected to a principal component analysis (PCA) using the prcomp procedure in R statistical software (version 2.15.2; R Development Core Team, 2008), and the first two principal components were used to represent floral morphology. Floral reward was measured as the amount of sugar production per inflorescence per day calculated by multiplying sugar concentration of the nectar and volume secreted by a single inflorescence (Sakai et al., 1999). We did not consider pollen as reward because pollen collection was only observed in the *Amegilla*-pollinated *Zingiber longipedunculatum* Ridley, whose amount of sugar production was not significantly different from those of other species mainly visited by *Amegilla* (Sakai et al., 1999). Flower color was assessed by human eye, and treated as a categorical variable with five levels: white, yellow, orange, pink, and red. Although independent contrasts cannot be calculated directly for categorical variables, they can be converted into explanatory variables through the use of s–1 dummy binary variables, where s is the number of states, using subsequent multiple regression analysis (Martins, 2004; see paragraphs immediately below). Floral measurements and sugar production data were log-transformed before analysis, thus converting data to an additive scale from the previous multiplicative scale.

Prior to performing the regression, we determined the extent by which related species shared similar floral characters by testing the degree of phylogenetic signal in each of the above variables using the AOT module in Phylocom 4.0.1 (Webb et al., 2008). AOT computes the variance of standardized independent contrasts (Felsenstein, 1985), which is tested against the expectations of a null model by randomly swapping trait values across the tips of the phylogenetic tree. If related species share similar trait values, the magnitude of independent contrasts will generally be similar across the tree, resulting in smaller variance. We used the phylogeny obtained by ML analysis as input, and significance was tested based on 999 randomizations. We also computed Blomberg's K, a standardized metric that measures the level of phylogenetic signal, using the picante package in R statistical software (version 2.15.2; R Development Core Team, 2008).

Then we tested for correlations between the evolution of pollination systems and floral traits using the independent contrast approach (Felsenstein, 1985). We employed the Brownian motion model of character evolution, which assumes that the amount of character change is proportional to time. Correlations were assessed by single regressions (pollination system vs. floral morphology or sugar production) or multiple regressions (pollination system vs. flower color) of standardized independent contrasts, with pollination system as the response variable. Because these analyses suggested significant association between pollination system and floral morphology/color, we subsequently conducted post hoc regression analyses to determine which individual morphology component or color contributes most to the overall correlation. Floral measurement data were tested individually by single regressions. Flower color was also analyzed independently using pairwise single regressions to evaluate the relative

contribution of individual flower color to the overall correlation. We excluded *Boesenbergia hirta* (Ridley) Merr. and *B. parva* (Ridley) Merr. from the analysis because they were separated from *B. lambirensis* S. Sakai & Nagam. by branch lengths of zero, making calculations of standardized independent contrasts for nodes connecting these three species impossible. Zero-length branches may be resolved using negligible branch lengths (e.g., 0.000001), but this in turn produced outlying contrast values that would have violated an assumption of independent contrast analysis (i.e., nonsignificant correlation between absolute values of standardized independent contrasts and their standard deviations; Garland et al., 1992). Using the ML tree as input, we calculated standardized independent contrasts and performed single regressions with Compare 4.6b software (Martins, 2004). Multiple regressions of flower color contrasts were conducted with the R statistical software package (version 2.8.1; R Development Core Team, 2008). To account for uncertainty in phylogenetic estimation, we repeated the above analysis on 100 trees randomly sampled from the posterior distribution of the Bayesian phylogenetic analysis.

RESULTS

***Phylogenetic analysis*—**The combined data matrix comprised 1255 and 742 aligned nucleotide sites for *matK* and ITS, respectively, of which 192 and 190 were parsimony informative.

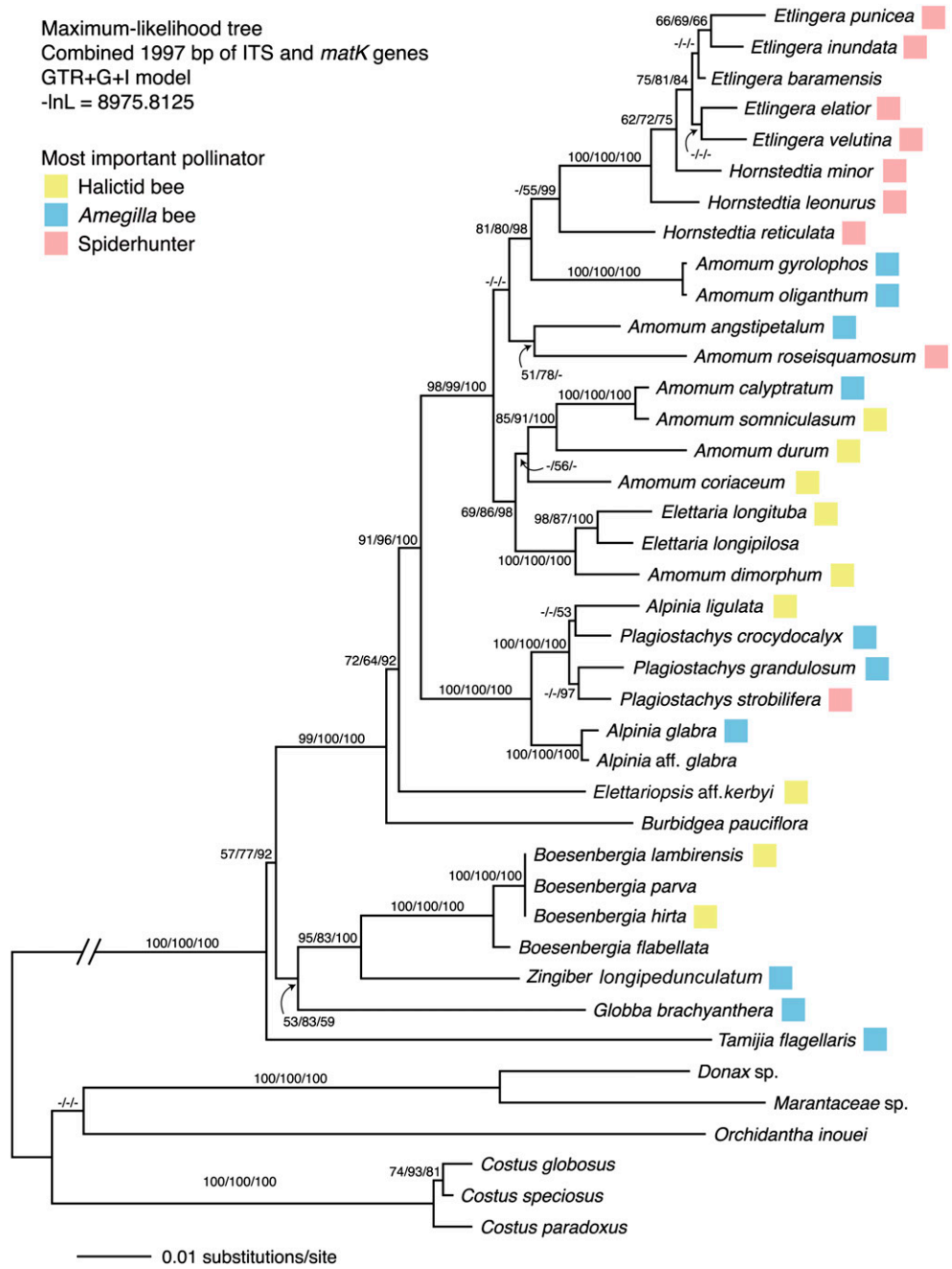

Fig. 3. Phylogenetic relationships among ginger species inferred from combined data for *matK* and ITS sequences. A maximum-likelihood (ML) tree is shown with parsimony bootstrap values and likelihood bootstrap values, followed by Bayesian posterior probabilities above the branches. Colors of squares to the right of species names indicate the dominant pollinator among the three.

MP heuristic searches of the combined nucleotide data resulted in 44 most-parsimonious trees of 1106 steps (consistency index excluding uninformative characters: 0.594; retention index: 0.818). ML analysis identified a single tree (ln-likelihood, –8975.8125) that was topologically identical to one of the most-parsimonious (MP) trees. Largely similar results were also produced by the Bayesian analysis, with conflicts occurring only at weakly supported nodes (Fig. 3). Three species-rich genera (*Alpinia*, *Amomum*, and *Hornstedtia*) were recovered as non-monophyletic, an outcome consistent with a previous molecular phylogenetic analysis of the Zingiberaceae (Kress et al., 2002).

Correlated evolution of pollination systems and floral traits— We found that related species tend to share same pollination system and similar floral-trait variables (Table 2, Fig. 3). Spiderhunter importance, red flower color, and most floral measurements had significant phylogenetic autocorrelation. Thus, independent contrasts were appropriate for testing correlations between the evolution of pollination system and floral traits (Abouheif, 1999).

Analysis among pollination system and three principle components of floral morphology detected some significant associations. The first principle component was dimension of floral size being equally weighted by all seven measurements (Fig. 4), and was not correlated with pollination system (Table 3). The second component described morphological variation independent from floral size (Fig. 4), and was positively and negatively correlated with *Amegilla* and halictid importance, respectively (Table 3). Pairwise analysis of pollination system and floral morphology indicated that lip length and stamen length were positively and negatively correlated, respectively, with the importance of both *Amegilla* and halictids. (Table 3). There was also a negative correlation between anther length and halictid importance. Sugar production was positively correlated with spiderhunter importance and negatively correlated with halictid importance. These significant results were found for >70% of the Bayesian trees tested, suggesting that our results were largely unaffected by stochastic error in phylogenetic estimation. An exception was the correlation between lip length and halictid importance, which was significant in only 51% of the trees tested.

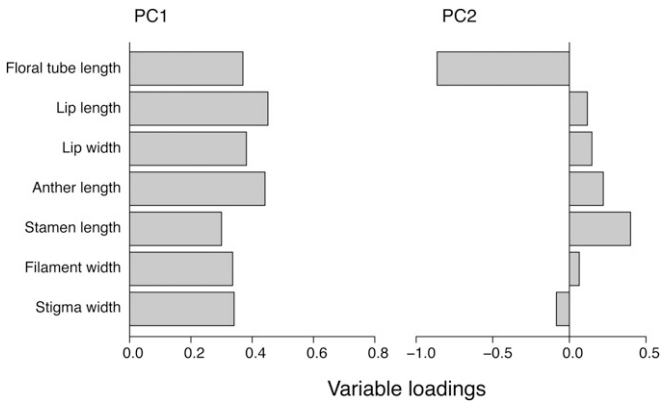

Fig. 4. Loadings on the standardized seven morphological variables of the first (PC1) and second (PC2) principal component analysis.

Multiple regressions of flower color on pollinator importance demonstrated significant correlations among flower color and all three pollination modes. The results were consistent across >80% of the Bayesian trees tested. Pairwise regressions of individual flower color on pollinator importance showed that pink flowers were positively correlated with spiderhunter importance ($r = 0.536$, $P = 0.004$), whereas orange flower color was positively correlated with *Amegilla* and negatively with halictid importance (*Amegilla*, $r = 0.392$, $P = 0.043$; halictid, $r = -0.401$, $P = 0.038$). Additionally, white and yellow flower colors were positively correlated with halictid importance (white, $r = 0.438$, $P = 0.022$; yellow, $r = 0.444$, $P = 0.020$).

TABLE 2. Significance of phylogenetic signal for pollinator importance and floral trait variables. Variances of standardized independent contrasts calculated for each variable were tested against the null expectation by randomizing tips of the phylogenetic tree 999 times. Bold font indicates *P*-values <0.05.

Pollinator importance / floral trait variables	No. of species[a]	Variance of standardized contrasts	Blombe rg's *K*	*P*-value
Spiderhunter	**27**	**10.28**	**0.744**	**0.003**
Amegilla	27	24.8	0.286	0.301
Halictid	27	20.96	0.234	0.331
Floral tube length	**30**	**7.29**	**0.475**	**0.001**
Lip length	**30**	**6.72**	**0.422**	**0.013**
Lip width	**30**	**5.95**	**0.317**	**0.016**
Anther length	**30**	**4.88**	**0.626**	**0.001**
Stamen length	**30**	**6.82**	**0.303**	**0.024**
Filament width	30	6.7	0.369	0.100
Stigma width	**30**	**2.92**	**0.863**	**0.001**
Sugar production	19	481.01	0.377	0.418
Orange	30	47.73	0.054	0.960
Pink	30	13.87	0.188	0.531
Red	**30**	**12.44**	**0.448**	**0.020**
Yellow	30	10.11	0.176	0.598
White	30	62.66	0.110	0.610

[a]The numbers of the species included in the test for each character.

DISCUSSION

The constructed phylogeny showed that none of the three pollination systems were monophyletic. Although our sampling is geographically limited, it is safe to say that at least two of the three pollination systems have evolved more than twice within a family. The three pollinator groups studied mostly forage periodically for scattered floral resources. Hence, the habits of ginger plants, such as low-density growth and patchy distribution, may exert an ecological constraint on the types of pollinators that may visit. Repeated shifts between the same sets of pollinator guilds have also been suggested in other groups (e.g., *Penstemon* and segregate genera [Plantaginaceae], Wilson et al., 2007 and *Aquilegia* [Ranunculaceae], Whittall and Hodges, 2007). The plant pollinator interactions observed in Bornean gingers are comparable with the neotropical sister group of Costaceae (Zingiberales) mostly pollinated by euglossine bees (medium-sized bee) and hummingbirds (Kay et al., 2005). However, diversity of bird pollinators of gingers in Borneo is much lower than those of neotropical Costaceae, among which further specialization to territorial and nonterritorial hummingbirds may occur (Kay and Schemske, 2003). On the other hand, species primarily pollinated by small-sized bees may be absent in neotropical Costaceae. Presence of hummingbirds,

TABLE 3. Correlations among pollinator contrasts and floral trait contrasts. Significant correlations ($P < 0.05$) are indicated in bold (*, < 0.05; **, < 0.01). Ranges in brackets represent 95% intervals across 100 Bayesian trees. Degrees of freedom are 25 for floral measurements and 17 for sugar production.

Pollinator	Spiderhunter		*Amegilla*		Halictid	
Floral morphology						
PC1	$r = 0.03$	[−0.11–0.19]	$r = 0.37$	[0.20–0.54]	$r = -0.36$	[−0.52–−0.22]
PC2	$r = -0.06$	[−0.19–0.03]	**$r = 0.45$***	[0.28–0.63]	**$r = -0.44$***	[−0.62–−0.27]
Floral tube length	$r = 0.073$	[0.04–0.17]	$r = -0.047$	[−0.11–0.01]	$r = 0.04$	[−0.02–0.08]
Lip length	$r = -0.102$	[−0.18–0]	**$r = 0.461$***	[0.33–0.6]	**$r = -0.407$***	[−0.55–0.28]
Lip width	$r = -0.119$	[−0.24–0.01]	$r = 0.378$	[0.26–0.48]	$r = -0.241$	[−0.36–0.12]
Anther length	$r = 0.111$	[0.01–0.21]	$r = 0.316$	[0.19–0.41]	**$r = -0.399$***	[−0.47–0.33]
Stamen length	$r = 0.039$	[−0.06–0.13]	**$r = 0.563$****	[0.42–0.68]	**$r = -0.591$****	[−0.69–0.46]
Filament width	$r = 0.115$	[0.02–0.21]	$r = 0.158$	[0.06–0.24]	$r = -0.200$	[−0.27–0.13]
Stigma width	$r = 0.106$	[0.05–0.19]	$r = 0.029$	[−0.04–0.09]	$r = -0.05$	[−0.1–0.01]
Sugar production	**$r = 0.505$***	[0.38–0.61]	$r = 0.232$	[−0.05–0.54]	**$r = -0.608$****	[−0.77–0.44]
Flower color	**$r = 0.561$****	[0.44–0.644]	**$r = 0.361$***	[0.3–0.55]	**$r = 0.43$***	[0.35–0.6]

which are highly specialized to nectar feeding, associated with higher proportion of bird-pollinated plants in the plant community (Momose et al., 1998) may be related with these differences. Comparison of evolution of pollination system and floral traits in these sister lineages may be an interesting subject for future studies.

Among the floral traits examined, flower color was most strongly associated with pollination vectors, followed by quantity of floral reward. Color is considered a major predictor or determinant of pollinators at higher taxonomic levels (e.g., bees, flies, beetles, moths, birds, and bats), although it may not be as important at finer taxonomic scales (Fenster et al., 2004). The association between red or orange flowers and bird pollination is ubiquitous (e.g., Bradshaw and Schemske, 2003; Wilson et al., 2004). Gingers in Lambir also fit this pattern, i.e., all species pollinated by birds have red or pink flowers except for *Amomum roseisquamosum* Nagam. & S. Sakai, which has white flowers with pink bracts. Our molecular phylogeny suggests that this species was derived from white-flowered, bee-pollinated species. This evolutionary background may explain the anomalous bird pollination of a white-flowered species.

In contrast to bird pollination, color differentiation among different bee groups has rarely been reported. Nevertheless, there might be some differentiation among gingers in Lambir. Orange flowers were borne on *Amegilla*-pollinated plants, and yellow flowers on halictid-pollinated plants, while the majority of bee-pollinated species had white flowers. One possible explanation for partial flower color correlation with pollinator bees in the gingers may lie in color perception differences between *Amegilla* and halictids. Another potential explanatory mechanism may be floral mimicry among species sharing pollinators. As a result of convergence on a common advertising display, different plant species may gain collective advantage. To date, this phenomenon has been poorly studied (Roy and Widmer, 1999; Schaefer and Ruxton, 2009). Application of a phylogenetic approach to sympatric and allopatric relatives would be relevant to an investigation of these issues. To confirm correlations between the evolution of flower color and pollination systems in future studies, quantitative color measurement across the full spectral ranges of pollinators will be important.

Evolutionary changes in the quantity of floral reward were correlated with pollinator body size. In all plant species we studied, nectar was the exclusive reward for pollinators, with the exception of one species (see Materials and Methods). High sugar production was associated with the largest pollinator (spiderhunter bird), and low production with the smallest (halictid bee). The amount produced by *Amegilla* bee-pollinated plants was in the middle, thus no significant trend was detected (Table 3). The result is consistent with expectation from pollinator energetics (Heinrich, 1975).

As for morphological floral characters, evolutionary changes correlated with pollination vectors were found only in the two bee-pollination systems. We propose that this correlation exists because bee-pollination in gingers requires closer morphological matching between floral structures and pollinator bodies than does spiderhunter-pollination. Spiderhunters simply insert their bills into floral tubes, i.e., they do not land on or crawl into the flower. In the analysis using the first two principal components (PC1 and PC2), significant correlation was found only in PC2. Since PC1 was an axis of whole floral size, the result suggests that relative size of each morphological character, rather than whole flower size, is more closely related with pollination. Among the seven morphological floral characters, the strongest correlation was for stamen length, followed by lip length. In most bee-pollinated flowers observed in this study, pollen is deposited on the dorsal surface of the thorax and abdomen of the pollinators. Stamen length adjustment is required for successful pollen deposition when a pollinator shift occurs between bee groups that have large size differences. The flower lip functions as a platform for pollinators, i.e., they land on it before crawling under the stamen and inserting their proboscis into the floral tube. The correlation between the lip length and pollination system may reflect the fact that larger visitors require larger landing platforms.

Lengths of the floral structures bearing rewards generally correspond well to the lengths of pollinators' probing structures (Fenster et al., 2004). Nevertheless, we did not detect a significant correlation for floral tube length even though there was considerable variation in the lengths of probing structures among pollinator groups (halictid bee: 3.9–8.7 mm; *Amegilla*: 11.0–12.2 mm; spiderhunter: 35.9–55.6 mm; Sakai et al., 1999; Yumoto et al., 1997). *Boesenbergia ischnosiphon* and *Elettaria longituba* (Ridley) Holt. had the longest floral tubes (> 60 mm), but they were pollinated by halictid bees with the shortest proboscis. The floral tube of *Plagiostachys strobilifera* (Bak.) Ridley (8.8 mm) was much shorter than the bills of their spiderhunter pollinators, and nectar of this ginger is consumed by *Amegilla* bees that do not effect pollination (Sakai et al., 1999). This contrasts with observations by Whittall and Hodges (2007), who demonstrated that spur lengths of columbine flowers are highly

correlated with probe length of pollinators, i.e., bees, hummingbirds, and hawkmoths. The discrepancy between their study and ours may be attributable in part to the multiple functions of floral tubes in gingers. In columbine flowers, nectar spurs are floral parts specialized for presenting nectar to pollinators, whereas floral tubes of gingers also function to keep the ovules away from herbivores by increasing the distance between anthers/stigmas and ovules. The anthers/stigmas of columbine flowers must be exposed to make contact with pollinators, whereas those of gingers are better concealed and kept away from herbivores.

In this study, we found considerable variation in the strengths of association among pollinator groups and floral traits, and presented potential explanations for this variability. Flower color and the quantity of floral reward may be important traits in all three pollination systems. Floral morphology, on the other hand, was significantly correlated only in the case of bee pollination, probably because bees make closer physical contact with flower structures than do bird pollinators. Of the floral morphological characters measured, there was a significant association only for a subset of traits, and floral tube length was not among them. Floral functions other than pollination may obscure the correlations in apparently important characters. Effects of indirect selection on floral traits (selection on the pleiotropically related nonfloral traits) are still largely unknown (Armbruster, 2002). Lack of perfect correspondence or discrepancy between pollination systems and expected floral traits may not be uncommon (e.g., Smith et al., 2008). Further studies on the variation in the strengths of association would contribute to a better understanding of plant–pollinator interactions, one of the most intriguing mutualisms in the natural world.

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APPENDIX 1. Materials used in the phylogenetic analyses and GenBank accession numbers. All samples were collected at Lambir, Sarawak (see Materials and Methods), and voucher specimens were deposited at KYO.

Zingiberaceae

TAXON; GenBank accession no.: *matK*; ITS; *voucher specimen*.

Alpinia glabra Ridley; JF715463; AB097221; *S. Sakai 388*. ***Alpinia* aff. *glabra***; JF715464; AB097223; *S. Sakai 377*. ***Alpinia ligulata*** K. Schum.; JF715465; AB097222; *S. Sakai 375*. ***Amomum angustipetalum*** S. Sakai & Nagam.; JF715466; AB097245; *S. Sakai 389*. ***Amomum calyptratum*** Nagam. & S. Sakai; JF715467; AB097239; *S. Sakai 363*. ***Amomum coriaceum*** R.M. Smith; JF715468; AB097240; *S. Sakai 357*. ***Amomum dimorphum*** M.F. Newman; JF715469; AB097244; *S. Sakai 372*. ***Amomum durum*** S. Sakai & Nagam.; JF715470; AB097241; *S. Sakai 362*. ***Amomum gyrolophos*** R.M. Smith; JF715471; AB097242; *S. Sakai 352*. ***Amomum oliganthum*** K. Schum.; JF715472; AB097243; *S. Sakai 370*. ***Amomum roseisquamosum*** Nagam. & S. Sakai; JF715473; AB097246; *S. Sakai 188*. ***Amomum somniculosum*** S. Sakai & Nagam.; JF715474; AB097247; *S. Sakai 373*. ***Boesenbergia flabellata*** S. Sakai & Nagam.; JF715475; AB097226; *S. Sakai 378*. ***Boesenbergia hirta*** (Ridley) Merr.; JF715476; AB097227; *S. Sakai 366*. ***Boesenbergia lambirensis*** S. Sakai & Nagam.; JF715477; AB097224; *S. Sakai 367*. ***Boesenbergia parva*** (Ridley) Merr.; JF715478; AB097225; *S. Sakai 379*. ***Burbidgea pauciflora*** Val.; JF715479; AB097253; *S. Sakai 241*. ***Elettaria longipilosa*** S. Sakai & Nagam.; JF715480; AB097229; *S. Sakai 380*. ***Elettaria longituba*** (Ridley) Holt.; JF715481; AB097228; *S. Sakai 201*. ***Elettariopsis* aff. *kerbyi***; JF715482; AB097249; *S. Sakai 359*. ***Etlingera baramensis*** S. Sakai & Nagam.; JF715483; AB097234; *S. Sakai 390*. ***Etlingera elatior*** (Jack) R.M. Smith; JF715484; AB097230; *S. Sakai 351*. ***Etlingera inundata*** S. Sakai & Nagam.; JF715485; AB097233; *S. Sakai 355*. ***Etlingera punicea*** (Roxb.) R.M. Smith; JF715486; AB097232; *S. Sakai 226*. ***Etlingera velutina*** (Ridley) R.M. Smith; JF715487; AB097231; *S. Sakai 376*. ***Globba brachyanthera*** K. Schum.; JF715488; AB097235; *S. Sakai 369*. ***Hornstedtia leonurus*** (J. König) Retz.; JF715489; AB097237; *S. Sakai 365*. ***Hornstedtia minor*** (Bl.) K. Schum.; JF715490; AB097238; *S. Sakai 381*. ***Hornstedtia reticulata*** (K. Schum.) K. Schum.; JF715491; AB097236; *S. Sakai 387*. ***Plagiostachys crocydocalyx*** (K. Schum.) B.L. Burtt & R.M. Smith; JF715492; AB097250; *S. Sakai 360*. ***Plagiostachys glandulosum*** S. Sakai & Nagam.; JF715493; AB097251; *S. Sakai 374*. ***Plagiostachys strobilifera*** (Bak.) Ridley; JF715494; AB097252; *S. Sakai 361*. ***Tamijia flagellaris*** S. Sakai & Nagam.; JF715495; AB097248; *S. Sakai 373*. ***Zingiber longipedunculatum*** Ridley; JF715496; AB097254; *S. Sakai 358*.

Outgroups

TAXON; GenBank accession no.: *matK*; *Voucher specimen*.

Donax sp. (Marantaceae); JF715497; *S. Sakai 305*. ***Stachyphrynum*** sp. (Marantaceae); JF715498; *S. Sakai 80*. ***Orchidantha inouei*** Nagam. & S. Sakai (Lowiaceae); JF715499; *S. Sakai 356*. ***Costus globosus*** Bl.-complex by Maas (1979) (Costaceae); JF715500; *S. Sakai 353*. ***Costus paradoxus*** K. Schum. (Costaceae); JF715501; *S. Sakai 364*. ***Costus speciosus*** (J. König) J.E. Smith (Costaceae); JF715502; *S. Sakai 371*.

APPENDIX 2. Primers used for this study.

Primer	Sequence (5′ to 3′)	Reference
matK AF	CTATATCCACTTATCTTTCAGGAGT	Ooi et al. (1995)
matK 8R	AAAGTTCTAGCACAAGAAAGTCGA	Ooi et al. (1995)
matK ZMF	CTCACAATTTACAATCTAGTCATTCA	Designed for this study
matK ZLR	TTACGGAGAAAGAGGAAGCCAGATT	Designed for this study
matK ZPF	AATCTTATTTTTATCTGTGGTCTCA	Designed for this study
matK ZNR	CGAAGAATTCATTAGTACACTTGAAA	Designed for this study
ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)