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Molecular phylogeny of the *cristata* species group of the genus *Colocasiomyia* (Diptera: Drosophilidae)

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Flies of the *Colocasiomyia cristata* species group depend their life cycles on specific host plants of the family Araceae and play important roles as species-specific pollinators in return. A pair of ‘stamenicolous’ and ‘pistillicolous’ species cohabit in the same inflorescence and such pairs have been reported from different host species and different geographical regions. To understand the evolution of host selection and cohabitation and the divergence between stamenicolous and pistillicolous species in the *C. cristata* group, we conducted molecular phylogenetic analyses. The mitochondrial *ND2* sequences (maximum 858 base pairs) of 27 OTUs were analysed. Phylogenetic relationships reconstructed by the neighbour-joining, maximum parsimony and maximum likelihood methods were summarised in a consensus network. The resolution in the inferred phylogeny was higher than those in the previous cladistic studies based on the morphological characteristics. We recognised three main clades: the *C. colocasiae-alocasiae*, *C. diconica-xenolocasiae* and *C. cristata-sulawesiana* clades. Comparisons of ecological traits among species on their phylogenetic relationships revealed that the origins of cohabitation and stamenicolous and pistillicolous breeding habits are more complicated than thought in the previous hypothesis. Since the number of the species is limited in this study, more biogeographic information and comparative ecological studies based on reliable phylogenetic trees with comprehensive taxon sampling of both *Colocasiomyia* flies and their host plants will be necessary.

1. Introduction

The genus *Colocasiomyia* de Meijere, 1914 (Diptera: Drosophilidae) comprises 26 described species and approximately 46 undescribed species distributed in the Oriental and Papuan regions (Sultana et al., 2006; Toda et al., unpubl. data) and is classified into five species groups (Okada, 1990; Sultana et al., 2002; Sultana et al., 2006; Yafuso et al., 2008). The mem-

bers of each species group visit a specific taxonomical group or groups of host plants in the families Araceae, Arecaceae and Magnoliaceae (Sultana et al., 2006) (Fig. 1). Flies of the *C. cristata* species group exhibit especially high host specificity: each species usually utilises only one or two host plant species.

In addition to the host specificity, particular breeding habits and larval development in their host inflorescences and infructescences were reported for certain *Colocasiomyia* species. It is sharing of the same inflorescence by a pair of *Colocasiomyia* species with microallopatric breeding niche separation: one species, referred to as “pistillicolous species”, mainly utilises the lower pistillate region (female-inflorescence) of spadix for oviposition and larval development, whereas the other, referred to as “stamenicolous species”, the upper staminate region (male-inflorescence). This phenomenon was first

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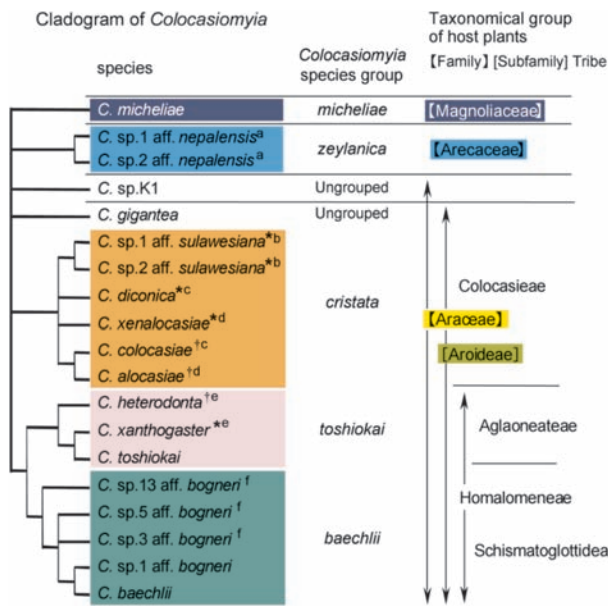


Figure 1 : A hypothesis on *Colocasiomyia* phylogeny and species groups corresponding to the taxonomical groups of their host plants [after Sultana et al. (2006) with modification]. The cladogram was obtained based on 62 morphological characters. The members of the *Colocasiomyia cristata* species group visit inflorescences of the tribe Colocasieae (sensu Mayo et al., 1997, Araceae), the *C. baechlii* species group to the tribe Schismatoglottideae (sensu Mayo et al., 1997, Araceae), the *C. crassipes* species group to *Michelia* species (Magnoliaceae), the *C. toshiokai* species group to the tribe Homalomeeneae (sensu Mayo et al., 1997, Araceae) and the *C. zeylanica* species group to *Pinanga* species (Arecaceae). †Stamencolous species; * pistillicolous species; each alphabet (a-f) indicates cohabiting species in the same host plant.

regarded as “synhospitalic pairs” or “synhospitalism” (Eichler, 1966) by Okada (1980) for *Colocasiomyia* flies, but is referred to as “cohabitation” in the present study.

Such cohabiting traits have been reported in different host plants, from different geographic areas and on different species groups of *Colocasiomyia* flies. For example, some species pairs of the *C. cristata* group have been reported to breed together on inflorescences of the genera *Colocasia* and *Alocasia* (Carson and Okada, 1980; Honda-Yafuso, 1983; Toda and Okada, 1983; Okada and Yafuso, 1989). *Colocasiomyia stamencicola* (Carson & Okada, 1980) and *C. pistillicola* (Carson & Okada, 1980) have been recorded from inflorescences of *Colocasia esculenta* (L.) Schott (Carson and Okada, 1980), and *C. alocasiae* (Okada, 1975) and *C. xenalocasiae* (Okada, 1980) from *Alocasia odora* (Roxb.) K. Koch (Honda-Yafuso, 1983; Yafuso, 1994).

In some species, and presumably in most species, *Colocasiomyia* flies seem to depend on inflorescences

and infructescences of host plants almost throughout their entire life cycles (Takenaka, 2006). In return, some *Colocasiomyia* species have been confirmed to play important roles as species-specific pollinators for their host plants (Carson and Okada, 1980; Kramadibrata and Hambali, 1983; Yafuso, 1993; Mori and Okada, 2001; Miyake and Yafuso, 2003; Takenaka et al., 2006). *Colocasiomyia* flies and their host plants may have co-evolved through such intimate relationships. Further, these pollination mutualisms may have increased species-diversity of both *Colocasiomyia* flies and their host plants.

During the last decade, a considerable number of new species have been found in the genus *Colocasiomyia* (Sultana et al., 2002; Sultana et al., 2006; Toda and Lakim, in press; Toda et al., unpubl. data). These discoveries lead us to a new phase of study on the taxonomy and ecology of this genus, because the observed variation in ecological traits of some new species cannot be explained in accordance with so far proposed hypotheses for cohabitation of stamencolous and pistillicolous species: in some systems one fly species monopolizes the host inflorescences (Takenaka et al., 2006), while others may cohabit with up to seven other species (Toda and Lakim, in press).

Okada (1980, 1986b, 1988, 1990) carried out phenetic analyses and proposed the classification of species groups within the genus *Colocasiomyia*. Grimaldi (1991) was the first to examine phylogenetic relationships among species of this genus by a cladistic analysis using 22 morphological characters. Sultana et al. (2006) conducted another cladistic analysis for 21 *Colocasiomyia* species based on 62 morphological characters and revised the classification of species groups within this genus (Fig. 1). The cladograms resulting from these two analyses were consistent in supporting the monophyly of the *C. zeylanica* and *C. toshiokai* species groups but incongruent with each other at many points. The *C. cristata* group was suggested as a paraphyletic group in Grimaldi (1991). Sultana et al. (2006) recovered its monophyly, supporting the proposition of this species group by Okada (1990), but remained phylogenetic relationships among and within species groups largely unresolved (Fig. 1). As to the relationships within the *C. cristata* group, stamencolous species such as *C. colocasiae* (Duda, 1924), *C. alocasiae* and *C. stamencicola* formed a clade in both cladograms, but the phylogenetic positions of

pistillicolous species such as *C. xenalocasiae*, *C. diconica* (Toda & Okada, 1983) and *C. sulawesiana* Okada & Yafuso, 1989 were unstable or remained unresolved.

To understand the evolutionary processes of mutualism between *Colocasiomyia* flies and their host plants and cohabitation between stamenicolous and pistillicolous species, we must know precise phylogenetic relationships among the species. In this study, we aimed to resolve the phylogenetic relationships within the *C. cristata* group based on a molecular phylogenetic analysis using the mitochondrial NADH dehydrogenase subunit 2 (*ND2*) gene sequences and to consider the evolutionary origins of the breeding habits, cohabitation and host selection in this group.

2. Materials and methods

Twenty-six operational taxonomic units (OTUs) were selected as the ingroup (Table 1). On the basis of the research by Sultana et al. (2006), we included the DNA sequences of *Scaptodrosophila lebanonensis* (Wheeler, 1949) (the GenBank accession number HQ110598), *Drosophila immigrans* Sturtevant, 1921 (HQ110577), *D. virilis* Sturtevant, 1916 (HQ110586) and *D. funebris* (Fabricius, 1787) (HQ110575) as the outgroup. The sequences of *C. alocasiae* and *C. xenalocasiae* from Okinawa were provided by R. Segawa of Tokyo Metropolitan University. Adults of other *Colocasiomyia* flies were collected in the field between 2003 and 2007 and were preserved in 99.5% ethanol until DNA extraction. DNA was extracted from one adult fly by the method of Boom et al. (1990) with some modification (Kobayashi et al., 2009). The *ND2* fragment was amplified by the polymerase chain reaction (PCR) technique. Nucleotide sequences for the primers were as follows: *ND2* HEAD, 5'-AAGCTACTGGGTTCATACC-3' (tRNA-Trp); *ND2* TAIL, 5'-ATATTTACAGCTTTGAAGG-3' (tRNA-Met). PCR was done in 25 μ L reaction volume with mixture contained 2 mM Tris-HCl pH 8.0, 10 mM KCl, 0.01 mM EDTA, 0.1 mM DTT, 0.05% Tween 20, 0.05% Nonidet P-40, 0.2 mM of each dNTP, 5–10 μ M of each primer, approximately 0.5 μ g template DNA and 0.65 units Blend Taq polymerase™ (TOYOBO, Japan). PCR amplification were performed for 35 cycles using ATC 201 thermal cycler (Apollo, Belgium) under the following parameters: 94°C for 30 s, 52°C for 30 s, 72°C for 1 min, except for the first cycle

of 94°C for 3 min and the last cycle of 72°C for 10 min. DNA sequencing reaction was carried out using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) and the nucleotide sequences were determined using ABI PRISM® 3100-Avant Genetic Analyzer according to the protocol supplied by the manufacturer. DNA sequences were analyzed by Sequencing Analysis Software v3.7 (Life Technologies) and MEGA software version 5 beta 7 (Tamura et al., 2007). Finally, we retrieved maximum 858 base pairs of the coding region for each resulting sequence (DDBJ Accession numbers AB609013–AB609038, Table 1). We aligned the sequences using ClustalW program with codon model implemented in MEGA 5 with gap opening penalty 10 and gap extension penalty 0.1 for pairwise alignment and gap opening penalty 10 and gap extension penalty 0.2 for multiple alignment following the default setting of the program.

We performed the neighbour-joining (NJ) and maximum parsimony (MP) methods using MEGA software. All codon positions were included, but all indels and ambiguous sites were treated as missing data. In the NJ phylogenetic reconstruction, the evolutionary distances were computed using the Tamura-Nei (1993) model and pairwise deletion option. In the MP phylogenetic reconstruction, the most parsimonious trees were obtained using the close-neighbour-interchange (CNI) algorithm at search level 3, in which the initial trees were obtained by random addition of sequences (50 replicates). There were 858 sites in the final dataset, of which 337 were parsimony informative. The strict consensus tree was generated from the two most parsimonious trees. We also performed the maximum likelihood (ML) phylogenetic reconstruction. The KAKUSAN software (Tanabe, 2007) version 4 was used to determine the appropriate model of DNA substitution; as a result, CodonProportional with TVM+Gamma model was selected by the corrected Akaike Information Criterion (AICc4). ML analyses were performed using Treefinder (Jobb, 2008) with the 100 iterations of the likelihood ratchet implemented in Phylogears software version 2 (Tanabe, 2010).

To assess the branch confidence on the NJ, MP and ML trees, the bootstrap test (Felsenstein, 1985) was performed with 1,000 replicates. The resultant trees were rooted using the outgroup. To help the visualisation of conflicting or ambiguous phylogenetic

Table 1 : Operational taxonomic units (OTUs) used for the molecular phylogenetic analyses

	Subfamily	Genus	Subgenus	Species group	Species-subgroup	Species	ID	Locality	Accession No.
1	Drosophiliinae	<i>Scaphodrosophila</i>				<i>lebanonensis</i>			HQ110598
2		<i>Drosophila</i>	(<i>Drosophila</i>)	<i>immigrans</i>		<i>immigrans</i>			HQ110577
3			(<i>Drosophila</i>)	<i>virilis</i>		<i>virilis</i>			HQ110586
4			(<i>Drosophila</i>)	<i>funebri</i>		<i>funebri</i>			HQ110575
5		<i>Colocasiomyia</i>		ungrouped		<i>gigantea</i>	K2-19	Bogor Botanical Garden, West Java, Indonesia	AB609013
6						<i>gigantea</i>	K2-20	Bogor Botanical Garden, West Java, Indonesia	AB609014
7						sp.X1	GaoG1	Xishuangbanna Tropical Botanical Garden, Yunnan, China	AB609015
8				<i>cristata</i>	<i>colocasiae</i>	<i>colocasiae</i>	K7-2	Situ Tengah, Bogor, West Java, Indonesia	AB609016
9					<i>colocasiae</i>	<i>colocasiae</i>	K4-8	Kg. Apin Apin, Keningau, Sabah, Borneo, Malaysia	AB609017
10					<i>colocasiae</i>	<i>colocasiae</i>	K7-3	Kinabalu Park Head Quarter, Sabah, Borneo, Malaysia	AB609018
11					<i>colocasiae</i>	<i>iskandari</i>	2006v04-26	Kerinci, Sumatra, Indonesia	AB609019
12					<i>colocasiae</i>	sp.1 aff. <i>iskandari</i>	2006v04-27	Probolinggo, Madakaripura, East Java, Indonesia	AB609020
13					<i>colocasiae</i>	<i>steudnerae</i>	2006v04-17	Xishuangbanna Tropical Botanical Garden, Yunnan, China	AB609021
14					<i>colocasiae</i>	<i>alocasiae</i>	Segawa	Okinawa, Japan	AB609022
15					<i>colocasiae</i>	<i>alocasiae</i>	K8-2	Xishuangbanna Tropical Botanical Garden, Yunnan, China	AB609023
16						sp. aff. <i>stamenicola</i>	K8-12	Xishuangbanna Tropical Botanical Garden, Yunnan, China	AB609024
17						sp. aff. <i>stamenicola</i>	2006v04-18	Xishuangbanna Tropical Botanical Garden, Yunnan, China	AB609025
18						<i>xenolocasiae</i>	Segawa	Okinawa, Japan	AB609026
19					<i>xenolocasiae</i>	<i>xenolocasiae</i>	2006v04-16	Xishuangbanna Tropical Botanical Garden, Yunnan, China	AB609027
20					<i>diconica</i>	<i>diconica</i>	K8-6	Situ Tengah, Bogor, West Java, Indonesia	AB609028
21					<i>diconica</i>	<i>diconica</i>	K8-3	Kg. Apin Apin, Keningau, Sabah, Borneo, Malaysia	AB609029
22					<i>diconica</i>	<i>diconica</i>	K8-7	Padan-Sukalami, West Sumatra, Indonesia	AB609030
23						sp.1 aff. <i>diconica</i>	K8-4	Bogor Botanical Garden, West Java, Indonesia	AB609031
24						sp.1 aff. <i>diconica</i>	K8-8	Madakaripura, East Java, Indonesia	AB609032
25						<i>crislata</i>	K2-6	Bogor Botanical Garden, West Java, Indonesia	AB609033
26						<i>crislata</i>	K2-15	Bogor Botanical Garden, West Java, Indonesia	AB609034
27						<i>crislata</i>	K4-2	Ciamis, West Java, Indonesia	AB609035
28						<i>sulawesiana</i>	K4-3	Enrekang, South Sulawesi, Indonesia	AB609036
29						sp.1 aff. <i>sulawesiana</i>	K4-4	Kota Kinabalu, Sabah, Borneo, Malaysia	AB609037
30						sp.2 aff. <i>sulawesiana</i>	K7-1	Kota Kinabalu, Sabah, Borneo, Malaysia	AB609038

signals among the NJ, MP and ML trees, a consensus network (Holland et al., 2004) was computed using the SplitsTree4 package (Huson and Bryant, 2006) without edge weights (Holland et al., 2006). The consensus network was modified to a species tree (network) and compared with information on cohabiting pairs, host plant and breeding characteristics.

3. Results

All conspecific OTUs formed monophyletic

clades except for *C. alocasia* in the ML tree (Fig. 2C). The branching patterns of the NJ, MP and ML trees mostly agreed with each other except five branches that were associated with relatively lower bootstrap values (Figs 2A-C). Such topologies with signals of phylogenetic uncertainty were represented by box structures with parallel edges in the consensus network, which was computed from the NJ tree, the two most parsimonious trees and the ML trees as input (Fig. 2D). One of the five box structures was located at the clade connecting *C. sulawesiana*, *C. sp.1* aff.

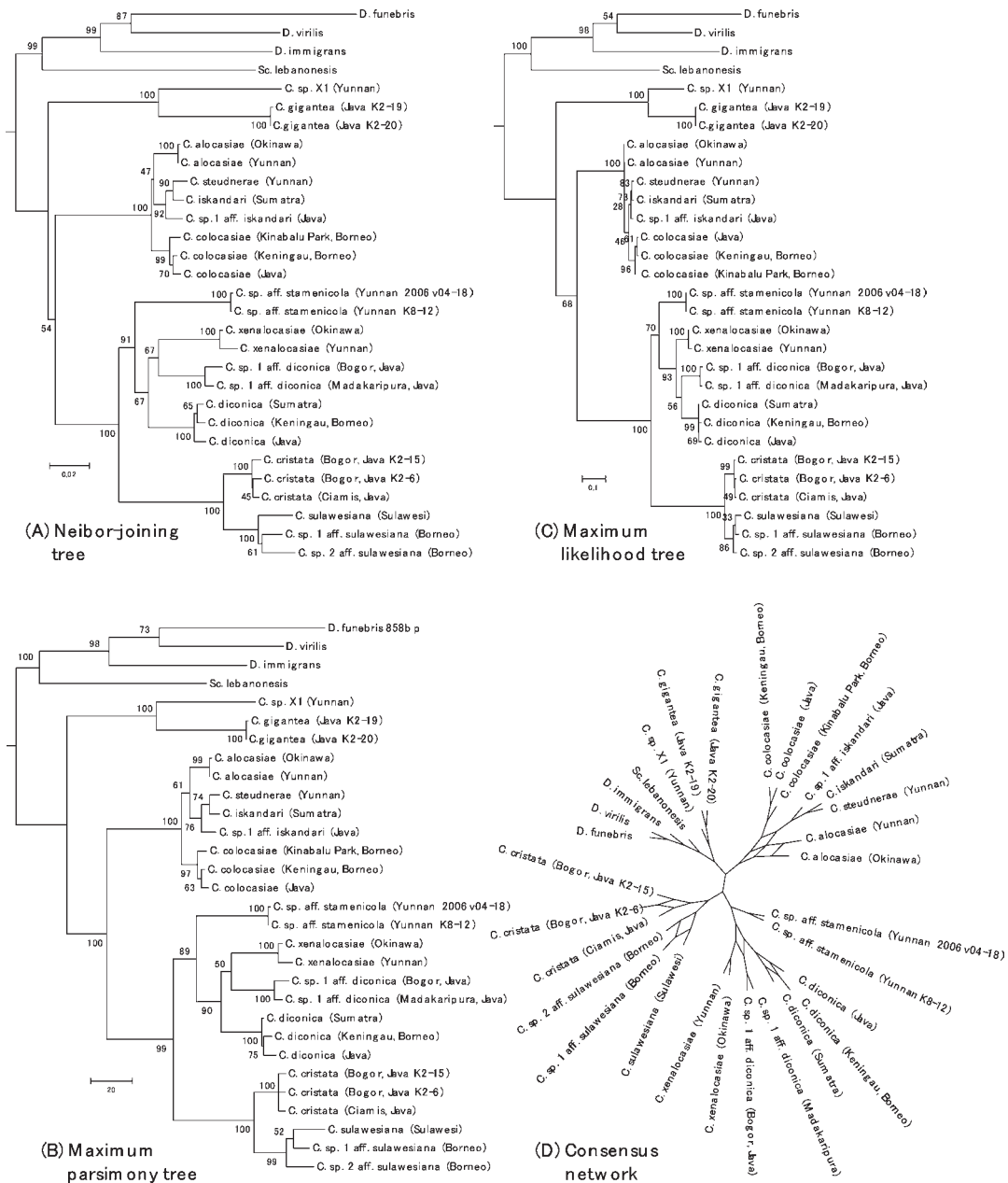


Figure 2 : The phylogenetic relationships among OTUs in the *Colocasiomyia cristata* species group and related taxa. (A) The neighbour-joining tree drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. (B) The consensus tree of two most parsimonious trees; branches without consensus are collapsed; except for the multifurcated branches, the tree is drawn to scale with branch lengths calculated using the average pathway method in the units of the number of changes over the whole sequence. (C) The maximum likelihood tree. (D) The consensus network. See the text for more details.

sulawesiana and *C. sp.2* aff. *sulawesiana* and the other remaining OTUs. This structure simultaneously represents two different topologies: (*C. sulawesiana*, (*C. sp.1* aff. *sulawesiana*, *C. sp.2* aff. *sulawesiana*)) in the NJ tree and ((*C. sulawesiana*, *C. sp.1* aff. *sulawesiana*), *C. sp.2* aff. *sulawesiana*) in the MP and ML trees. Likewise, two other box structures were located between external branches in each conspecific clade of *C. diconica* and *C. cristata* de Meijere, 1914. For the remaining boxes, each box was located at a node that connects three different species or clades: (*C. diconica*, *C. sp.1* aff. *diconica* and *C. xenalocasiae*) and (*C. colocasiae*, *C. alocasiae* and (*C. steudnerae* Takenaka & Toda, in Takenaka et al., 2006, *C. iskandari* (Okada, 1986) and *C. sp.1* aff. *iskandari*)). The *C. colocasiae*-*alocasiae* clade exhibited a reticulated structure because of the paraphyletic topology of *C. alocasiae* in the ML tree.

We redrew the consensus network (Fig. 2D) as a modified rooted species network (i.e. species tree) for the ingroup (Fig. 3). The clade consisting of *C. gigantea* (Okada, 1987) and *C. sp.X1* was placed as the sister to the *C. cristata* group. The *C. cristata* group was first divided into two clades associated with high bootstrap values (99–100%, Figs 3A–C), one comprising *C. colocasiae*, *C. alocasiae*, *C. steudnerae*, *C. iskandari* and *C. sp.1* aff. *iskandari* and the other comprising the remaining species. The latter further diverged into two clades associated with moderate to high bootstrap values (70–100%): (*C. sp. aff. stamenicola*, *C. xenalocasiae*, *C. diconica* and *C. sp.1* aff. *diconica*) and (*C. cristata*, *C. sulawesiana*, *C. sp.1* aff. *sulawesiana* and *C. sp.2* aff. *sulawesiana*). Within each clade, *C. sp. aff. stamenicola* first diverged from (*C.*

xenalocasiae, *C. diconica* and *C. sp.1* aff. *diconica*) and *C. cristata* first diverged from (*C. sulawesiana*, *C. sp.1* aff. *sulawesiana* and *C. sp.2* aff. *sulawesiana*).

4. Discussion

4.1 Phylogenetic relationships among species

In the cladistic analysis of Sultana et al. (2006), *C. gigantea* was not assigned to any species group in the genus *Colocasiomyia*. The results of the present study suggested that *C. gigantea* formed a monotypic lineage together with *C. sp.X1*, a taxonomically undescribed species, which had not been discovered at the time of the study by Sultana et al. (2006). However, the phylogenetic position of the clade (*C. gigantea* + *C. sp.X1*) within the genus remained unresolved because no species group other than the *C. cristata* group was included in our phylogenetic analyses.

Consistent results between Sultana et al. (2006) and the present study were that both of the *C. cristata*-*sulawesiana* clade and the *C. colocasiae*-*alocasiae* clade were recognized within the *C. cristata* group, although Sultana et al. (2006) included only *C. colocasiae* and *C. alocasiae* for the former clade and only *C. sp.1* aff. *sulawesiana* and *C. sp.2* aff. *sulawesiana* for the latter in their analysis. Morphologically, all the species of the *C. colocasiae*-*alocasiae* clade in the present study shared five stout spines on the foreleg second tarsomere and the conical process on the male sternite VI (Grimaldi, 1991; Sultana et al., 2006), and therefore the *C. colocasiae*-*alocasiae* clade corresponded to the *C. colocasiae* species subgroup (Takenaka et al., 2006).

In Sultana et al. (2006) the relationships among

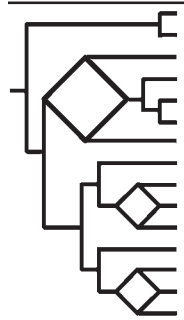
Phylogenetic relationship of <i>Colocasiomyia</i> flies	Typical combination of species sharing the same host inflorescence	Breeding habit	Ovipositor	Host plant	Area of distribution	Reference
		No breeding on the plant	-	<i>Rhaphidophora hookeri</i>	Yunnan	Unpublished
<i>C. sp.X1</i>			-	<i>Colocasia gigantea</i>	Java	Okada, 1987; Takenaka, 2006
<i>C. gigantea</i>		Stamenicolous	Narrow	<i>Colocasia esculenta</i>	The Oriental Region	Toda and Okada, 1987
<i>C. colocasiae</i>		No data	Wide	<i>Colocasia esculenta</i>	Java	Takenaka, 2006
<i>C. sp.1</i> aff. <i>iskandari</i>		No data	Wide	<i>Colocasia esculenta</i>	Java	Takenaka, 2006
<i>C. iskandari</i>		Intermediate	Wide	<i>Steudnera colocasiifolia</i>	Yunnan	Takenaka et al., 2006
<i>C. steudnerae</i>		Stamenicolous	Narrow	<i>Alocasia odora</i>	Ryukyu–Yunnan	Okada, 1980
<i>C. alocasiae</i>		Intermediate	Wide	<i>Alocasia odora</i>	Yunnan	Takano et al., Unpublished
<i>C. aff. stamenicola</i>		Pistillicolous	Wide	<i>Alocasia odora</i>	Ryukyu–Yunnan	Okada, 1980
<i>C. xenalocasiae</i>		Pistillicolous	Wide	<i>Colocasia gigantea</i>	Java and Sumatra	Takenaka, 2006
<i>C. sp.1</i> aff. <i>diconica</i>		Pistillicolous	Wide	<i>Colocasia esculenta</i>	Myanmar–Indonesia	Toda and Okada, 1987
<i>C. diconica</i>		Pistillicolous	Wide	<i>Alocasia alba</i>	Java	Takenaka, 2006
<i>C. cristata</i>		Pistillicolous	Wide	<i>Alocasia macrorrhizos</i>	Sulawesi	Okada and Yafuso, 1989; Takenaka, 2006
<i>C. sulawesiana</i>		Pistillicolous	Wide	<i>Alocasia macrorrhizos</i>	Borneo	Takenaka, 2006
<i>C. sp.1</i> aff. <i>sulawesiana</i>		Pistillicolous	Wide	<i>Alocasia macrorrhizos</i>	Borneo	Takenaka, 2006
<i>C. sp.2</i> aff. <i>sulawesiana</i>						

Figure 3 : Phylogenetic relationships among *Colocasiomyia* species and their breeding characteristics, host plants and geographical distributions.

the *C. colocasiae-alocasiae* clade, the *C. cristata-sulawesiana* clade and two independent species, *C. xenalocasiae* and *C. diconica*, were not resolved (Fig. 1). In the present analysis the two independent species formed a clade together with *C. sp. aff. stamenicola* and *C. sp.1 aff. diconica* and the relationships among the three monophyletic groups were resolved: the *C. cristata-sulawesiana* and the *C. xenalocasiae-diconica* clades are sister groups and the combined (*C. cristata-diconica*) clade is sister to the *C. colocasiae-alocasiae* clade (Fig. 2).

4.2 Evolution of the stamencolous and pistillicolous breeding habits

The two sister groups, the *C. colocasiae-alocasiae* and the *C. cristata-diconica* clades, included 'typical' stamencolous and pistillicolous species, respectively (Fig. 3). Okada (1986a) and Yafuso and Okada (1990) hypothesised the evolutionary origin and the dispersion of cohabitation between a stamencolous and a pistillicolous species as follows: a couple of ancestral species of *Colocasiomyia* had established cohabitation on a certain plant of Araceae in a certain area of the Oriental Region, probably the southern part of Angara land. Tightly keeping the cohabiting relationship, they then evolved into *C. alocasiae* and *C. xenalocasiae* and dispersed in the direction of Taiwan and Okinawa on the one hand, and into *C. colocasiae* and *C. diconica* in Myanmar, Indochina to Java as well as into *C. stamenicola* and *C. pistillicola* (Carson & Okada, 1980) in New Guinea on the other.

The results of the present study seem to partially support their hypothesis for the relationship between the cohabiting pair of *C. alocasiae* and *C. xenalocasiae* and the pair of *C. colocasiae* and *C. diconica*: the divergence between stamencolous and pistillicolous species may have occurred once at the base of the *C. cristata* group and the ancestral cohabiting pair radiated to several pairs on different host plants and at different locations: *C. alocasiae* and *C. xenalocasiae* on *Alocasia odora* in the area between Yunnan and Okinawa, *C. colocasiae* and *C. diconica* on *Colocasia esculenta* in Malesia and so on.

However, the *C. colocasiae-alocasiae* clade also includes *C. steudnerae*, *C. iskandari* and *C. sp.1 aff. iskandari*. *Colocasiomyia steudnerae* exhibits intermediate breeding habit between the stamencolous and pistillicolous species (Takenaka et al., 2006) (Fig. 3). The breeding habits of *C. iskandari* and *C. sp.1 aff. iskandari* are unknown, but both species possess the

wide ovipositors that are widely shared morphological characteristics among pistillicolous species, whereas stamencolous species possess the narrow ovipositors that are presumably an adaptation for laying eggs in the narrower spaces between stamens. Taking these into account, the evolutionary pattern of the stamencolous and pistillicolous breeding habits may have been more complicated than hypothesised by Okada (1986a) and Yafuso and Okada (1990).

4.3 Evolution of cohabiting pairs

Yafuso and Okada (1990) reported another pair of cohabiting species, *C. heterodonta* Yafuso & Okada, 1990 (stamencolous species) and *C. xanthogaster* Yafuso & Okada, 1990 (pistillicolous species), from inflorescences of *Homalomena* spp. (Araceae). According to Sultana et al. (2002), the *C. toshiokai* group, which includes these two species, is regarded as an independent lineage of the *C. cristata* group (Fig. 1). Thus, cohabitation seems to have evolved repeatedly in the genus *Colocasiomyia*. In this sense, a cohabiting pair of *C. sp.1 aff. sulawesiana* and *C. sp.2 aff. sulawesiana* might give another example of parallelism of cohabitation (Figs 1 and 3). Although these two cohabiting species are generally pistillicolous in their breeding habits, they show a tendency of differentiation in reproductive nature and traits parallel to that between stamencolous and pistillicolous species (Takenaka, 2006). This implies that they are in the initial phase of cohabitation with micro-allopatric niche differentiation. On the other hand, some pistillicolous *Colocasiomyia* species have been observed monopolizing their host plant inflorescences without cohabiting stamencolous species: *C. cristata* on *Alocasia alba* (Araceae), *C. sulawesiana* on *Alocasia macrorrhizos* (but see Okada and Yafuso, 1989) and *C. sp.1 aff. diconica* on *Colocasia gigantea* (Fig. 3). The reason for the lack of cohabiting stamencolous species is unclear yet, but it is notable that *C. cristata* and *C. sulawesiana* are intermediate in the female reproductive traits between *C. sp.1 aff. sulawesiana* and *C. sp.2 aff. sulawesiana* of the same clade (Takenaka, 2006).

4.4 Evolution of host selection

The members of the *C. xenalocasiae-diconica* and the *C. colocasiae-alocasiae* clades are widely distributed in the Oriental and Papuan regions and visit inflorescences of *Colocasia*, *Alocasia* and *Steudnera* species.

Colocasia esculenta is cultivated pan-tropically as an important crop plant today and is used as the host

plant by a number of *Colocasiomyia* species in the Oriental and Papuan regions. The region of its origin is still in dispute, but some researchers (Yen and Wheeler, 1968; Plucknett, 1976; Kurvilla and Singh, 1981; Matthews, 1995) believe that it is South-East Asia, probably within the Indo-Malayan region (but see Lebot, 1999). The host shifts between *Colocasia esculenta* and other plants such as of *Alocasia* and *Steudnera* may have occurred in the periphery of this region, e.g., southern China and the Sunda Islands.

For example, *C. alocasiae* and *C. xenalocasiae* might have sifted or expanded their host plants in the peripheral range of original host plant, probably *Colocasia esculenta*, because they breed on *Alocasia odora* in Taiwan and Ryukyu where inflorescences of *Colocasia esculenta* are not available, but utilize both plants in South China (M. J. Toda, pers. comm.).

The members of the *C. cristata-sulawesiana* clade exclusively utilise *Alocasia* species as host plants. At present the known distribution of this group is restricted to Borneo, Java and Sulawesi, which are known as the diversity centre of *Alocasia*. In addition to the species assigned to the *C. cristata-sulawesiana* clade in this study, *C. sp.3* aff. *diconica* and *C. sp.4* aff. *diconica*, which seem to be included in this clade (Toda et al., unpubl. data), have also been found monopolizing *Alocasia* inflorescences (Sultana et al., 2006). In the *C. cristata-sulawesiana* clade, the host shift from *Colocasia* to *Alocasia* might have occurred in couple with monopolization of host plant without cohabiting stamencolous species (but see Okada and Yafuso, 1989).

5. Concluding remarks

This study showed that the evolutionary origin of the cohabitation, the diversification of the stamencolous and pistillicolous breeding habits and the evolutionary route of the host selection in the *C. cristata* group are not so simple as previously thought by Okada (1986a) and Yafuso and Okada (1990). The plants so far recorded as hosts of the *C. cristata* group are still limited to about ten species of a few genera. Much more potential host plant species exist in the distribution range of the *C. cristata* group: for example, approximately ten *Colocasia* species are known from Indo-Malaya to the Sunda Islands (Mayo et al., 1997; Long and Liu, 2001; Cao and Long, 2003; Yin et al., 2004), 60–70 *Alocasia* species in the Oriental and

Papuan regions to Northeastern Australia (Hay et al., 1997; Mayo et al., 1997; Hay, 1998; Yuzammi and Hay, 1998; Hay, 1999) and eight species of *Steudnera* in Indochina excluding Malay Peninsula (Mayo et al., 1997). Given the high host specificity of each species in the *C. cristata* group, a number of *Colocasiomyia* species might have been undiscovered. Biogeographic information and comparative ecological studies based on a reliable phylogenetic hypothesis with comprehensive taxon sampling of both *Colocasiomyia* flies and their host plants will give us deeper insights into the evolution and diversity of this highly mutualistic insect-plant interactions.

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