Molecular phylogenies of figs and fig-pollinating wasps in the Ryukyu and Bonin (Ogasawara) islands, Japan

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The interaction between figs (*Ficus*, Moraceae) and fig-pollinating wasps (Chalcidoidea, Agaonidae) is one of the most specific mutualisms, and thus is a model system for studying coevolution and cospeciation. In this study we focused on figs and their associated fig-wasps found in the Ryukyu and Bonin (Ogasawara) Islands, Japan, because it has been suggested that breakdown in the specificity may occur in islands or at edge of a species' distribution. We collected 136 samples of 15 native fig species and 95 samples of 13 associated fig-wasps from all major islands in the Ryukyu Islands, including two fig species and one fig-wasp species endemic to the Bonin Islands. We performed molecular phylogenetic analyses using plastid DNA and nuclear ITS sequences for the figs and nuclear 28S rRNA and mitochondrial COI genes for the fig-wasps to investigate the inter-specific phylogenies and intraspecific variation within the mutualism. Our phylogenetic analyses using multiple samples per species show the single clade of each fig (except the Bonin endemic species) and fig-pollinating wasp species. Fig species belonging to the same subgenera formed well-supported clades in both plastid and ITS trees, except for the subgenus *Urostigma*. Likewise, fig wasps emerging from host fig species belonging to the same subgenera formed mostly well supported clades in both 28S and COI trees. Host specificity between the figs and fig-wasps functions strictly in these islands. There was very little sequence variation within species, and that no major geographic structure was found. The two Bonin endemic species (*F. boninsimae* and *F. nishimurae*) or their common ancestor and the associated fig-wasps (*Blastophaga* sp.) are apparently derived from *F. erecta* and its associated fig-wasps (*B. nipponica*), respectively, and probably migrated from the Ryukyu Islands.

Key words: *Ficus*, plastid DNA, nuclear ITS, 28S rDNA, mitochondrial COI

INTRODUCTION

The genus *Ficus* (Moraceae) is composed of about 750 species mainly distributed in tropical and subtropical regions of the world, exhibiting various life forms of tree, shrub, climber, and (hemi-)epiphyte (Corner, 1965; Berg, 1989, 1990). *Ficus* is one of the most diversified genera of woody plants in the tropics (Harrison, 2005), and because of the year-round production of the fruits, fig species play an important role as keystone plant resources

for many terrestrial organisms (Lambert and Marshall, 1991; Shanahan and Compton, 2001; Shanahan et al., 2001; Kissling et al., 2007). Symbiotic interaction between plants and animals is considered as one of important factors contributing the biodiversity of the terrestrial ecosystem especially in tropical and subtropical regions, therefore, it is interesting to know how the interaction among *Ficus* and their associated animals has arisen and is being maintained (Janzen, 1979; Frank, 1989; Kjellberg and Maurice, 1989; McKey, 1989; Windsor et al., 1989; Berg, 1990; Herre, 1996; Nason et al., 1998; Harrison, 2003; Harrison et al., 2003; Herre et al., 2008).

In contrast to the diversity of organisms feeding on fig fruit, the pollination of figs is entirely dependent on the members of a single clade of Chalcid wasps (Agaonidae) (Ramírez, 1970, 1974; Janzen, 1979; Wiebes, 1979;

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Corner, 1985). The genus *Ficus* is taxonomically defined by their unique, closed inflorescence (syconium), which bears many small unisexual flowers on the inside. The pollen-carrying female wasps enter via a small entrance (ostiole), protected by bracts, at the top of the syconium. Inside the syconium, the female wasps pollinate the flowers and oviposit in some of the ovules. Wasp larval development is entirely completed within the galled ovaries. It was previously thought that the interaction was predominantly species-specific, and therefore that lineages of figs and fig-wasps should have co-evolved and co-specified in isolation. Consequently, it was suggested that both groups should have basically identical phylogenetic relationships (Herre, 1996; Weiblen, 2001).

Recent advances in molecular phylogenetics afford us the opportunity to trace the co-evolutionary histories of figs and fig-wasps and to elucidate evolutionary trends of specific traits that contribute to the mutualism (e.g., Yokoyama, 1995, 2003; Herre et al., 1996; Machado et al., 1996, 2001, 2005; Kerdelhue et al., 1999; Weiblen, 2000, 2001, 2004; Weiblen and Bush, 2002; Cook and Rasplus, 2003; Jousset et al., 2003; Datwyler and Weiblen, 2004; Jackson, 2004; Rønsted et al., 2005; Su et al., 2008). These studies reveal a broad scale pattern of co-speciation, but at a finer scale within clades of closely related species there is little support for strict sense co-speciation (e.g., Weiblen and Bush, 2002; Machado et al., 2005; Su et al., 2008).

The degree of host specificity in fig wasps is fundamental to understanding the co-evolutionary biology of the interaction (Cook and Rasplus, 2003; Machado et al., 2005). Examples of two or more fig wasp utilizing the same host and of multiple host use by a single fig wasp have been known (Wiebes, 1966; Rasplus, 1994). However, they were thought to be exceptions to "the one-to-one rule". Recent studies, predominately from Central America, involving intense intraspecific sampling indicate that the breakdown in specificity is more prevalent, occurring in over a third of the species investigated. Population level molecular studies in both figs and fig-wasps have revealed high levels of intraspecific genetic heterogeneity, which may be a prerequisite for speciation, and suggest the presence of cryptic wasp species and hybridization among figs (Molbo et al., 2003; Machado et al., 2005; Haine et al., 2006; Su et al., 2008). It is thus important to conduct broad sampling throughout the distribution of each fig and fig-wasp species to understand the degree of intraspecific genetic variation.

Janzen (1979) proposed that the specificity between figs and fig-pollinating wasps might break down on islands or in very harsh environments towards the edge of a species' distribution, where pollinator populations are likely to suffer periodic extirpations. Indeed there is some evidence of this from the volcanic Krakatau Islands (Parrish et al., 2003). In this study, therefore, we focus on fig and

fig-wasp interactions found in the Ryukyu Islands to understand the specificity of fig and fig-wasp communities on islands in the northern edge of the species' distribution. In addition, we included the Bonin (Ogasawara) Islands because they are oceanic islands (ca. 1000 km from the Japan mainland and ca. 1200–1600 km from the Ryukyu Islands) and only endemic fig species (plus one introduced species) are found there.

The purposes of this study were to investigate (1) the inter and intraspecific sequence variation of Japanese figs and fig-wasps and (2) whether the molecular phylogenies of figs and fig-wasps collected from various localities in the Ryukyu and Bonin Islands are congruent or not, and finally to know whether the phenomenon of breakdown of the strict relationship between figs and fig-pollinating wasps in the Ryukyu Islands has occurred. We conducted molecular phylogenetic analyses of Japanese figs and their associated fig-pollinating wasps using sequence data of six non-coding plastid regions and nuclear ribosomal internal transcribed spacer (ITS) region for the fig phylogeny, and those of the nuclear 28S rRNA gene and mitochondrial COI gene for the wasp phylogeny.

MATERIALS AND METHODS

Sampling Sixteen species of *Ficus* are recognized as native figs in Japan (Fig. 1; Corner, 1965; Walker, 1976; Hatusima and Amano, 1994; Shimabuku, 1997; Yokoyama, 2006), nine of which (*F. superba* var. *japonica*, *F. caulocarpa*, *F. microcarpa*, *F. ampelas*, *F. irisana*, *F. virgata*, *F. benguetensis*, *F. septica*, and *F. variegata*) are widely distributed in the Southeast and East Asia (Corner, 1965). Four species (*F. pumila*, *F. thunbergii*, *F. nipponica*, and *F. erecta*) are mainly found in Japan, Korea, and China (Corner, 1965), and three species (*F. boninsimae*, *F. nishimurae*, and *F. iidaiana*) are endemic to the Bonin (Ogasawara) Islands.

The list of samples used in this study and their locality maps are given in Table 1 and Fig. 1, respectively. In the field, fresh leaves were collected and dried by silica gel for DNA analyses. The mature but unopened syconia (2 to 5) were also collected simultaneously from the same fig tree and kept in a plastic tube (50–100 mL). After the wasps had emerged from the syconia, we preserved them in 99.5% ethanol solution. We tried to collect all native figs and their fig-wasps. However, we failed to collect *F. iidaiana* in the Bonin Islands and the fig-pollinating wasps for *F. nipponica* and *F. nishimurae*, and were only able to collect one sample of fig-pollinating wasp for *F. irisana*, *F. pumila*, and *F. thunbergii*. In addition, we collected leaf sample and their associated fig-wasps from introduced *F. microcarpa* in the Bonin Islands. In total, we collected 136 leaf samples (individuals) from 15 species of *Ficus* for plastid DNA phylogeny (of which 49 samples were used for ITS phylogenetic analysis), and 95

Fig. 1. Locality maps of the samples used in this study. The locality numbers (sample ID numbers) correspond to those in Table 1 and all figures. Approximate northern limits of the distribution of all Japanese fig species are also presented by lines. *Ficus boninsimae*, *F. nishimurae*, and *F. iidaiana* are endemic to the Bonin Islands. Taxonomy of Japanese *Ficus* was followed by Yokoyama (2006).

Table 1. List of materials used in this study and their accession numbers

Fig species (subgenus / section) ^a / known pollinating wasp ^b	Locality	Voucher ^c or source	Abbr. of locality ^d	Sample ID	Accession numbers			
					Fig		Wasp	
					Plastid	ITS	28S	COI
<i>F. superba</i> var. <i>japonica</i> Miq. (<i>Urostigma</i> / <i>Urostigma</i>) / <i>Platyscapa ishii</i>ana (Grandi)								
	Anbo, Yaku-cho, Kagoshima Pref.	AZ096	YAK	119	T-2 ^e	–	–	–
	Yoshida to Iso, Kamiyaku-cho, Kagoshima Pref.	AZ098	YAK	121	T-2	–	–	–
	Koniya, Setouchi-cho, Kagoshima Pref.	AZ090	AMA	113	T-2	–	–	–
	Ikenosawa, Minamidaito-son, kinawa Pref.	AZ012	MDA	37	T-2	–	–	–
	Kita, Minamidaito-son, Okinawa Pref.	AZ015	MDA	40	T-2	–	–	–
	Oku, Kunigami-son, Okinawa Pref.	AZ029	OKI	54	T-2	–	–	–
	Asato, Gushikami-son, Okinawa Pref.	AZ032	OKI	57	T-2	AB485811	AB489204	AB488704
	Ikema, Hirara-shi, Okinawa Pref.	AZ068	MIY	93	T-2	–	–	–
	Taketomi-cho, Okinawa Pref.	RDH589	IRI	R589	T-2	AB485812-3	AB489204	AB488705
	Taketomi-cho, Okinawa Pref.	ZHS 000	IRI	00	T-2	–	–	–
	Taketomi-cho, Okinawa Pref.	ZHS 010	IRI	10	T-2	–	–	–
	Taketomi-cho, Okinawa Pref.	ZHS 024	IRI	24	T-2	–	–	–
<i>F. caulocarpa</i> Miq. (<i>Urostigma</i> / <i>Urostigma</i>) / <i>Platyscapa fischeri</i> Wiebes								
	Mt. Kuburadake, Yonaguni-cho, Okinawa Pref.	AZ045	YON	70	T-2	AB485818-21	AB489205	AB488708
	Isobe, Ishigaki-shi, Okinawa Pref.	AZ055	ISH	80	T-2	AB485822	AB489205	AB488709
	Taketomi-cho, Okinawa Pref.	ZHS 008	IRI	08	T-2	AB485814	AB489205	AB488706
	Taketomi-cho, Okinawa Pref.	ZHS 015	IRI	15	T-2	AB485815-7	AB489206	AB488707
<i>F. microcarpa</i> L.f. (<i>Urostigma</i> / <i>Conosycea</i>) / <i>Eupristina verticillata</i> Waterston								
	Kojima, Yaku-cho, Kagoshima Pref.	AZ099	YAK	122	T-2	AB485841-2	AB489207	AB488710
	Ayamaru-misaki, Kasari-cho, Kagoshima Pref.	AZ080	AMA	103	T-2	AB485838	–	–
	Gamo-zaki, Kasari-cho, Kagoshima Pref.	AZ084	AMA	107	T-2	AB485839-40	AB489207	–
	Kosyuku, Naze-shi, Kagoshima Pref.	AZ091	AMA	114	T-2	–	AB489207	–
	Shintosh, Minamidaito-son, Okinawa Pref.	AZ005	MDA	30	T-2	AB485828	–	–
	Ikenosawa, Minamidaito-son, Okinawa Pref.	AZ009	MDA	34	T-2	AB485829-32	AB489207	AB488711
	Ikenosawa, Minamidaito-son, Okinawa Pref.	AZ010	MDA	35	T-2	–	–	–
	Ikenosawa, Minamidaito-son, Okinawa Pref.	AZ013	MDA	38	T-2	–	AB489207	AB488712
	Ikema-jima, Hirara-shi, Okinawa Pref.	AZ069	MIY	94	T-2	–	–	–
	Thindahanata, Yonaguni-cho, Okinawa Pref.	AZ046	YON	71	T-2	AB485833	–	–
	Kubura to Hikawa, Yonaguni-cho, Okinawa Pref.	AZ048	YON	73	T-2	AB485834-5	AB489207	AB488713
	Mt. Kinbudake, Ishigaki-shi, Okinawa Pref.	AZ056	ISH	81	T-2	AB485836-7	AB489207	–
	Taketomi-cho, Okinawa Pref.	ZHS 009	IRI	09	T-2	AB485823	–	–
	Taketomi-cho, Okinawa Pref.	ZHS 018	IRI	18	T-2	AB485824-7	AB489207	AB488710
	Funaura, Taketomi-cho, Okinawa Pref.	AZ033	IRI	58	T-2	–	–	–
	Taketomi-cho, Okinawa Pref.	ZHS 013	IRI	13	–	–	AB489207	–
	Taketomi-cho, Okinawa Pref.	RDH581	IRI	R581	–	–	–	AB488710
	Taketomi-cho, Okinawa Pref.	RDH002	IRI	R2	–	–	AB489208	AB488714
	Taketomi-cho, Okinawa Pref.	ZHS 012	IRI	12	–	–	AB489208	AB488714
	Taketomi-cho, Okinawa Pref.	RDH591	HAT	R591	T-2	–	–	AB488710
	Okimura, Ogasawara-mura, Tokyo Pref.	ZHS OGS01	HAH	OGS01	T-2	AB485843-4	AB489207	AB488710
	Mt. Chibusayama, Ogasawara-mura, Tokyo Pref.	ZHS OGS04	HAH	OGS04	T-2	AB485845-8	AB489207	AB488710
	Harbor, Chichijima, Ogasawara-mura, Tokyo Pref.	ZHS OGS19	CHI	OGS19	T-2	AB485849-52	AB489207	AB488710
<i>F. ampelas</i> Burm.f. (<i>Sycidium</i> / <i>Sycidium</i>) / <i>Kradibia sumatrana</i> (Grandi)								
	Ishihara, Sumiyo-mura, Kagoshima Pref.	AZ087	AMA	110	T-2	AB485886-7	AB489209	AB488719
	Higashinakasonezoe, Hirara-shi, Okinawa Pref.	AZ073	MIY	98	T-2	–	–	–
	Mt. Kuburadake, Yonaguni-cho, Okinawa Pref.	AZ042	YON	67	T-2	AB485881-3	AB489209	AB488716
	Inoda, Ishigaki-shi, Okinawa Pref.	AZ052	ISH	77	T-2	AB485884-5	AB489209	AB488717
	Nozoko, Ishigaki-shi, Okinawa Pref.	AZ064	ISH	89	T-2	–	AB489209	AB488718
	Taketomi-cho, Okinawa Pref.	ZHS 025	IRI	25	T-2	–	–	–
	Funaura, Taketomi-cho, Okinawa Pref.	AZ036	IRI	61	T-2	AB485877-80	AB489209	AB488715
	Taketomi-cho, Okinawa Pref.	RDH584	IRI	R584	T-2	–	AB489209	AB488720
<i>F. irisana</i> Elmer (<i>Sycidium</i> / <i>Sycidium</i>) / <i>Kradibia commuta</i> Wiebes								
	Ikenosawa, Minamidaito-son, Okinawa Pref.	AZ014	MDA	39	T-2	–	–	–
	Ohtake, Ishigaki-shi, Okinawa Pref.	AZ058	ISH	83	T-2	AB485888-9	AB489210	AB488721
	Idona to Hoshino, Ishigaki-shi, Okinawa Pref.	AZ067	ISH	92	T-2	–	–	–
<i>F. virgata</i> Reinw. ex Blume (<i>Sycidium</i> / <i>Palaeomorpha</i>) / <i>Liporrhopalum philippinensis</i> Hill								
	You, Kasari-cho, Kagoshima Pref.	AZ081	AMA	104	T-2	–	–	–
	Akina, Tatsugo-cho, Kagoshima Pref.	AZ085	AMA	108	T-2	AB485895-7	AB489211	AB488723
	Nakano, Kitadaito-son, Okinawa Pref.	AZ020	KDA	45	T-2	–	AB489211	AB488724
	Kyutoh, Minamidaito-son, Okinawa Pref.	AZ002	MDA	27	T-2	–	–	–
	Minami, Minamidaito-son, Okinawa Pref.	AZ003	MDA	28	T-2	AB485891-2	AB489211	AB488722
	Ikenosawa, Minamidaito-son, Okinawa Pref.	AZ018	MDA	43	T-2	–	AB489211	AB488723
	Ada, Kunigami-son, Okinawa Pref.	AZ027	OKI	52	T-2	–	–	–
	Higashinakasone, Hirara-shi, Okinawa Pref.	AZ071	MIY	96	T-2	–	–	–
	Sonai, Yonaguni-cho, Okinawa Pref.	AZ041	YON	66	T-2	AB485893-4	AB489211	AB488722
	Nagura, Ishigaki-shi, Okinawa Pref.	AZ060	ISH	85	T-2	–	–	–
	Taketomi-cho, Okinawa Pref.	ZHS 001	IRI	01	T-2	–	–	–
	Taketomi-cho, Okinawa Pref.	ZHS 014	IRI	14	T-2	AB485890	AB489211	–
	Taketomi-cho, Okinawa Pref.	ZHS 022	IRI	22	T-2	–	AB489211	AB488722
	Taketomi-cho, Okinawa Pref.	RDH588	IRI	R588	T-2	–	AB489211	AB488725

Continued

Table 1. Continued

<i>F. benguetensis</i> Merr. (<i>Sycomorus</i> / <i>Sycocarpus</i>) / <i>Ceratosolen cornutus</i> Wiebes								
Kamiya, Sumiyo-mura, Kagoshima Pref.	AZ092	AMA	115	T-2	—	—	—	
Mihara, Nago-shi, Okinawa Pref.	AZ028	OKI	53	T-2	—	—	—	
Mt. Kuburadake, Yonaguni-cho, Okinawa Pref.	AZ043	YON	68	T-2	AB485856, AB485919	AB489212	AB488726	
Kubura to Hikawa, Yonaguni-cho, Okinawa Pref.	AZ047	YON	72	T-2	—	—	AB488727	
Inoda, Ishigaki-shi, Okinawa Pref.	AZ053	ISH	78	T-2	AB485857-60, AB485920	AB489212	AB488728	
Ohtake, Ishigaki-shi, Okinawa Pref.	AZ066	ISH	91	T-2	—	AB489213	AB488726	
Taketomi-cho, Okinawa Pref.	ZHS 021	IRI	21	T-2	—	AB489212	AB488726	
Taketomi-cho, Okinawa Pref.	RDH590	IRI	R590	T-2	—	AB489212	AB488726	
Taketomi-cho, Okinawa Pref.	ZHS 005	IRI	05	T-2	AB485853-5	AB489212	AB488726	
Funaura, Taketomi-cho, Okinawa Pref.	AZ034	IRI	59	T-2	—	AB489212	—	
Taketomi-cho, Okinawa Pref.	RDH004	IRI	R4	—	—	AB489212	AB488726	
<i>F. septica</i> Burm.f. (<i>Sycomorus</i> / <i>Sycocarpus</i>) / <i>Ceratosolen bisulcatus jucundus</i> Grandi								
Kyutoh, Minamidaito-son, Okinawa Pref.	AZ004	MDA	29	T-2	AB485863-6	AB489214	AB488732	
Minami, Minamidaito-son, Okinawa Pref.	AZ017	MDA	42	T-2	—	AB489214	AB488732	
Shirahama, Ohgimi-son, Okinawa Pref.	AZ024	OKI	49	T-2	AB485867	AB489214	AB488733	
Ada, Kunigami-son, Okinawa Pref.	AZ026	OKI	51	T-2	—	AB489214	AB488734	
Higashinakasonozoe, Hirara-shi, Okinawa Pref.	AZ072	MIY	97	T-2	—	—	—	
Agarizaki, Yonaguni-cho, Okinawa Pref.	AZ040	YON	65	T-2	AB485868-9	AB489214	AB488730	
Mt. Kuburadake, Yonaguni-cho, Okinawa Pref.	AZ044	YON	69	T-2	—	AB489214	AB488736	
Nozoko, Ishigaki-shi, Okinawa Pref.	AZ057	ISH	82	T-2	AB485870	AB489214	AB488735	
Moriyama, Ishigaki-shi, Okinawa Pref.	AZ063	ISH	88	T-2	—	AB489214	AB488736	
Taketomi-cho, Okinawa Pref.	ZHS 003	IRI	03	T-2	AB485861-2	AB489214	AB488729	
Taketomi-cho, Okinawa Pref.	ZHS 016	IRI	16	T-2	—	AB489214	AB488730	
Taketomi-cho, Okinawa Pref.	ZHS 017	IRI	17	T-2	—	—	AB488731	
Funaura, Taketomi-cho, Okinawa Pref.	AZ037	IRI	62	T-2	—	AB489214	AB488735	
Taketomi-cho, Okinawa Pref.	RDH585	IRI	R585	T-2	—	AB489214	AB488738	
Taketomi-cho, Okinawa Pref.	RDH586	IRI	R586	T-2	—	AB489214	AB488731	
Taketomi-cho, Okinawa Pref.	RDH580	IRI	R580	—	—	AB489214	AB488737	
<i>F. variegata</i> Blume (<i>Sycomorus</i> / <i>Sycomorus</i>) / <i>Ceratosolen appendiculatus</i> (Mayr)								
Mt. Kuburadake, Yonaguni-cho, Okinawa Pref.	AZ049	YON	74	T-2	—	—	—	
Mt. Bannadake, Ishigaki-shi, Okinawa Pref.	AZ054	ISH	79	T-2	—	AB489215	—	
Nagura, Ishigaki-shi, Okinawa Pref.	AZ061	ISH	86	T-2	—	AB489215	AB488740	
Taketomi-cho, Okinawa Pref.	ZHS 006	IRI	06	T-2	AB485871-2	AB489215	AB488739	
Taketomi-cho, Okinawa Pref.	ZHS 007	IRI	07	T-2	—	AB489215	AB488740	
Taketomi-cho, Okinawa Pref.	ZHS 019	IRI	19	T-2	AB485873-6	AB489215	AB488740	
Funaura, Taketomi-cho, Okinawa Pref.	AZ038	IRI	63	T-2	—	AB489215	—	
Taketomi-cho, Okinawa Pref.	RDH583	IRI	R583	T-2	—	AB489215	AB488741	
<i>F. pumila</i> L. (<i>Synoecia</i> / <i>Rhizocladus</i>) / <i>Wiebesia pumilae</i> (Hill)								
Nagata-misaki, Kamiyaku-cho, Kagoshima Pref.	AZ094	YAK	117	T-2	AB485898-900	AB489216	AB488742	
You, Kasari-cho, Kagoshima Pref.	AZ082	AMA	105	T-2	—	—	—	
Maehida, Kasari-cho, Kagoshima Pref.	AZ093	AMA	116	T-2	—	—	—	
Kyutoh, Minamidaito-son, Okinawa Pref.	AZ006	MDA	31	T-2	—	—	—	
Agarizaki, Yonaguni-cho, Okinawa Pref.	AZ050	YON	75	T-2	—	—	—	
Hirakubozaki, Ishigaki-shi, Okinawa Pref.	AZ059	ISH	84	T-2	—	—	—	
<i>F. thunbergii</i> Maxim. (<i>Synoecia</i> / <i>Rhizocladus</i>) / <i>Wiebesia</i> sp.								
Taketomi-cho, Okinawa Pref.	ZHS 020	IRI	20	T-2	—	—	—	
Taketomi-cho, Okinawa Pref.	RDH587	IRI	R587	T-2	AB485902	AB489217	—	
<i>F. nipponica</i> Franch. et Sav. (<i>Synoecia</i> / <i>Rhizocladus</i>) / <i>Wiebesia callida</i> (Grandi)								
Nakama, Yaku-cho, Kagoshima Pref.	AZ095	YAK	118	T-2	AB485901	—	—	
<i>F. erecta</i> Thunb. (<i>Ficus</i> / <i>Ficus</i>) / <i>Blastophaga nipponica</i> Grandi								
Kitamidorigaoka, Toyonaka-shi, Osaka Pref.	AZ001	OSA	26	T-2	—	AB489218	AB488744	
Ama, Nantan-cho, Hyogo Pref.	AZ078	AWA	101	T-2	—	—	—	
Nagata, Kamiyaku-cho, Kagoshima Pref.	AZ097	YAK	120	T-2	AB485911-3	AB489218	AB488752	
You, Kasari-cho, Kagoshima Pref.	AZ083	AMA	106	T-2	—	—	—	
Asato, Naze-shi, Kagoshima Pref.	AZ086	AMA	109	T-2	—	AB489218	AB488745	
Koniya, Setouchi-cho, Kagoshima Pref.	AZ089	AMA	112	T-2	—	AB489218	AB488745	
Minami, Kitadaito-son, Okinawa Pref.	AZ019	KDA	44	T-2	—	—	AB488745	
Kyutoh, Minamidaito-son, Okinawa Pref.	AZ007	MDA	32	T-2	—	—	—	
Kyutoh, Minamidaito-son, Okinawa Pref.	AZ008	MDA	33	T-2	—	—	—	
Ikenosawa, Minamidaito-son, Okinawa Pref.	AZ011	MDA	36	T-2	—	—	—	
Shintosh, Minamidaito-son, Okinawa Pref.	AZ016	MDA	41	T-2	—	AB489218	AB488745	
Nago, Nago-shi, Okinawa Pref.	AZ023	OKI	48	T-2	—	AB489218	AB488747	
China, Chinen-son, Okinawa Pref.	AZ030	OKI	55	T-2	AB485906	AB489218	AB488748	
China, Chinen-son, Okinawa Pref.	AZ031	OKI	56	T-2	—	AB489218	AB488749	
Afuso, Onna-son, Okinawa Pref.	AZ021	OKI	46	T-2	AB485904-5	AB489218	AB488745	
Afuso, Onna-son, Okinawa Pref.	AZ022	OKI	47	T-2	—	AB489218	AB488745-6	
Ada, Kunigami-son, Okinawa Pref.	AZ025	OKI	50	T-2	—	AB489218	AB488745	
Aragusuku, Gusukube-cho, Okinawa Pref.	AZ070	MIY	95	T-2	—	—	—	
Mt. Urabudake, Yonaguni-cho, Okinawa Pref.	AZ039	YON	64	T-2	AB485907-8	AB489218	AB488750	
Inoda, Ishigaki-shi, Okinawa Pref.	AZ051	ISH	76	T-2	—	—	—	
Mt. Omotodake, Ishigaki-shi, Okinawa Pref.	AZ062	ISH	87	T-2	AB485909-10	AB489218	—	

Continued

Table 1. Continued

Ohtake, Ishigaki-shi, Okinawa Pref.	AZ065	ISH	90	T-2	–	AB489218	AB488751
Taketomi-cho, Okinawa Pref.	ZHS 023	IRI	23	T-2	AB485903	AB489218	AB488743
Funaura, Taketomi-cho, Okinawa Pref.	AZ035	IRI	60	T-2	–	AB489218	AB488745
Taketomi-cho, Okinawa Pref.	RDH582	IRI	R582	T-2	–	AB489218	–
<i>F. boninsimae</i> Koidz. (<i>Ficus</i> / <i>Ficus</i>) / <i>Blastophaga</i> sp.							
Mt. Tyuohzan, Ogasawara-mura, Tokyo Pref.	Seo 5510	CHI	126	T-2	AB485917-8	AB489219	–
Mt. Chibusayama, Ogasawara-mura, Tokyo Pref.	Seo 5529	HAH	127	T-2	–	AB489219	–
Mt. Chibusayama, Ogasawara-mura, Tokyo Pref.	Seo 5530	HAH	128	T-2	–	AB489219	–
Mt. Chibusayama, Ogasawara-mura, Tokyo Pref.	Seo 5532	HAH	130	T-2	–	AB489219	–
Mt. Chibusayama, Ogasawara-mura, Tokyo Pref.	ZHS OGS05	HAH	OGS05	T-2	–	AB489219	–
Tamagawa Dam, Ogasawara-mura, Tokyo Pref.	ZHS OGS09	HAH	OGS09	T-2	–	AB489219	–
Mt. Mikkatsukiyama, Ogasawara-mura, Tokyo Pref.	ZHS OGS13	CHI	OGS13	T-2	–	AB489219	–
<i>F. nishimurae</i> Koidz. (<i>Ficus</i> / <i>Ficus</i>) / <i>Blastophaga</i> sp.							
Okumura, Ogasawara-mura, Tokyo Pref.	Seo 5494	CHI	123	T-2	–	–	–
Mt. Tyuohzan, Ogasawara-mura, Tokyo Pref.	Seo 5495	CHI	124	T-2	AB485914-6	–	–
OUTGROUPS (for plastid DNA of <i>Ficus</i>)							
<i>F. insipida</i> Willd. (<i>Pharmacosycea</i> / <i>Pharmacosycea</i>)	Liesner 1373	–	133	T-2	–	–	–
<i>F. tonduzii</i> Standl. (<i>Pharmacosycea</i> / <i>Pharmacosycea</i>)	Smith 2737	–	136	T-2	–	–	–
<i>Broussonetia papyrifera</i> Vent.	AZ077	–	100	T-2	–	–	–
<i>Castilla elastica</i> Sessé in Cerv.	Azuma s.n.	–	123b	T-2	–	–	–
OUTGROUPS (for nuclear ITS region of <i>Ficus</i>)							
<i>F. insipida</i> Willd. (<i>Pharmacosycea</i> / <i>Pharmacosycea</i>)	Ref-1 ^f	–	–	–	AY063592	–	–
<i>F. maxima</i> Mill. (<i>Pharmacosycea</i> / <i>Pharmacosycea</i>)	Ref-1	–	–	–	AY063595	–	–
OUTGROUPS (for fig wasps)							
<i>Tetrapus americanus</i> Mayr (host fig: <i>F. maxima</i>)	Ref-2 ^f	–	–	–	–	AB576184	AB308327
<i>T. costaricanus</i> Grandi (host fig: <i>F. insipida</i>)	Ref-2	–	–	–	–	AB576185	AB308328
<i>T. ecuadoranus</i> Grandi (host fig: <i>F. yoponensis</i>)	Ref-2	–	–	–	–	AB576183	AB308322

^aafter Berg and Corner (2005) for subgeneric and sectional classification.

^bafter Yokoyama (2006).

^cspecimens deposited at KYO except for “ZHS” series which are at BRH (Biohistory Research Hall).

^dsee Fig. 1.

^esee Table 2.

^fRef-1; Joussetin et al. (2003), Ref-2; Su et al. (2008).

samples (one wasp per fig tree) of the associated fig-pollinating wasps from 13 fig species (Table 1).

DNA extraction, PCR and sequencing of Figs

Total DNA was isolated from the silica gel-dried leaves (1–2 g) using modified CTAB (Cetyltrimethylammonium Bromide) method of Doyle and Doyle (1987). The following six non-coding plastid regions were amplified; *rps16* intron (primers; *rps16/1F* and *rps16/2R*), *trnG* intron (UCC2/F and UCC/1R), *petB* intron (*petB/1F* and *petB/2R*) (Nishizawa and Watano, 2000), *trnL* intron (c and d), *trnL-trnF* spacer region (e and f) (Taberlet et al., 1991), and *atpB-rbcL* spacer region (AT1 and RB) (Azuma et al., 1999).

The PCR mixture (20 µL) contained 1 µL (100–150 ng) of template DNA, 200 µmol/L of each dNTP, 1 µmol/L each primer, 2.5 mmol/L MgCl₂, 2 µL of 10X *Taq* buffer, 1U of *Taq* polymerase (TaKaRa Ex *Taq*, TaKaRa BIO INC., Japan). The PCR was performed by a GeneAmp PCR System 9700 (Applied Biosystems) starting at 94°C (5 min), followed by 35 cycles of denaturation at 94°C (60 sec), annealing at 50°C (90 sec), and extension at 72°C (2 min), and final extension at 72°C (7 min). After checking a single band by electrophoresis on 1% agarose gel, the PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN K. K., Tokyo, Japan). Direct sequencing of both strands were conducted on an ABI 3100 Genetic Analyzer (Applied Biosystems) using BigDye

Terminator version 3.1 Cyclic Sequencing Ready Reaction Kit (Applied Biosystems) under conditions in manufacturer's protocol.

For sequencing of the nuclear ITS region, primers ITS-Y5 and ITS-Y4 were used for the amplification under the condition described in Kita and Ito (2000). The thermal conditions for the PCR were as follows: denaturation at 94°C (5 min); followed by 35 cycles of denaturation at 94°C (30 sec), annealing at 50°C (30 sec), and extension at 72°C (1 min); and final extension at 72°C (7 min). When direct sequencing suggested polymorphism of ITS sequences, molecular cloning was conducted in such cases, and more than five colonies were applied for sequencing.

DNA extraction, PCR and sequencing of Fig-wasps

Total DNA was extracted from a single wasp using a QIAamp DNA Mini Kit (QIAGEN K. K., Tokyo, Japan). PCR amplification and sequencing of the cytochrome oxidase subunit I (COI) and nuclear 28S rRNA genes were conducted according to Su et al. (2001, 2008). A fragment of the mitochondrial DNA containing an approximately 1000 bp 5'-region of COI gene was amplified by a primer set “COI1-1-Ple” and “COI-2M” (Su et al., 2008), and a fragment comprising the variable domains D1 and D2 of 28S rRNA gene were amplified by a primer set “28S-01” and “28SR-01” (Kim et al., 2000; Su et al., 2001).

Alignment and phylogenetic analysis Alignments of the DNA sequences were carried out by MAFFT ver.6 with FFT-NS-2 methods (Kato et al., 2002; <http://mafft.cbrc.jp/alignment/server/>), which were then manually checked. Neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) criteria were used in the phylogenetic analyses. All analyses were performed by PAUP* 4.0b10 (Swofford, 2003), with heuristic search, 10 replications of random addition sequence, and TBR (tree bisection reconnection) branch swapping option (for MP and ML). One thousand (MP and NJ) and/or one hundred (ML) bootstrap replications were performed. Because of the huge amount of time required for the bootstrap analysis under the ML criterion, the bootstrap values were not estimated for ITS and COI trees. In MP analysis of ITS sequence data, the Maxtree was set to 10,000. For ML analyses, the nucleotide substitution model was selected by Akaike Information Criterion in Modeltest3.7 (Posada and Crandall, 1998). Samples which showed identical sequence were represented by one sample, ignoring differences in number of single-nucleotide repeats.

The sequence data of six non-coding plastid regions from each fig sample were combined into one sequence. Two species from different genera (*Broussonetia papyrifera* and *Castilla elastica*) in Moraceae were used as outgroups.

Two species in *Ficus* subgenus *Pharmacosycea* (*F. insipida* and *F. tonduzii*) were also included in the plastid phylogenetic analysis to confirm the *Pharmacosycea* species is sister to all other *Ficus* species, because they were designated as outgroups in ITS phylogenetic analysis. For ITS phylogenetic analysis, the sequences of two *Pharmacosycea* species (*F. insipida* and *F. maxima*) were used as outgroups. For the phylogenetic analyses of fig-wasps, three species of genus *Tetrapus* (*T. costaricanus*, *T. americanus*, and *T. ecuadoranus*) which pollinate *Pharmacosycea* species were used as outgroups (Rønsted et al., 2005). Prior to doing this, we confirmed that these *Tetrapus* species were sister to the collected fig-wasps by using non-pollinating or parasitic wasps as outgroups.

RESULTS

Plastid DNA phylogeny of *Ficus* A combined data matrix of six non-coding regions of plastid DNAs of *Ficus* and two genera (*Broussonetia* and *Castilla*) was composed of 3922 characters after alignment (total 24 OTUs) (accession numbers of new sequences are AB445493–AB445616, Table 2). There were 271 (6.9%) variable sites, of which 111 (2.8%) characters were parsimony-informative, among all OTUs, and 93 (2.3%) variable sites, of which 60

Table 2. List of accession numbers and haplotypes of plastid DNA of *Ficus*

Species sample ID	<i>rps16</i> intron	<i>trnG</i> intron	<i>petB</i> intron	<i>trnL</i> intron	<i>trnL-trnF</i> spacer	<i>atpB-rbcL</i> spacer	combined haplotype
Japanese Figs							
<i>F. superba</i> var. <i>japonica</i> (all samples)	AB445493	AB445513	AB445533	AB445554	AB445573	AB445593	A
<i>F. caulocarpa</i> (all samples)	AB445494	AB445514	AB445534	AB445555	AB445574	AB445594	A
<i>F. microcarpa</i> (all samples)	AB445495	AB445515	AB445535	AB445556	AB445575	AB445595	B
<i>F. ampelas</i> (all samples)	AB445496	AB445516	AB445536	AB445557	AB445576	AB445596	C
<i>F. irisana</i> (all samples)	AB445497	AB445517	AB445537	AB445558	AB445577	AB445597	C
<i>F. virgata</i> (all samples)	AB445498	AB445518	AB445538	AB445559	AB445578	AB445598	D
<i>F. benguetensis</i> (115)	AB445499	AB445519	AB445539	AB445560	AB445579	AB445599	E
(53, 78, 91)	AB445499	AB445519	AB445540	AB445560	AB445579	AB445599	F
(68, 72, 21, R590)	AB445499	AB445519	AB445540	AB445560	AB445579	AB445600	G
(05, 59)	AB445499	AB445519	AB445541	AB445560	AB445579	AB445600	H
<i>F. septica</i> (all samples)	AB445500	AB445520	AB445542	AB445561	AB445580	AB445601	I
<i>F. variegata</i> (74, 79, 86, 06, 07, 19, R583)	AB445501	AB445521	AB445543	AB445562	AB445581	AB445602	J
(63)	AB445502	AB445521	AB445543	AB445562	AB445581	AB445602	K
<i>F. pumila</i> (117, 105, 116, 31, 84)	AB445503	AB445522	AB445544	AB445563	AB445582	AB445603	L
(75)	AB445503	AB445522	AB445544	AB445563	AB445582	AB445604	M
<i>F. thunbergii</i> (all samples)	AB445504	AB445523	AB445545	AB445564	AB445583	AB445605	N
<i>F. nipponica</i> (all samples)	AB445505	AB445524	AB445546	AB445565	AB445584	AB445606	O
<i>F. erecta</i> (26, 101, 120, 106, 109, 112, 44, 32, 33, 36, 41, 48, 55, 56)	AB445506	AB445525	AB445547	AB445566	AB445585	AB445607	P
(46, 47)	AB445506	AB445525	AB445547	AB445566	AB445585	AB445608	Q
(50)	AB445506	AB445526	AB445547	AB445566	AB445585	AB445607	R
(64)	AB445506	AB445525	AB445547	AB445566	AB445585	AB445610	S
(76)	AB445506	AB445525	AB445547	AB445566	AB445586	AB445609	T
(95, 87, 90, 23, 60, R582)	AB445506	AB445525	AB445547	AB445566	AB445585	AB445609	U
<i>F. boninsimae</i> (all samples)	AB445507	AB445527	AB445548	AB445567	AB445587	AB445611	U
<i>F. nishimurae</i> (all samples)	AB445508	AB445528	AB445549	AB445568	AB445588	AB445612	U
American Figs							
<i>F. insipida</i>	AB445509	AB445529	AB445550	AB445569	AB445589	AB445613	V
<i>F. tonduzii</i>	AB445510	AB445530	AB445551	AB445570	AB445590	AB445614	W
OUTGROUPS							
<i>Broussonetia papyrifera</i>	AB445511	AB445531	AB445552	AB445571	AB445591	AB445615	X
<i>Castilla elastica</i>	AB445512	AB445532	AB445553	AB445572	AB445592	AB445616	Y

(1.5%) characters were parsimony-informative, within the ingroup (*Ficus*). The MP analysis generated two equally most parsimonious trees with tree length 294, CI (consistency index) excluding uninformative characters = 0.886, RI (retention index) = 0.929. The best-fit model for the ML analysis of plastid sequences was K81uf + G with gamma shape 0.5078. Topology of the strict consensus MP tree and NJ tree were identical to that of the ML tree (Fig. 2).

The MP, NJ and ML trees showed that the subgenus *Pharmacosycea* was sister to the rest of *Ficus* species, which comprised subgenera *Ficus*, *Synoecia*, *Sycidium*, *Sycomorus*, and *Urostigma*, although bootstrap values supporting the clade were low [61(ML) / < 50(NJ) / 64(MP)]. Judging from this and previous studies (Weiblen, 2000; Datwyler and Weiblen, 2004; Zerega et al., 2005), subgenus *Pharmacosycea* appears to have sister relationship with the other *Ficus* examined. Intraspecific sequence variations were observed in *F. erecta* (6 haplotypes), *F. pumila* (2), *F. benguetensis* (4), and *F. variegata* (2). While, following four pairs of species did not show interspecific sequence difference: one haplotype of *F. erecta* and the Bonin endemic species (*F. boninsimae* and *F. nishimurae*); *F. nipponica* and *F. thunbergii*; *F. ampelas*

and *F. irisana*; *F. superba* var. *japonica* and *F. caulocarpa* (Fig. 2).

There are five well [97/99/99 (subgenus *Ficus*), 100/99/100 (subg. *Synoecia*), 89/84/85 (subg. *Urostigma*) and 80/88/83 (subg. *Sycomorus*)] or moderately [61/55/59 (subg. *Sycidium*)] supported clades containing more than one species, which are basically concordant with circumscriptions of the subgenera (Berg and Corner, 2005) (Fig. 2).

Nuclear ITS phylogeny of *Ficus* An aligned sequence matrix of nuclear ITS sequences of *Ficus* was composed of 703 characters [total 84 OTUs from 49 leaf samples plus outgroups], in which *F. insipida* and *F. maxima* (subgenus *Pharmacosycea*) were set as outgroups (accession numbers of new sequences are AB485811–AB485920, Table 1). There are 198 (28.2%) variable sites, of which 130 (18.5%) characters were parsimony-informative, within the ingroup (Japanese *Ficus*), and 220 (31.3%) variable sites, of which 153 (21.8%) characters were parsimony-informative, among all OTUs. The MP analysis generated 10,000 (maxtree) equally parsimonious trees with tree length 388, CI excluding uninformative characters = 0.583, RI = 0.929. The best-fit model for the ML analyses was TVM + I + G with gamma shape

Fig. 2. Molecular phylogenetic tree of Japanese *Ficus* constructed by ML criterion using six plastid non-coding regions. The strict consensus tree by MP analysis also showed the same topology. Numbers on branches indicate bootstrap values (ML/NJ/MP > 50%). Number in parentheses indicates sample ID number (see Table 1), but for example, “*F. virgata* (all;14)” means all samples (14 individuals) of *F. virgata* showed an identical sequence. Subgeneric classification is also presented.

Figs (ITS)

Fig. 3. Molecular phylogenetic tree of Japanese *Ficus* constructed by ML criterion using nuclear ITS sequences. Branches which also appeared in the NJ tree and the strict consensus tree of 10000 MP trees were denoted by thick lines. Numbers on branches indicate bootstrap values (NJ/MP > 50%). Bootstrap analysis under ML criterion was not performed. Each OTU indicates sample ID (see Table 1) and clone ID (sample-clone), but “-a” at clone ID indicates that the sequence was determined by direct sequencing. Names of species and subgenera are presented at right side of OTUs. The sequences of two *Pharmacosyceae* species were retrieved from the DNA Data Bank of Japan (DDBJ).

0.8720. Clades that appeared in the strict consensus tree of 10,000 MP trees and NJ tree are denoted by thick lines in the ML tree (Fig. 3). The bootstrap values noted on the branches were estimated by bootstrap analyses using NJ and MP criteria (Fig. 3). Major discordance between the MP, NJ and ML trees was found in phylogenetic relationship among subgeneric clades.

Molecular cloning of amplified ITS sequences gave multiple copies in some samples but all copies derived from one species grouped together as a single clade with relatively high bootstrap values in MP, NJ and ML analyses, except for the following: copies of *F. boninsimae* and *F. nishimurae* (Bonin endemics) were nested with each other in a single clade within the clade of *F. erecta*, as was found in the plastid DNA phylogeny (clade A in Fig. 3 and see Fig. 2). Copies of *F. caulocarpa* formed a clade but the bootstrap value was low (clade E2 in Fig. 3). As in the plastid DNA sequences, ITS sequences of *F. thunbergii*

and *F. nipponica* showed no difference (clade B in Fig. 3).

In MP, NJ and ML analyses, close affinity among species belonging to the same subgenus was supported again. Phylogenetic resolution at interspecific level was better in the ITS phylogeny than in the plastid DNA phylogeny.

Nuclear 28S phylogeny of fig-wasps An aligned sequence matrix of the nuclear 28S gene of fig-wasps was composed of 959 characters (total 19 OTUs; 89 samples plus outgroups) (accession numbers of new sequences are AB489204–AB489219 and AB576183–AB576185, Table 1). There were 330 (34.4%) variable sites, of which 246 (25.7%) characters were parsimony-informative, within the ingroup, and 366 (38.2%) variable sites, of which 296 (30.9%) characters were parsimony-informative, among all OTUs. The MP analysis generated one most parsimonious tree with tree length 876, CI excluding uninformative characters = 0.602, RI = 0.709. Clades which

Fig. 4. Molecular phylogenetic tree of fig-pollinating wasps constructed by ML criterion using nuclear 28S gene sequences. Branches which also appeared in the NJ tree and the MP tree were denoted by thick lines. Numbers on branches indicate bootstrap values (ML/NJ/MP > 50%). Number in parentheses indicates sample ID (see Table 1), but for example, "*Ceratosolen bisulcatus* (all;14)" means all samples (14 individuals) of *C. bisulcatus* showed an identical sequence. Host figs from which the fig-wasps were collected and the subgeneric classification are also presented.

appeared in the MP and NJ trees are denoted by thick lines in the ML tree (Fig. 4). The best-fit models were GTR + I + G with gamma shape 0.4973 for 28S. The topologies of MP, NJ and ML trees were basically congruent, but the clade of *Kradibia* + *Liporrhopalum* branched first in the consensus MP and NJ trees. Species belonging to the same genera were grouped together in a single clade with high bootstrap values (Fig. 4).

Intraspecific variation was observed in *Ceratosolen cornutus* (from *F. benguetensis*) and *Platyscapa* sp.-2 (from *F. caulocarpa*). While, the other twelve species of fig-wasps did not show intraspecific variation. Fig-wasps emerged from fig species belonging to the same subgenera formed a single clade without exception. However, phylogenetic relationships among these clades were still unclear. Although *F. boninsimae* (Bonin endemic) showed no dif-

Fig. 5. Molecular phylogenetic tree of fig-pollinating wasps constructed by ML criterion using mitochondrial COI gene sequences. Branches which also appeared in the NJ tree and the strict consensus tree of MP trees were denoted by thick lines. Numbers on branches indicate bootstrap values (NJ/MP > 50%). Bootstrap analysis under ML criterion was not performed. OTUs indicate sample ID (see Table 1). Names of fig-wasps, host figs and their subgeneric classification are also presented. The sequences of outgroup species (*Tetrapus americanus*, AB308327; *T. costaricanus*, AB308328; *T. ecuadoranus*, AB308322) were analyzed in previous study (Su et al., 2008).

Fig. 6. Summarized interspecific relationship of Japanese *Ficus* (left) and their associated fig-wasps (right). Branches which appeared in both trees (plastid and ITS or 28S and COI) are presented with bootstrap values (plastid/ITS or 28S/COI). Dashed lines indicate the branches collapsed in the plastid phylogenetic tree. Arrowed lines indicate interaction between the fig and their associated fig-wasps. COI sequences of *Wiebesia* sp. and *Blastophaga* sp. were not determined, therefore, the bootstrap values for clades of *W. sp.* and *W. pumilae*, and *B. nipponica* and *B. sp.* were not indicated. *Ficus boninsimae* and *F. nishimurae* have nested within a clade of *F. erecta*. Because fig-wasps from *F. nipponica* and *F. nishimurae* were not collected, they were placed near the closest relatives.

ference with one haplotype of *F. erecta* in the plastid DNA tree (Fig. 2) and nested within a clade of *F. erecta* in the ITS tree (Fig. 3), the fig-wasp (*Blastophaga* sp.) emerging from *F. boninsimae* is clearly diverged from *B. nipponica* (*F. erecta*) (Fig. 4). The wasp species pollinating *F. superba* and *F. caulocarpa* appear to be morphologically different from the known pollinating wasp species of these two figs (Wiebes, 1966). Hence, we tentatively treated them as *Platyscapha* sp.-1 and *P. sp.-2* in this study (Figs. 4–6). To confirm this result, further molecular and morphological analyses are in progress.

Mitochondrial COI phylogeny of fig-wasps An aligned sequence matrix of mitochondrial COI genes of fig-wasps was composed of 868 characters (total 52 OTUs; 75 samples plus outgroups) (accession numbers of new sequences are AB488704–AB488752). No indels (insertion or deletion) were required for the sequence alignment. There are 407 (46.9%) variable sites, of which 376 (43.3%) characters were parsimony-informative, within the ingroup, and 419 (48.3%) variable sites, of which 384 (44.2%) characters were parsimony-informative, among all OTUs. The MP analysis generated 550 equally most parsimonious trees with tree length 1111, CI excluding uninformative characters = 0.522, RI = 0.896. Clades which appeared in the strict consensus MP tree and NJ tree are denoted by thick lines in the ML tree (Fig. 5). The best-fit models were GTR + I + G with gamma shape 0.6710. The bootstrap values noted on the

branches were estimated by bootstrap analyses using NJ and MP criterions. The interspecific topologies of MP, NJ and ML trees were basically concordant, but *Wiebesia pumilae* was sister to the *Ceratosolen* clade in the consensus MP tree, and to the clade with *Ceratosolen*, *Kradibia* and *Liporrhopalum* in the NJ tree.

Although all fig-wasp species showed intraspecific sequence variation (except *Kradibia commuta* and *W. pumilae* for which only one sample was examined), all species clearly formed a single clade with 100% bootstrap values. In addition to the 28S tree (Fig. 4), the COI tree also showed that fig-wasps emerging from figs belonging to the same subgenera formed a single clade. In the 28S phylogeny (Fig. 4), *Ceratosolen cornutus* and *Platyscapha* sp.-2 showed intraspecific sequence variation, however, the two species did not show the same pattern of variation in the COI phylogeny (Figs. 4 and 5).

DISCUSSION

Interspecific phylogeny of figs and their associated fig-wasps Molecular phylogenetic analysis of the non-coding plastid sequences indicated the sister relationship between two lineages (sects. *Urostigma* and *Conosycea*) in subgenus *Urostigma* (Fig. 2). In contrast, the ITS phylogeny suggested that the two lineages were distantly related (clades E1 and E2 in Fig. 3), which were concordant with the results in previous ITS phylogenetic studies (Weiblen, 2000; Jousset et al., 2003; Rønsted et al.,

2005). Although the two lineages belong to different sections, the subgenus *Urostigma* is well characterized as being monoecious (the other subgenera studied here are all dioecious), producing aerial roots, and the presence of a single waxy gland (Berg and Corner, 2005). Moreover, it should be noted that it was shown that the fig-wasps collected from these host fig species were closely related to each other in both 28S and COI phylogenetic trees (*P. sp.-1*, *P. sp.-2*, *Eupristina verticillata*, and *E. sp.* in Figs. 4 and 5), consistent with previous studies (Machado et al., 2001; Weiblen, 2001; Jiang et al., 2006; Lopez-Vaamonde et al., 2009). These results suggest that monophyletic relationship of members in subgenus *Urostigma* detected in the plastid phylogeny may be a more reliable phylogenetic hypothesis than that found in the ITS tree, although more extensive analysis should be conducted.

On the whole, the interspecific relationships of figs were better resolved in the ITS tree than the plastid tree, indicating a high performance of the ITS sequence in phylogenetic inference of the lower-level phylogeny of *Ficus*. For example, relationships among members of subgenus *Sycomorus* (*F. benguetensis*, *F. variegata*, and *F. septica*) were not resolved (polytomous) in the plastid tree (Fig. 2), but they were clear in the ITS tree (Fig. 3). Likewise, *F. ampelas* and *F. irisana* in subgenus *Sycidium* showed an identical sequence of plastid DNA, but they were distinct from each other in the ITS sequences.

Although the inter-subgeneric relationships of figs were generally unclear, species taxonomically belonging to the same subgenus formed a single clade in both plastid and ITS trees (except for subgenus *Urostigma* in the ITS tree) with relatively high bootstrap supports (Figs. 2 and 3). As was expected, the same pattern was observed in the phylogenies of the associated fig-wasps, that is, the fig-wasps collected from the host figs belonging to the same subgenus formed a well-supported clade without exception, but the relationships among these clades are still uncertain in most cases (Figs. 4 and 5).

These results indicate that the fig and fig wasp lineages found in the Ryukyu Islands have been reproductively isolated throughout their evolutionary history and suggests that the host specificity of these fig-pollinators remains very high today, despite being at the northern edge of the species' range and scattered across an island archipelago. There was no evidence of host switching among fig-wasps, as has been reported from elsewhere (e.g., Machado et al., 2005; Su et al., 2008). The nuclear ITS sequence data is sometimes used as a molecular marker to detect a historical or current hybridization event in plants, as it provides multiple copies (or a chimeric copy) from both maternal and paternal progenitor lineages (e.g., Sang et al., 1995; Barkman and Simpson, 2002; Siripun and Schilling, 2006). However, we found that all ITS copies derived from one species of fig were gathering in a single clade and supported by high boot-

strap values (except for *F. caulocarpa*). Thus, it seems that hybridization among closely related *Ficus* species has not occurred in the Ryukyu Islands (Fig. 3).

Interestingly, *F. nipponica* and *F. thunbergii* (in subgenus *Synoecia*) are the only species which showed an identical ITS sequence, even if they are morphologically distinct. However, because both species are relatively rare in the Ryukyu Islands (one and two samples collected, respectively) and we did not perform molecular cloning procedures, more critical analysis should be conducted. It should also be noted that both species reproduce vegetatively.

In this study, we did find one example of two wasp species (*E. verticillata* and *E. sp.*) pollinating one fig species (*F. microcarpa*) (Figs. 4 and 5). These two species can be easily distinguished from each other by their morphological features. One appears to be an undescribed species. As seen in the phylogenetic trees (Figs. 4 and 5), *E. sp.* is closely related to the known pollinating wasp species (*E. verticillata*) of *F. microcarpa*, suggesting it may be a case of breakdown of the host specificity or recent speciation at one fig species. In this study we were able to analyze the pollinating wasps from a total of 14 fig trees of *F. microcarpa*. *Eupristina sp.* was only collected from two trees inhabiting the Iriomote island, where *E. verticillata* was also distributed.

Intraspecific variations of figs and fig-wasps in the Ryukyu Islands To understand the evolutionary process of co-speciation between the fig and fig-wasp mutualism, it may be important to reveal intraspecific genetic variation (or structure) in both fig and fig-wasp, which sometimes leads to finding cryptic species (Molbo et al., 2003; Haine et al., 2006).

Molecular cloning produces multiple copies of ITS sequences from a single leaf in most cases and these copies scattered and nested with other copies originated from different individuals of the same species (Fig. 3). It is therefore difficult to infer intraspecific variation of each fig species based on the ITS sequence data. On the other hand, plastid DNA has often been used to detect intraspecific variation and indeed gave a clear geographic structure in some plant species native in Japan (e.g., Fujii et al., 1997; Ohi et al., 2003a, 2003b).

Phylogenetic analysis of six non-coding plastid DNA sequences of *Ficus* revealed that there was relatively large intraspecific variation in *F. erecta* (six haplotypes) (Fig. 2). Three haplotypes formed a subclade with low bootstrap values (63/62%), and the other three haplotypes showed an unclear relationship. All samples constituting the subclade were collected from northern area of the Ryukyu Islands and the mainland (i.e., OKI, KDI, MDI, AMA, YAK, AWA, and OSA; Fig. 1), and the rest samples were collected from the southern islands in the Ryukyu Islands (MIY, YON, ISH, and IRI; Fig. 1), suggesting that

genetic differentiation between the northern and southern areas has occurred possibly by a geographical isolation, although the associated fig-wasp (*B. nipponica*) did not show clear intraspecific variation and geographic structure.

Ficus benguetensis and its pollinator, *C. cornutus*, showed intraspecific variations (Figs. 2, 4, and 5). However, a clear geographical structure was not evident from the phylogenetic trees, and the phylogenetic relationships among the haplotypes were not concordant between the fig and the fig-wasp, and between the 28S and COI trees of the wasp. Likewise, although there was no intraspecific variation in the plastid DNA of the host figs, the COI phylogenies indicated that *K. sumatrana* (*F. ampelas*) and *L. philippinensis* (*F. virgata*) each divided into two well-supported clades. However, the clades did not correspond to the island geography (Figs. 1 and 5).

Because the number of individuals analyzed and molecular approaches used in this study may be insufficient to detect or infer the intraspecific genetic structure of each species, we need to use more proper DNA markers for population genetic analysis to reveal intraspecific variation in figs and fig-wasps.

Origin of species endemic to the Bonin Islands and relationship with wasps Because the Bonin (Ogasawara) Islands are typical oceanic islands, located ca. 1000 km south of the mainland of Japan and ca. 1200 km east of the Ryukyu Islands (Fig. 1), plants and animals living on the islands (or their ancestors) must be migrated from adjacent continents or continental islands. There are three endemic fig species (*F. boninsimae*, *F. nishimurae*, and *F. iidaiana*) in the Bonin Islands, all of which belong to section *Ficus* (Corner, 1965; Berg and Corner, 2005; Yokoyama, 2006). Although *F. iidaiana* could not be included in this study, a phylogenetic analysis using plastid non-coding sequences clearly indicated that all samples of *F. boninsimae* and *F. nishimurae* were identical to each other and to one haplotype of *F. erecta* (Fig. 2). All samples of *F. erecta* showing the identical haplotype with *F. boninsimae* and *F. nishimurae* were collected from the southern Ryukyu Islands (i.e., MIY, ISH, IRI, Fig. 1). Likewise, the ITS phylogenetic tree also showed that *F. boninsimae* and *F. nishimurae* were not distinct, but formed a single clade with lineages of *F. erecta* (Fig. 3).

Morphologically, *F. boninsimae* and *F. nishimurae* are considered to be closely related and are sometimes treated as a single species (Corner, 1965; Yokoyama, 2006), but there are a few morphological and ecological differences (Yokoyama, 2006). However, when the two species are compared with *F. erecta*, there are few good diagnostic characters (Yokoyama, 2006). The molecular phylogenetic analyses conducted here evidently suggest that a common ancestor of the two species originated from *F. erecta* in the southern Ryukyu Islands (a possible migration from Taiwan or southern China cannot be

rejected). Having established in the Bonin Islands the ancestor diversified morphologically into the two species in response to the isolated island environments without genetic differentiation at plastid sequence level, and with little differentiation in ITS sequence or in the RAPD analysis by Yokoyama (2003). This situation is considered to be a consequence of rapid radiation, a typical phenomenon in oceanic islands (Carlquist, 1974; Crawford and Stuessy, 1997; Baldwin et al., 1998; Ito, 1998).

We were only able to collect fig-wasps from *F. boninsimae* in the Bonin Islands, and we were only able to determine the nuclear 28S sequence for phylogenetic analysis. The analysis of the 28S sequences of all fig-wasps collected clearly indicated that *Blastophaga* sp. from *F. boninsimae* was sister to *B. nipponica* (from *F. erecta*) (Fig. 4). There was no intraspecific sequence variation in either species and 2.92% base difference was observed between them. This value is almost equivalent to that between *K. sumatrana* (*F. ampelas*) and *K. commuta* (*F. irisana*) (3.99%) and between *W. pumilae* (*F. pumila*) and *W. sp.* (*F. thunbergii*) (2.19%), suggesting that *B. sp.* (*F. boninsimae*) and *B. nipponica* (*F. erecta*) have differentiated enough to establish reproductive isolation each other, even if the host-figs (*F. boninsimae* and *F. erecta*) have not clearly diverged into the two lineages.

Species introduced in the Bonin Islands A few individuals of *F. microcarpa* without its pollinating wasps were introduced into the Bonin Islands in the early Meiji era (more than one hundred years ago), but have produced a number of mature syconia in recent years. We were interested in which wasps pollinated the introduced *F. microcarpa* on the Bonin Islands.

All of the fig-wasps collected from *F. microcarpa* in the Bonin Islands are morphologically considered to be the pollinating fig-wasp, *E. verticillata*, and indeed phylogenetic analyses of the wasp clearly indicated that they were identical to a lineage found in the Ryukyu Islands in both 28S and COI phylogenies (OGS01, 04, 19 in Figs. 4 and 5), suggesting long distance dispersal, more than 1200 km, of the fig-wasp from the Ryukyu Island to the Bonin Islands. It may be considered that the recent migration was effected by human activities. However, there is no direct transportation between the Ryukyu and Bonin Islands and no regular airport in the Bonin Islands. Hence, human assisted migration is considered unlikely.

Conclusion Summary of results of the phylogenetic analyses and interaction between figs and fig-wasps found in the Ryukyu and Bonin Islands is illustrated in Fig. 6. The host specificity between the figs and fig-pollinators is tightly maintained in the Ryukyu and Bonin Islands. There is no or little intraspecific variation in both figs and fig-wasps among islands, suggesting the dispersal within the Ryukyu Islands occurs easily,

although in *F. erecta* there is some divergence between northern and southern areas. The two endemic species (*F. boninsimae* and *F. nishimurae*) or their common ancestor in the Bonin (Ogasawara) Islands and the associated fig-wasps (*Blastophaga* sp.) are apparently derived from *F. erecta* and its associated fig-wasp (*B. nipponica*), respectively, which probably migrated from the Ryukyu Islands (ca. 1200–1600 km) or a more distant area.

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