Doctoral Thesis

Tohoku University

The Phylogenetic and Evolutionary Study of Japanese Asclepiadoideae (Apocynaceae)

（日本産ガガイモ亜科（キョウチクトウ科）の系統・進化学的研究）

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CONTENTS

ABSTRACT

The subfamily Asclepiadoideae (Apocynaceae) comprises approximately 2,000 species, and mainly occurs tropical and subtropical regions through the world. In Japan, 35 species belonging to nine genera have been recorded. Although many taxonomic studies have been conducted so far, the studies treating ecological and phylogenetical aspects are quite few. Therefore, I first conducted taxonomic re-examination for Japanese Asclepiadoideae based on the morphological observation of herbarium specimens and living plants. Furthermore cytotaxonomic study, pollinator observations, breeding system analysis of an autogamous species, and molecular phylogenetic analysis on *Vincetoxicum* and its allied genera were performed. Then, I discussed evolutionary trends and histories for *Vincetoxicum* and its allied genera.

From the result of taxonomic re-examination for Japanese Asclepiadoideae species based on the morphological observations, I proposed two new combinations, i.e., *Tylophora matsumurae* and *Vincetoxicum yonakuniense*, and described two new *Vincetoxicum* species, i.e., *Vincetoxicum izuense* and *V. hoyoense*.

Somatic chromosome numbers were examined for 28 species and two varieties of seven genera in Asclepiadoideae collected in Japan. The chromosome numbers of 21 species and two varieties were newly reported here. All taxa examined had chromosome numbers of 2n = 22 except for 2n = 44 in *Cynanchum caudatum* var. *caudatum*, *Vincetoxicum ambiguum*, *V. sublanceolatum* var. *sublanceolatum*, *V. sublanceolatum* var. *macranthum* and 2n = 24 for *Cynanchum boudinieri*. This result indicates that the polyploidy is not important factor for diversification in Japanese Asclepiadoiedeae.

Pollinators for 28 species belonging to seven genera of Asclepiadoideae were observed in the field. Pollination systems found in 28 species observed are follows: i) moth pollination, ii) generalized insect pollination, iii) wasp pollination, iv) dipteran pollination, v) dipteran and moth pollination, vi) autogamy.

Reproductive characters and genetic diversities were investigated for an autogamous insular endemic species of *Tylophora matsumurae* and its progenitor species of *T. tanakae*. In *T. matsumurae*, the anther sacs were not dehisced even at anthesis. Pollen tubes were germinated *in situ* in anther sacs and fertilized ovules of the same flower. These facts suggest a highly autogamous nature of *T. matsumurae*. No isozyme variations were detected in all seven populations *T. matsumurae* examined. A phenogram constructed using the neighbor-joining method based on Nei's unbiased genetic distance indicated that the *T. matsumurae* clustered with an Okinoerabu Island population of *T. tanakae*. It was suggested

that *T. matsumurae* has been derived from the predominated self-pollinating population of *T. tanakae* in central Ryukyu Islands, such as Okinoerabu Island, and rapidly enlarged its distribution area. The highly autogamous nature of *T. matsumurae* has probably played major role in its quick expansion.

Molecular phylogenetic analyses of *Vincetoxicum* and its allied genera were conducted based on the nucleotide sequences of cpDNA (two intergenic spacers of *trnL* (UAA)-*trnF* (GAA) and *psbA-trnH* and three introns, i.e., *atpF*, *trnG* (UCC) and *trnL* (UAA)), and nrDNA (ITS and ETS regions). From the result of the phylogenetic analysis, two monophyletic groups were recognized; one consisted of seven taxa of *Tylophora, Vincetoxicum inamoenum, V. magnificum* and *V. macrophyllum* (Clade I) and the other consisted of seventeen accessions of *Vincetoxicum* (Clade II). The monophyly of the genus *Vincetoxicum* was not supported. Although many nucleotide substitutions were observed in Clade I, the genetic differentiation within Clade II was small. Low genetic diversification but considerable morphological divergence suggests that the species in Clade II had undergone rapid diversification.

The phylogenetic distribution of pollinator types indicated that all species in Clade I were pollinated by Diptera, and that, Clade II were pollinated by the four insects orders. The most conspicuous pollination mode sifts found in Clade II was the changing from dipteran to moth pollinators. The most important character shared by four moth-pollinated species is relatively long guide rail. A switch from dipteran to moth may require only slight change, which is guide rail length in *Vincetoxicum* species.

A possible hypothesis for the process of rapid radiation in Clade II is as follow. A widely spread ancestral species partitioned its distribution areas and adapted to various environments such as edges of forest, sunny meadows, marsh and rocky beach. Then, they adapted to local dominant pollinators, and each population has isolated and diverged from other populations.

CHAPTER I. GENERAL INTRODUCTION

The subfamily Asclepiadoideae (Apocynaceae) comprises approximetely 2,000 species, and mainly occurs tropical and subtropical regions through the world (Zomlefer, 1994). The most of the species of the subfamily are twining perennial herbs, rarely trees, shrubs and succulents (Mabberley, 1997). On the one hand, the flowers show all the usual floral organs and their number is extremely stable among the members of the subfamily (five sepals, five petals, five stamens, and two carpels) (Endress, 1994). On the other hand, this subfamily has been discriminated from the other subfamilies of Apocynaceae by following three unique floral characters (Fig. 1). First, a pollinarium consisted of mass of pollen grains and translator apparatus, which are developed from stigmatic secretion and attaches the pollinarium to pollinator mechanically. Second, a gynostegium consists of post genitally fusion of the five stamens and an apex of a style. Third, corona composed one or more wholes of structure adnate to, or located between, the corolla and stamens. These elaborate structures of flowers of Asclepiadoideae enable highly adaptation in animal mediated pollination as well as Orchidaceae (Endress, 1994). The interactions between complicated flowers of asclepiads and animal pollinators have fascinated ecologists and the knowledge of the pollination mechanisms of asclepiads has been advanced through the North American members of the genus *Asclepias* (reviewed by Wyatt and Broyles, 1994).

For the last decade, the anatomy, morphology and phylogeny of the Asclepiadoideae are rapidly advanced (Ollerton and Liede, 1996, Endress and Stevens, 2001). In particular, recent molecular phylogenetic analyses rapidly increase the knowledge of relationships of the genera in the Asclepiadeae (e.g. Sennblad and Bremer, 1994, 2000; Civeryerl et al., 1998; Liede, 2001; Liede and Tauber, 2000; Liede et al., 2002).

In Japan, 35 species belonging to nine genera are so far known to be distributed and all of these genera are belonging to the tribe Asclepiadeae and Marsdenieae (Yamazaki 1993). Although many taxonomic studies have been conducted so far (e.g. Maximowicz, 1877; Matsumura, 1898; Naki, 1914, 1937; Koidozumi, 1932, 1938; Kitagawa, 1940, 1959; Ohwi 1943; 1965; Ohwi and Ohashi, 1973; Hatusima, 1963, 1977; Ohashi, 1966, 1990; Yamazaki 1968, 1993), the studies treating ecological and phylogenetical aspects are quite few.

In chapter II, I conducted taxonomic re-examination for Japanese Asclepiadoideae and proposed two new combinations and described two new species. Cytotxonomical study on Japanese Asclepiadoideae was carried out in chapter III. In chapter IV, I studied pollination biology of 28 Japanese asclepiad species. In chapter V, I examined ecological and genetic aspect of an autogamos species in this subfamily as a case study. In chapter VI, I performed

phylogenetic analysis on *Vincetoxicum* of which center of diversities is in temperate Asia and its allied genera. Then, I discuss the evolutionary trends for the *Vincetoxicum* and its allied genera by integrating the data of all chapters in chapter VII.

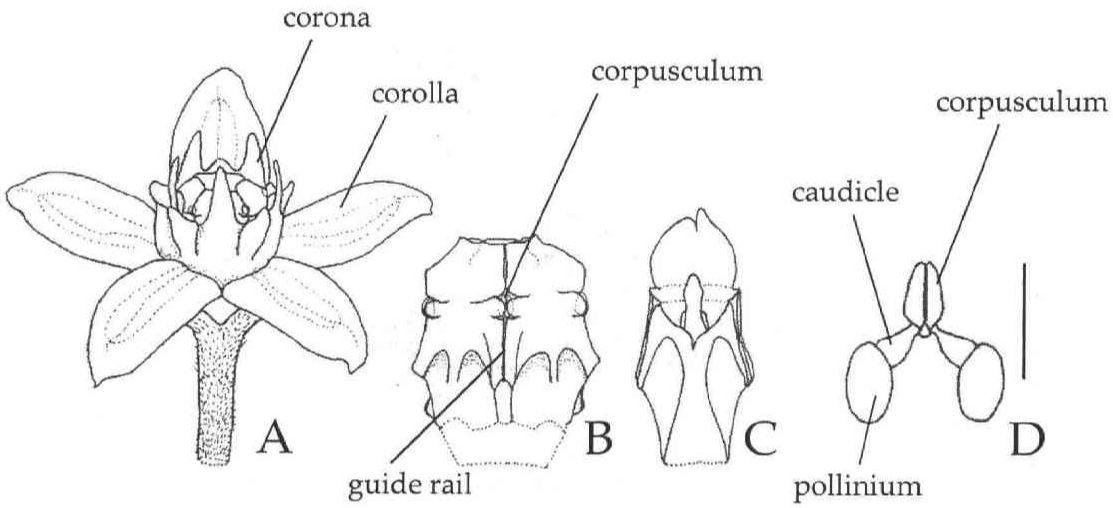

Fig. 1. General floral morphology of the Asclepiadeae (*Cynanchum liukiuense* Warb.). A. whole flower. B. gynostegium; corolla and corona are removed. C. adaxial view of stamen. D. porinarium. Scale bar = 2 mm for A; 1 mm for B, C; 0.6 mm for D.

CHAPTER II. TAXONOMIC REVISION ON SOME JAPANESE ASCLEPIADOIDEAE SPECIES

In Japan, 35 asclepiad species in nine genera are recorded, and all species belong to the tribe Asclepiadeae (three genera) or Marsdenieae (six genera) (Yamazaki, 1993). Recent morphological and phylogenetic studies provide new aspects for delimitation on several genera (Endress and Bryans, 2000; Swarupanandan et al., 1996; Liede, 1996a; Liede et all. 2002).

For example, the genus *Vincetoxicum* have been grouped with the closely related genus *Cynanchum* (e.g. Hooker, 1883; Tsiang and Li, 1977; Gilbert et al., 1995), although recent studies on morphology, chemical constituents and molecular phylogeny revealed that this genus is not monophyletic (Qiu et al., 1989; Liede, 1996a; Liede et al., 2002). Based on these evidences, *Vincetoxicum* is now considered to be an independent genus (e.g. Liede et al., 1996a; Endress and Bruyns, 2000). Moreover molecular phylogenetic studies revealed that *Vincetoxicum* is rather closely related to *Tylophora* than *Cynanchum* (Liede et al., 2002). Thus, taxonomic treatments of Japanese *Vincetoxicum* and its allied genera need further re-examination.

From the result of morphological observations on my own FAA fixed specimen collections and herbarium specimens deposited in KAG, KYO, MAK, OSA, RYU, SHO, TI, TNS, and URO, I propose two new combinations and describe two new species here.

Vincetoxicum yonakuniense (Hatus.) T. Yamashiro & Y. Tateishi **comb. nov.**

Cynanchum yonakuniense Hatus. in J. Phytogeo. Tax. **25**: 26 (1977). Hatus. and Amano, Fl. Ryukyus: 114 (1977), 2nd ed.: 176 (1994). Shimabuku, Vasc. Fl. Ryukyu Isls.: 370 (1990); rev. ed.: 430 (1997). T. Yamaz. in K. Iwats. et al. (eds.), Fl. Jap. **3a**: 175 (1993).

Tylophora sp. Hatus. & Kanai in Mem. Nat. Sci. Mus. Tokyo (7): 118 (1974).

Tylophora yonakuniensis Hatus., Fl. Ryukyus, rev. ed. 890 (1975), nom. nud. (descr. Japon.)

Type: Isl. Yonakuni, Ryukyus, 30 Sept. 1973, *S. Hatsusima 35657* (RYU non vidi, photocopy in KAG!)

A perennial twining herb up to 2 m long. Rhizome short and erect. Roots fascicled (Fig. 2A). Stems terete, 1-2 mm in diameter, sparsely pubescent along 1 or 2 lines with short curved hairs or glabrous. Leaves opposite, membranaceous to chartaceous; petiole 1-2.8 cm long, sparsely pubescent with short curved hairs above; blade ovate, 2.5-8 cm long, 1.5-5 cm wide, glabrous on both sides, sometimes sparsely ciliate with curved short hairs, apex acute to obtuse and shortly apiculate, base shallowly cordate, truncate or rounded. Inflorescence

axillary, umbellate-cymose, 1-5 flowered; rachis 6-19 mm long, glabrous. Pedicel slender 8-16 mm long, glabrous. Flower (Fig. 2B & 3) pale greenish yellow or sometimes pale reddish yellow. Calyx rotate, 5-6.5 mm across, deeply 5-lobed; lobes lanceolate, 2.2-3.2 mm long, ca. 1 mm wide, glabrous or sparsely pubescent on abaxial side. Corolla (Fig. 3A) rotate, deeply five-lobed, 18-25 mm across; lobes lanceolate, glabrous on both surfaces, 7.5- 11.5 mm long, 3-3.5 mm wide. Corona (Fig. 3D) shorter than gynostegium, deeply five-lobed; lobes fleshy, deltoid-ovate, ca. 1.2 mm long, 1 mm wide, adnate in 2/3 length with the abaxial side of the stamen, glabrous. Stamen (Fig. 3E-G) ca. 2 mm long, 0.7 mm wide; anther wings ca. 1.3 mm long; connective appendages 0.5 mm long, 0.5 mm wide, ovate. Pollinarium (Fig. 3H): corpuscula brownish, 0.24 mm long; caudicula 0.1 mm long, cylindrical; pollinia 0.22 mm long, 0.18 mm wide, subapically attached to the caudicles. Follicles (Fig. 4A-D) lanceolate in outline, 5.5-7 cm long, 0.9-1.4 cm wide, glabrous. Seeds (Fig. 4E-G) blackish brown, black-speckled, flat, ovate, 6.5-8 mm long, 4-5.2 mm wide, with comae ca. 25 mm long.

Distribution. *Ryukyus: Yonaguni and Uotsuri Islands. Endemic.*

Specimens examined. Ryukyus: Yonaguni Isl. near Tobaru (*S. Hatusima 35657*, 29 Sep.-3 Oct. 1973. KAG-Photocopy of the holotype specimen); at the foot of calcareous cliff south of air field, alt. 10 m (*S. Hatusima et al.* 2 Oct. 1973. TI & TNS); Mt. Kubura-dake (*T. Yamashiro & S. Ujiie 4188*, 12 Dec. 1998. URO); Tabaru (*T. Yamashiro & S. Ujiie 4199*, 12 Dec. 1998. URO). Uotsuri Isl. (*K. Shinjo 7272*, 29 May-7 Jun. 1979. URO).

Cynanchum yonakuniense has been known as a perennial twining herb endemic to Yonaguni Island in the Ryukyu Archipelago (Fig. 5). This species was described by Hatusima (1977) based on a flowering specimen. Since then, it has never been collected and its fruits and seeds have never been observed (Yamazaki 1993). This species is listed on the national list of threatened plants in Japan as "critically endangered" to extinction (Environment Agency of Japan, 2000).

Recently, I rediscovered *C. yonakuniense* from Yonaguni Island. Moreover, I found out a specimen of this species collected on Uotsuri Island, Senkaku Islands in the Ryukyu Archipelago.

In Yonaguni Island, I found *C. yonakuniense* at two localities: Tabaru and Mt. Kubura-dake (Fig. 5). At Tabaru, only three individuals were found on damp grassland dominated by *Pennisetum purpureum* near paddies. At Mt. Kubura-dake, I found 13 individuals growing on an edge of an evergreen forest along a road at altitudes 50-120 m. Flowering and fruiting plants were found on both sites.

In addition, I found out a specimen of *C. yonakuniense* (*K. Shinjo 7272*, URO) on Uotsuri

Island from the collection made by Mr. Shinjo. The flower morphology of the specimen agreed with those of *C. yonakuniense*.

So far, this species has taxonomically been classified in *Cynanchum* L. Recent studies on morphology and chemical constituents of *Cynanchum* s. l. by Qiu et al. (1989) and Liede (1996) revealed that this genus is not monophyletic. They divided *Cynanchum* s. l. to two genera, *Vincetoxicum* Wolf and *Cynanchum* s. s. Thus, transference of many Japanese species of *Cynanchum* to *Vincetoxicum* were carried out (e.g. Kitagawa 1959; Ohashi 1990). Yamazaki (1993) treated, however, *Vincetoxicum* as a section of *Cynanchum*, and included *C. yonakuniense* in the section *Vincetoxicum*.

Vincetoxicum characterized by a short and erect rhizome bearing fibrous roots and five lobed fleshy corona without triangular appendage. In contrast, *Cynanchum* has tuberous or ascending rhizome with roots not fibrous (Qiu et al. 1989; Liede 1996a). Corona of *Cynanchum* is tubular or deeply five lobed, and the lobes are membranaceous or fleshy, and if fleshy, they have a triangular appendage on adaxial side.

Cynanchum yonakuniense has a short and erect rhizome with fascicled roots (Fig. 2A). The corona of this species is deeply five lobed, and each lobe is fleshy, triangularly ovate without appendage on adaxial side, and is adnate at 2/3 length with the abaxial side of the stamen (Fig. 3D-E). I agree with Qiu et al. (1989) and Liede (1996a) in separating *Cynanchum* s. s. and *Vincetoxicum*, and thus I transfer *C. yonakuniense* to *Vincetoxicum*.

Tylophora matsumurae (Yamazaki) T. Yamashiro & Tateishi, comb. nov.

C. villosum Matsum. in Bot. Mag. Tokyo **12**: 39 (1898), non Roem. & Schult. (1982).

T. hispida Decne. var. *tanakae* Hatus. in J. Geobot. (Kanazawa) 12:10 (1963), pro parte excl. syn. *T. tanakae* Maxim.

C. matsumurae T. Yamaz. in J. Jap. Bot. **43**: 222 (1968).

V. matsumurae (T. Yamaz.) H. Ohashi in J. Jap. Bot. **65**: 277 (1990).

Type: Ryukyus. Okinawa Isl., Onna-son (*J. Matsumura*, 25 Apr. 1897, fl. in TI-Holotype).

Specimens examined

1. ***Tylophora matumurae*** (T. Yamaz.) T. Yamash. & Y. Tateishi

Kagoshima: Amami Isl., Kuzi-Yuwan (*G. Koidzumi*, 4 May 1923, KYO); Kakeroma Isl., Angyaba (*Z. Tashiro*, 11 Mar. 1924, KYO); Okinoerabu Isl. (*H. Ohba*, 1924, KYO); China-cho, Taminazaki (*T. Shimizu 85-449*, 6 Apr. 1985, fl. TI); Wadomari-cho, Wadomari (*T. Yamashiro 4061*, 6 Aug. 1996, fl. URO); Yoron Isl., Maehama (*T. Yamashiro & A. Yamashiro 7764*, 3 Nov. 2001, fr. TUS). **Okinawa**: Okinawa Isl., Kunigami-son, Hedomisaki (*T. Amano 6653*, 25 Jul. 1951, fr. RYU)(*E. H. Walker et al. s. n.*, 25 Jul. 1951, fl. TI); Onna-son (*J. Matsumura*, 25 Apr.

1897, fl. TI- Holotype); Manzamo (*T. Kanashiro*, 23 Nov. 1937, fr. RYU)(*F. Yamazaki et al.*, 1 Apr. 1968, fl. TI)(*A. Takushi*, 22 Sep. 1968, fr. TI)(*T. Yamazaki*, 24 Jun. 1971, fr. TI)(*Y. Miyagi 5721*, Aug. 1966, fr. RYU)(*Y. Tateishi & T. Yamashiro 45144*, 6 Jun. 1998, fl. URO); Katsuren-cho (*Y. Tashiro*, May 1887, fr. TI).

2. ***Tylophora tanakae*** Maxim.

Nagasaki: Goto Isls., Fukue Isl. (*Z. Tashiro s. n.*, fl. KYO). **Kumamoto**: Amakusa-gun, Hogashima (*Tamiyama s. n.*, 20 Aug. 1931, fl. KYO & TI); Akuneohshma Isl., (*S. Hayashimatsu s. n.*, 4 Aug. 1936. fl. & fr. KYO). **Miyazaki**: Toimisaki Cape (*K. Nagai s. n.*, 4 Aug. 1964, fl. KYO). **Kagoshima**: Kushikino-shi, Nagasakibana (*T. Yamashiro & A. Yamashiro 7801*, 24 Feb. 2002, fr. TUS); Yamagawa-cho, Nagasakibana (*Z. Tashiro s. n.*, 27 Aug. 1924, fl. KYO)(*G. Murata, M. Togashi & H. Kanai s. n.*, 23 Jan. 1965, fr. KYO); Kimotsuki-gun, Sata-cho, Isashiki (*G. Murata 12872*, 9 Aug. 1959, fl. KYO); Tajiri (*T. Yamashiro & A. Yamashiro 7775*, 5 Nov. 2001, fr. TUS); Tanegashima Isl. (*T. Makino s. n.*, anno 1938, fl. KYO). Yaku Isl. (*Z. Tashiro s. n.*, Aug. 1919, fl. KYO); Kurio (*J. Murata et al. 15743*, 22 Oct. 1983, fr. KYO, TI)(*M. Tagawa s. n.*, 31 Aug. 1933, fl. KYO); Kamiyaku-cho, Isso (*T. Yamashiro & A. Yamashiro 7818*, 28 Jan. 2002, fr. TUS); Kuchinoshima Isl. (*S. Sako 7243*. 18 Dec. 1968, fr. KYO, TI); Takara Isl. (*M. Hori 790*, 29 May 1953, fl. KYO); Amami Isl., Yamato-son, Odana (*Y. Tateishi & T. Yamashiro 45592*, 2 Aug. 1998, fl. URO); Kikai Isl. (*K. Yamaguchi s. n.*, 21 May 1919, fl. KYO); Tokunoshima Isl., Isen-cho, Intabumisaki (*K. Iwatsuki et al.*, 395, 26 Aug. 1975, fl. KYO); en route Ikema to Mt. Inokawa-dake (*M. Kato & E. Miki 245*, 27 Aug. 1978, fl. KYO); Okierabu Isl., China-cho, Tamina Cape (*T. Yamashiro 4052*, 5 Aug. 1998, fl.& fr. URO); Wadomari-cho, Hanzaki Cape (*T. Yamashiro 4033*, 4 Aug. 1998, fl. URO).

Okinawa: Iheya Isl. (*Y. Niiro 3064*, 28 Dec. 1958, st. RYU); Ie Isl., Waji (*H. Ogawa 717*, 4-5 Dec. 1994, fr.URO); Okinawa Isl., Nago (*Z. Tashiro s. n.*, 16 Jan. 1924. fr. KYO); Onna-son, Maeda-misaki Cape (*Y. Miyagi 9078*, 18 May 1980, fl. RYU); Manzamo (*E. Takamine 2382*, 4 Jul. 1974, fl. & fr. RYU); Naha-shi (*G. Koidzumi s. n.*, 14 May 1923, fl. KYO); Shuri (*S. Hatusima 34000*, 1 Oct. 1972, fl. RYU); Naminoue (*Y. Taira s. n.*, 16 May 1938, fl. KYO); Ohnoyama (*Y. Muramatsu & I. Furusawa s. n.*, 29 Sep. 1940, fl. TI); Tsuji (*T. Amano s. n.*, 20 Sep. 1951, fl. KYO); Itoman-shi, Gushikawajoshi (*T. Yamashiro 4003*, 2 Nov. 1997, fr. URO); Tokashiki Isl., Aharen (*Y. Miyagi 9160*, 6 Sep. 1980, fl. RYU); Aka Isl. (*T. Yamashiro & A. Kuwataka 4410*, 9 May 1999, fl. URO); Geruma Isl. (*Y. Miyagi 7873*, 9-12 Aug. 1977, fl. RYU)(*G. Ikeda 4029*, 9 Aug. 1977, fl. RYU); Aguni Isl. (*S. Hatusima & Y. Miyagi 38610*, 10-13 Aug. 1974, fl. RYU)(*K. Shinjo & Y. Tateishi 12031*, 9 May 1998, fl. URO); Tonaki Isl. (*T. Yamashiro 1384*, 3 Oct. 1995, fl. & fr. URO); Kume Isl. (*Y. Niiro 644*, 24 Nov. 1957, fr. RYU); Nakazato-son, I-fu beach (*T. Yamashiro 7836*, 3 Feb. 2002, fr. TUS); Miyako Isl. (*S. Sakaguchi s. n.*, anno 1922, fl. KYO)(*S. Nakasone s. n.*, Jun.

1929, fr. KYO); Karimata (*G. Koidozumi s. n.*, 16 Jun. 1923, fl. KYO)(*M. Furuse 4613*, 20 Nov. 1973, yfr. RYU); Hirara-shi, Nishizato (*T. Amano 5855*, 23 Nov. 1948, st. RYU); Gusukube-cho, Tomori (*S. Hatusima et al.* 36967, 4-9 Dec. 1973, fr. RYU); Higashihenna-zaki (*S. Hatusima et al. 38549*, 6-11 Jul. 1974, fr. RYU); Irabu Isl. (*S. Nakazone s. n.*, 13 Dec. 1936, fr. KYO); Nagayama (*M. Furuse 4621*, 21 Nov. 1973, fl. RYU); Ikema Isl. (*S. Hatusima et al. 3823*, 11 Jul. 1974, fl. RYU); Ohgami Isl. (*S. Hatusima 3844*, 11 Jul. 1974, st. RYU); Shimoji Isl. (*I. Kawakami s. n.*, 17 Aug. 1973, fl. RYU); Minna Isl. (*Y. Miyagi 7128*, 27 Sep. 1975, fr. RYU); Ishigaki Isl., Hirakubozaki (*S. Hatusima 33339*, 6 Aug. 1972, fl. RYU); Nosoko (*N. Fukuoka & M. Ito 132*, 2 Aug. 1981, fl. KYO); Yarabu Pen., Kannonzaki (*M. Furuse 666*, 7 Aug. 1972, fl. bud, RYU); Hirae (*S. Tawada s. n.*, 11 Aug. 1933, fl. KYO); Kabira beach (*N. Fukuoka & M. Ito 306*, 22 Oct. 1983, fl. KYO); North of Kawahara (*Y. Tateishi et al. 40277*, 16 Sep. 1994, fl. URO); Nakura (*M. Furuse 537*, 31 Jul. 1972, fl. RYU); Uganzaki (*S. Hatusima 33289*, 7 Aug. 1972, fl. bud, RYU); Iriomote Isl., Funauki (*S. Tawada s. n.*, 12 Aug. 1936, fl. KYO); Funaura (*M. Furuse 3969*, 8 Sep. 1973, fl. RYU); Shirahama (*E. H. Walker & S. Tawada 6520*, 17 Aug. 1951, fl. TI)(*G. Murata & H. Tabata 674*, 9 Aug. 1974, fl. KYO); Toyohara (*S. Hatusima 32910*, 25 Jul. 1972, fl. RYU); Taketomi Isl. (*M. Furuse 1456*, 9 Oct. 1972, fl. RYU); Kuroshima Isl. (*M. Furuse 932*, 27 Aug. 1972, fl. RYU)(*Y. Niiro & Y. Miyagi 5975*, 4 Nov. 1974, fr. RYU); Hatoma Isl. (*K. Shimabuku 3553*, Aug. 1976, fl. RYU); Aragusuku Isl. (*Y. Miyagi 10442*, 4 Aug. 1974, fl bud. RYU); Yonaguni Isl., Sanninodai (*Hatusima et al. 35877*, 29 Sep.-3 Oct. 1973, fl. RYU); Mt. Urabu-dake (*T. Yamashiro & S. Ujiie 4202*, 12 Dec. 1998, fl & fr. URO); North slope of Mt. Kubura (*T. Yamashiro & S. Ujiie 4197*, 12 Dec. 1998, fl & fr. URO); Hateruma Isl. (*M. Furuse 237*, 8 Jul., 1972, fl. bud. RYU) (*Y. Miyagi 6743*, 9-15 Jul. 1975, fl. bud. RYU); Kitadaito Isl., (*S. Hatusima 33714*, 4 Oct. 1972, fl. RYU)(*K. Shinjo 5078*, 12-21 Mar. 1967, fl. URO); Minamidaito Isl. (*S. Hatusima 33982*, 8 Oct. 1972, fl. RYU).

Cynanchum matsumurae T. Yamaz. is a short erect perennial herb growing in grasslands on windy headlands or exposed limestones near seashore, and is endemic to Amami, Kakeroma, Okinoerabu, Yoron, and Okinawa Islands in the Ryukyu Archipelago, Japan (Fig. 6) (Yamazaki 1968; T. Yamashiro unpublished data). This species is listed as endangered in the red list of Japanese vascular plants (Environment Agency of Japan, 2000).

Cynanchum matsumurae was originally described by Matsumura (1898) as *Cynanchum villosum* on Okinawa Island. Hatusima (1963) regarded, however, this species only as a dwarf ecotype of *Tylophora tanakae* Maxim., which is distributed from Kyushu Island to the Ryukyu Archipelago (Fig. 6). On the other hand, Yamazaki (1968) treated this species as a distinct species of *Cynanchum* section *Vincetoxicum* based on its suspended pollinia. Because of the

presence of the earlier homonym of *C. villosum*, he gave it new name, *C. matsumurae* T. Yamaz. Later, Ohashi (1990) raised this section to a genus and proposed several combinations under the promoted *Vincetoxicum*, and accordingly *C. matsumurae* was combined to *Vincetoxicum*. On the other hand, *Cynanchum matsumurae* sometimes resembles to *T. tanakae* and has often been misidentified as it.

The genus *Vincetoxicum* is distinguished from *Tylophora* based on corona morphology and orientation of pollinia (Li et al., 1995). While the corona lobes of *Vincetoxicum* are inserted at the base of gynostegium and its pollinia are pendulous, corona lobes of *Tylophora* are inserted on the backs of anthers and its pollinia are horizontal to erect (Li et al., 1995).

The coronas of *C. matsumurae* are inserted on the backs of anther and separate each other (Fig. 7A, d). A pollinarium of *C. matsumurae* is similar to that of *T. tanakae* (Fig. 8A, B). The corpusculum of *C. matsumurae* is trapezoid and the caudicles are ascending and filiform (Fig. 8A). The pollinarium is placed horizontally in anther sac.

Because the morphological characters of the corona and pollinarium of *C. matsumurae* agreed well with those of *Tylophora*, I propose new combination under *Tylophora* for this species.

Although *C. matsumurae* is morphologically similar to *T. tanakae*, there are some differences in several characters (Table 1).

Vincetoxicum izuense T. Yamashiro, **sp. nov.** (Figs. 9A, B & 10).

Haec species *V. japonico* et *V. sublanceolato* affinis, sed a priore caulibus volubilibus, laminis foliorum subtus dense pilosis, rachidibus inflorescentiarum brevioribus, et a posteriore laminis foliorum ellipticis (in illo lanceolatis vel angusti-triangularlibus), folliculis latioribus differt.

Type: JAPAN. Shizuoka Pref., Shimoda-shi, Mihogasaki, rocky beach, 20 Jul. 2000 (fl.), *T. Yamashiro 7435*, (holotype TUS; isotype TI).

A perennial twining herb. Rhizome short and erect with roots fascicled. Stem slender, terete, 0.7-1.5 mm in diameter, 0.5-1 m long, lower part densely pubescent with curved hairs, upper part puberulent along two lines. Leaves opposite; blades chartaceous, elliptic, oblong or ovate-oblong, 2.4-5.1 cm long, 1.1-3.1 cm wide, apex acute or rounded shortly apiculate, base rounded, margin and nerves pubescent above, densely pubescent below; petioles 2-5.7 mm long, grooved above, densely covered with curved hairs on both surfaces. Inflorescence axillary, umbellate-cymose, 4-5 flowered; peduncle 1.2-5.4 mm long or becoming sessile

toward the apical part of stem, densely covered with curved hairs. Flowers darkish-purple; pedicels slender, 3.5-6 mm long, puberulent. Calyx rotate, deeply 5 lobed; lobes lanceolate with acute apex, 2 mm long, 1 mm wide, puberulent outside. Corolla rotate, 12 mm across, deeply 5 lobed; lobes lanceolate, 5 mm long, 1.9-2.2 mm wide, retuse in apex, glabrous on both surface. Corona 1/2 shorter than gynostegium, dark-purplish, deeply 5 lobed; lobes adnate to abaxial side of filaments, triangular with obtuse apex, ca.1 mm long, 1 mm wide, fleshy. Stamen 1.8 mm long; anther wings ca. 0.5 mm long; connective appendage membranaceous, depressed ovate, 0.3 mm long, 0.7 mm wide. Pollinarium: corpusculum narrowly-ellipsoid, 0.2 mm long, 0.06 mm wide; caudicules 0.12 mm long, filiform; pollinia ellipsoid, 0.28 mm long, 0.15 mm wide. Follicles fusiform, 4.5 cm long, 1 cm wide, glabrous. Seeds flat, ovate, 6.5-7.2 mm long, 4 mm wide, blackish brown, black-speckled, with coma ca. 2 cm long. Chromosome numbers 2n=22 (Fig. 12A).

Nom. Jap.: Izu-kamomezuru (nov.)

Vincetoxicum izuense is a perennial herb growing on the rocky beach and margin of thickets near the sea and endemic to the south of the Izu Peninsula (Fig. 13). Flower morphology of the species quite resembles to those of *V. sublanceolatum* distributed from Tohoku to Kinki districts of Japan (Yamazaki, 1993). This species had been considered to be a maritime ecotype of *V. sublanceolatum* (Biological Laboratory Imperial Household, 1980). From my field and morphological investigations, I found ecological and morphological differences between these species. *Vincetoxicum sublanceolatum* prefers to grow on wet grounds such as paddy field, marsh or banks of rivers whereas *V. izuense* occurs on rocky beach. The leaf blades of the former are lanceolate, linear-lanceolate or narrowly triangular in shapes and membranaceous in texture, those of the latter are elliptic and charataceous. The chromosome numbers of *V. sublanceolatum* have been reported as 2n = 44 (in Chapter III), while I observed 2n=22 for *V. izuense* collected in Mihogasaki, Shimoda-shi, Shizuoka Prefecture (Fig. 12A), indicating that *V. izuense* is also distinguished from *V. sublanceolatum* at ploidy level.

Vincetoxicum izuense also resembles to *V. japonicum* distributed widely from Tohoku to Kyushu district (Yamazaki, 1993), in its elliptical leaves and habitats. Both species inhabit on rocky beach or in meadows near the sea and sometimes co-occurred, but *V. izuense* is easily distinguished from *V. japonicum* by its twining habit and lower surface of leaves clothed densely with curved hairs.

Other specimens examined. JAPAN. Honshu. Shizuoka Pref.: Shimoda-shi; Suzaki, 21 Jul.

1974, *J. Murata 31* (KYO); Goyotei, Maruyama, 21 Jun. 1979, *H. Hara s. n.* (TI); Ebisu Isl., anno 1964, *N. Tanaka s. n.* (KAG); Kakizaki, Nadegahara, 4 Aug. 1982, *T. Sato 2066* (TNS). Kamo-gun, Minamiizu-cho, Nakagi-Sashida, 22 Jun. 1958, *H. Noguchi 4539* (KAG); Hagachi, 13 Jul. 1957, *N. Noguchi 4539* (KAG).

Vincetoxicum hoyoense T. Yamashiro, **sp. nov.** (Figs. 9C, D & 11).

Haec species *V. japonico* et *V. sublanceolato* affinis, sed a priore caulibus volubilibus, foliis et floribus majoribus; et a posteriore foliis ellipticis, ovatis vel oblongis (in illo lanceolatis vel anguste-triangularibus), folliculis latioribus differt.

Type: JAPAN. Oita Pref.: Minamiamabe-gun, Tsurumi-cho, Kajiyoseura, rocky beach, 8 Jun. 2000, *T. Yamashiro & A. Yamashiro 7447* (holotype TUS; isotype KYO, TI, TNS).

A perennial twining herb up to 2 m long. Rhizome short and erect. Roots fascicled. Stems terete, 1-2.5 mm in diameter, sparsely pubescent along one or two lines with short curved hairs when young, later glabrescent, many-branched. Leaves opposite. Leaves on main stem: blade chartaceous, elliptic, ovate or oblong, 9-13.5 cm long, 5-7.5 cm wide, 5-7 reddish nerved, pubescent on nerves of both surfaces, more or less densely on lower one, margin ciliate, apex acuminate, base shallowly cordate; petiole grooved on upper surface, 0.8-2.2 cm long, pubescent with short curved hairs on both surfaces. Leaves on lateral shoots are 2-3 times smaller than those of main stems; blade chartaceous, narrowly ovate or oblong, 3-6 cm long, 1.3-2.5 cm wide, apex acuminate or acute, base rounded or truncate, petiole 5-8 mm long. Inflorescence axillary, 1-3 per axil, umbellate-cymose, 8-20 flowered, 4-8 flowers open at time; peduncle 1.5-6 cm long, 1-2 branched, pubescent on one line. Pedicel slender 8.5-15 mm long, pubescent on one side or densely covered with short hairs, purplish or greenish. Flowers dark purplish or rarely pale greenish yellow. Calyx rotate, purplish or greenish, deeply 5-lobed; lobes narrowly ovate, ca. 2 mm long, 1 mm wide, apex acute, densely clothed with curved hairs on abaxial surface. Corolla rotate, deeply five-lobed, 18-20 mm across; lobes lanceolate, 7-9.5mm long, 2.5-3 mm with retuse apex, glabrous on both surfaces. Corona 1/2 times shorter than gynostegium, deeply five-lobed; lobes fleshy, triangular, ca. 1mm long, 1mm wide, adnate to abaxial side of the stamen, glabrous. Stamen 2 mm long, 0.7 mm wide; anther wings ca. 0.5 mm long; connective appendages 0.7 mm long, 0.7 mm wide, broadly ovate. Pollinarium: corpuscula narrowly ellipsoidal, brownish, 0.26 mm long, 0.14 mm wide; caudicula filiform, 0.2 mm long; pollinia ellipsoidal, 0.3 mm long, 0.12 mm wide, subapically attached to the caudicles. Follicles fusiform, 5.5-7 cm long, 0.9-1.4

cm wide, glabrous. Seeds blackish brown, black-speckled, flat, ovate, 7.5-8.6 mm long, 4-5.7 mm wide, with coma 16.5-18.2 mm long. Chromosome numbers 2n=22 (Fig. 12B).

Nom. Jap.: Hoyo-kamomezuru (nov.)

Vincetoxicum hoyoense is a large perennial twining herb growing on the rocks or margin of thickets facing to the sea. This species is distributed around the Bungo channel and endemic to small islands and peninsulas of Ehime, Oita and Miyazaki Prefectures (Fig. 13).

This species resembles to *V. japonicum* f. *puncticulatum* and might have been misidentified as the latter. *Vincetoxicum hoyoense* is distinguished from *V. japonicum* by having twining stems and larger (over 10 cm long) leaf blades with shallowly cordate base. While corolla lobes of *V. hoyoense* are 7-9.5 mm long and lanceolate in shape, those of *V. japonicum* are 4-7 mm long and ovate or oblong in shape. Although flower characters are rather similar to *V. sublanceolatum* and *V. izuense* than *V. japonicum*, *V. hoyoense* can be easily distinguished by size and shape of leaves from *V. sublanceolatum* and *V. izuense*. *Vincetoxicum hoyoense* also resembles to *V. izuense* in flower morphology and leaf shapes, however it is easily distinguished from the latter by having the larger leaves ovate or elliptic with shallowly cordate base and the large corollas.

Other specimens examined. JAPAN. **Shikoku**. Ehime Pref.: Minamiuwa-gun, Jyohen-cho, Ategi Isl.: 14 Jun. 1923, *M. Ogata s. n.* (KYO); 17 Jul. 1942, *S. Sugaya s. n.* (TUS); *T. Yamashiro & A. Yamashiro 7445* (TUS). **Kyushu**. Oita Pref.: Minamiamabe-gun, Kamae-cho, Mishima Isl., 24 Jul. 1921, *Z. Tashiro s. n.* (KYO); 21 Oct. 1984, *M. Arakane s. n.* (KAG); Tsurumi-cho, Tsurumi Cape, *Z. Tashiro s. n.* (KYO); Tanga, 7 Nov. 2001, *T. Yamashiro & A. Yamashiro 7798* (TUS); Kajiyoseura, 3 Aug. 2000, *T. Yamashiro & A. Yamashiro 7230* (TUS), Ohshima Isl., 8 Jun. 2001 *T. Yamashiro & A. Yamashiro 7450* (TUS). —Miyazaki Pref.: Higashisuki-gun, Kitaura-cho, Shimaura-jima, 3 Aug. 1936, *Y. Samejima s. n.* (KYO).

Table 1. The morphological differences between *T. matsumurae* and *T. tanakae*.

Characters	*T. matsumurae*	*T. tanakae*
habit	erect	creeping, twining or erect
stem length (m)	0.1–0.4	0.2-3
leaf blade shape	elliptic	oblong, elliptic (at base of stems) or ovate (at lateral shoots)
leaf blade length x width (cm)	1.4-4 x 1-3.4	3-8 x 2.5-5
petiole length (mm)	1.3-2.9	4-18
inflorescence-rachis length (mm)	absent or 0.1-1	4-18
corolla lobe shape,	oblong	lanceolate
corolla lobes length x width (mm)	3.6 x 2.3	4.1 x 2.3
hairs of fruit	pubescent	pubescent or glabrous
fruit length x width (mm)	35-48 x 4.8-6.4	40-60 x 6.5-10
seed length x width (mm)	3.5-4.8 x 1.6-2.5	5.2-8 x 3-4

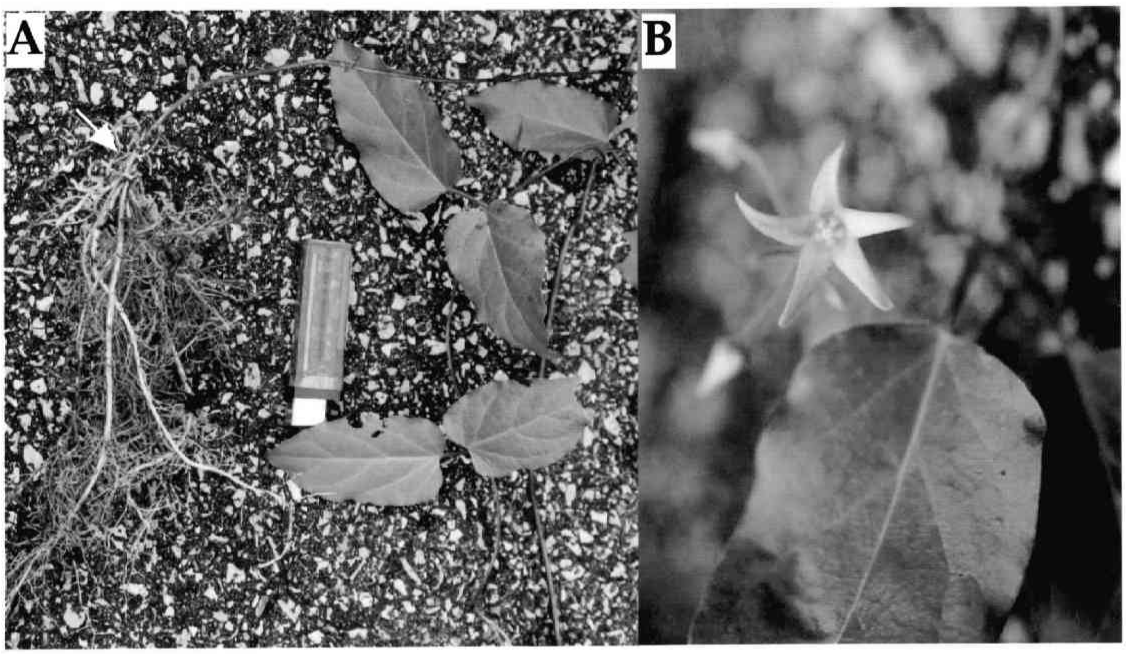

Fig. 2. Flowering individual of *Vincetoxicum yonakuniense*. A. Stem, rhizome and roots. B. Flowers. Arrow indicates rhizome.

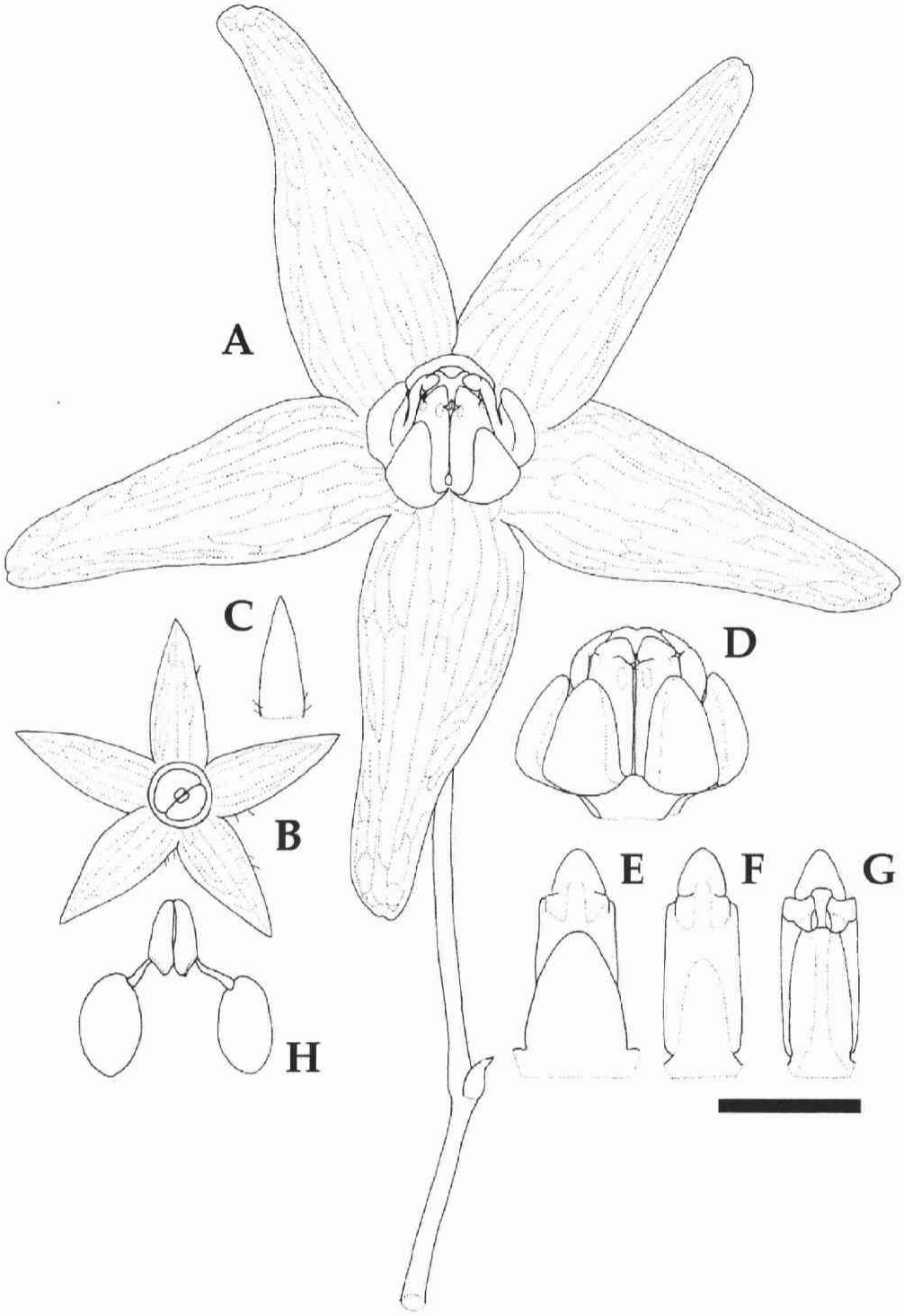

Fig. 3. Flower of *Vincetoxicum yonakuniense*. A. whole flower. B. adaxial view of calyx and pistil; corolla, corona and stamens are removed. C. abaxial view of calyx lobe. D. corona and gynostegium. E. abaxial view of stamen and corona lobe. F. abaxial view of stamen; corona lobe is removed. G. adaxial view of stamen. H. pollinarium. Scale bar represents 3 mm for A-C, 2 mm for D-G and 400É m for H.

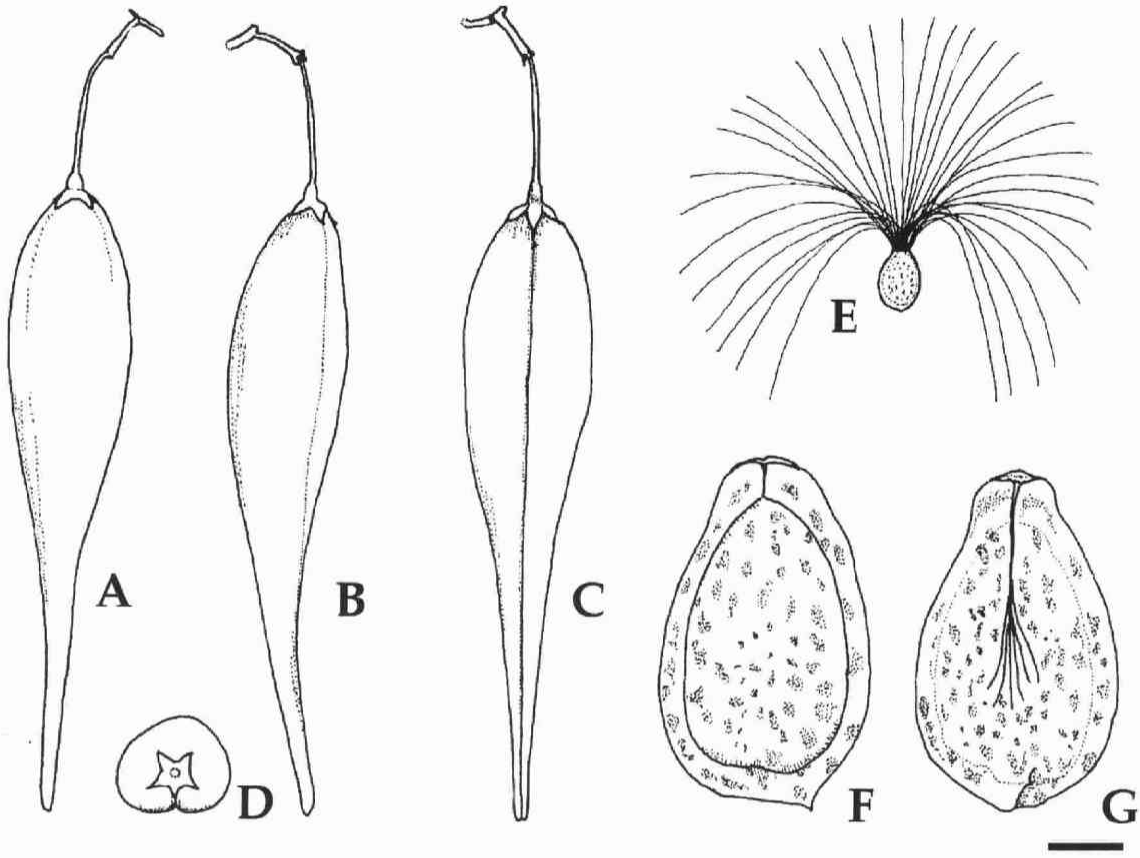

Fig. 4. Follicles and seeds of *Vincetoxicum yonakuniense*. A. abaxial view of follicle. B. lateral view of follicle. C. adaxial view of follicle. D. basal view of follicle. E. seed with coma. F. dorsal view of seed, coma is removed. G. ventral view of seed, coma is removed. Scale bar represents 1 cm for A-E and 2 mm for F, G.

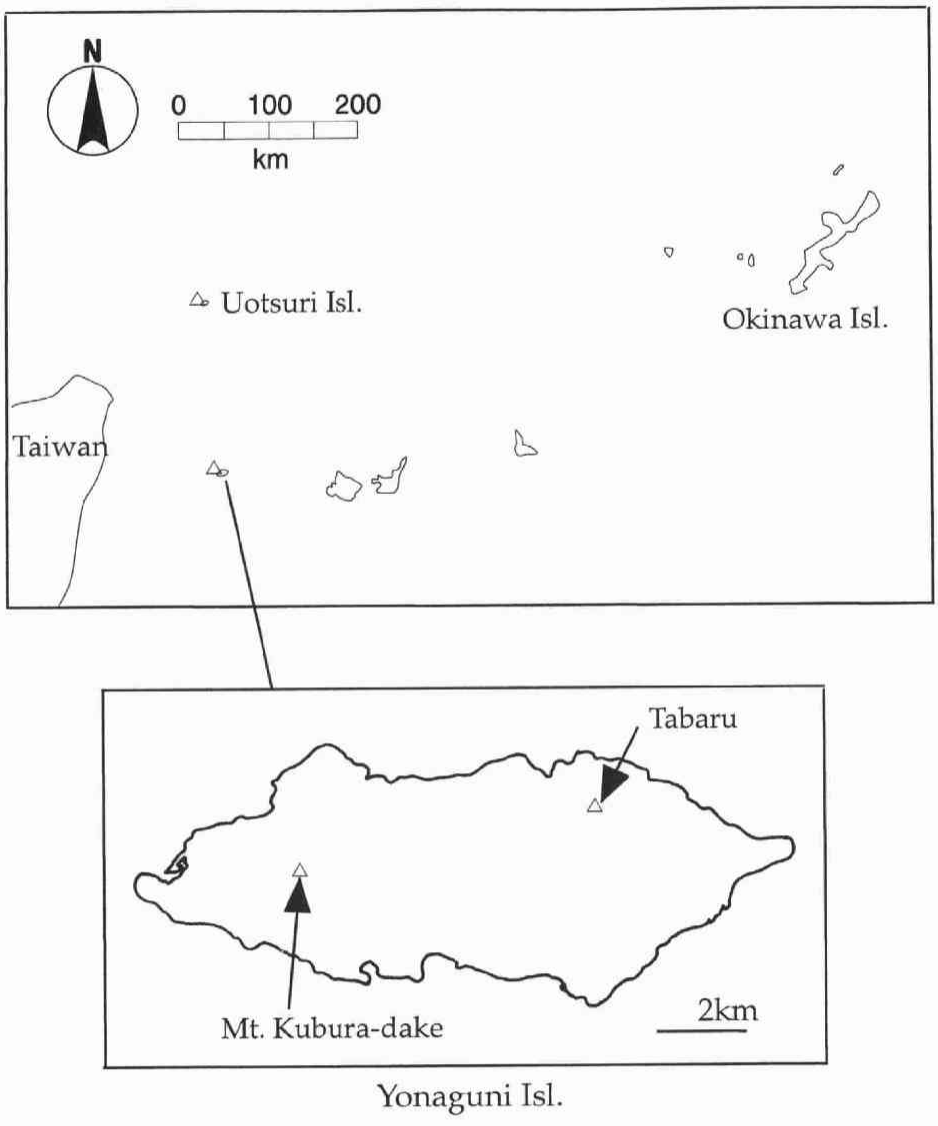

Fig. 5. Distribution map of *Vincetoxicum yonakuniense*. Open triangles indicate distribution of *V. yonakuniense*.

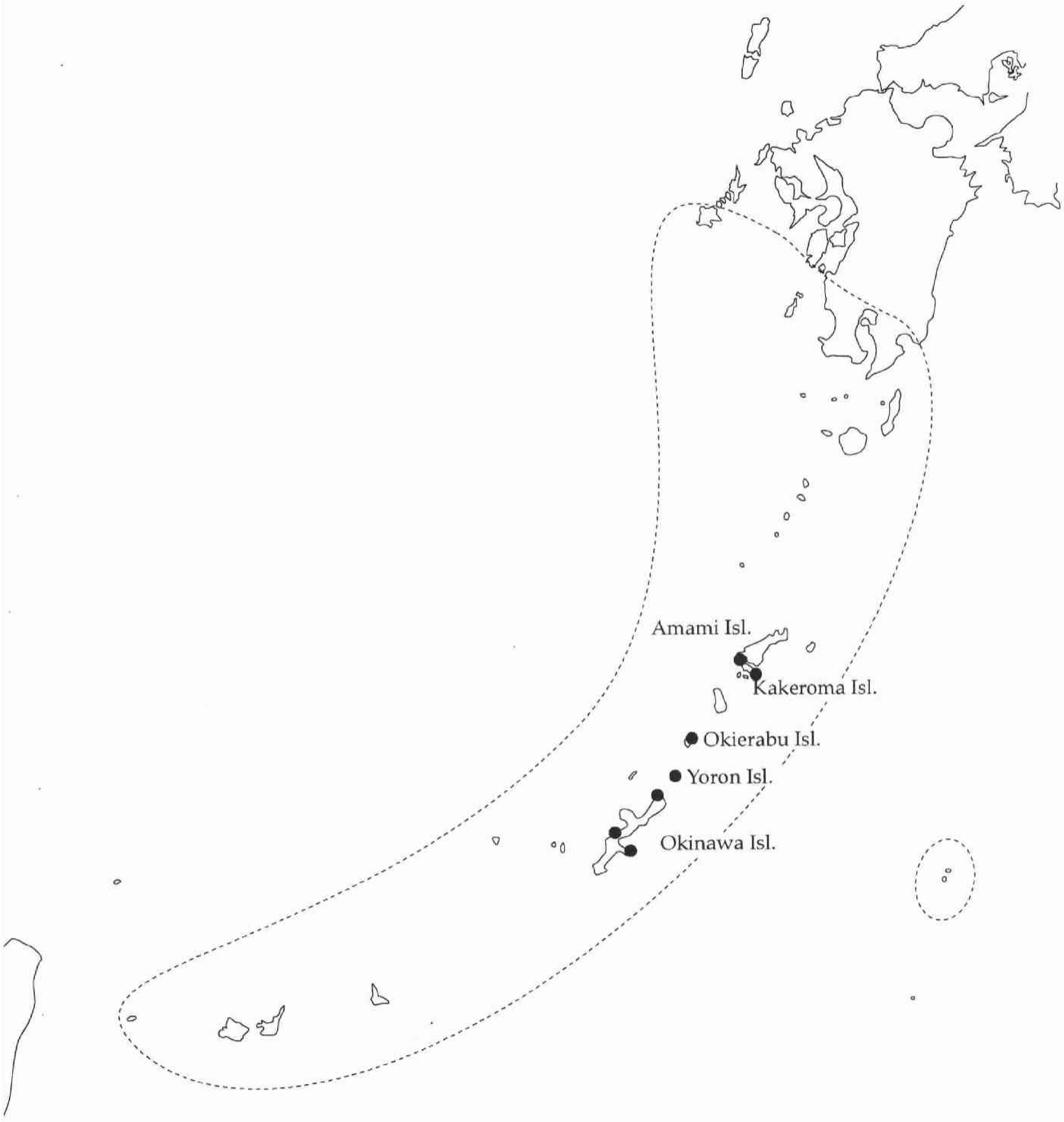

Fig. 6. Distribution map of *T. matsumurae* and *T. tanakae*. Solid circles indicate the distribution of *T. matsumurae* and dotted lines indicate the range of the distribution of *T. tanakae*.

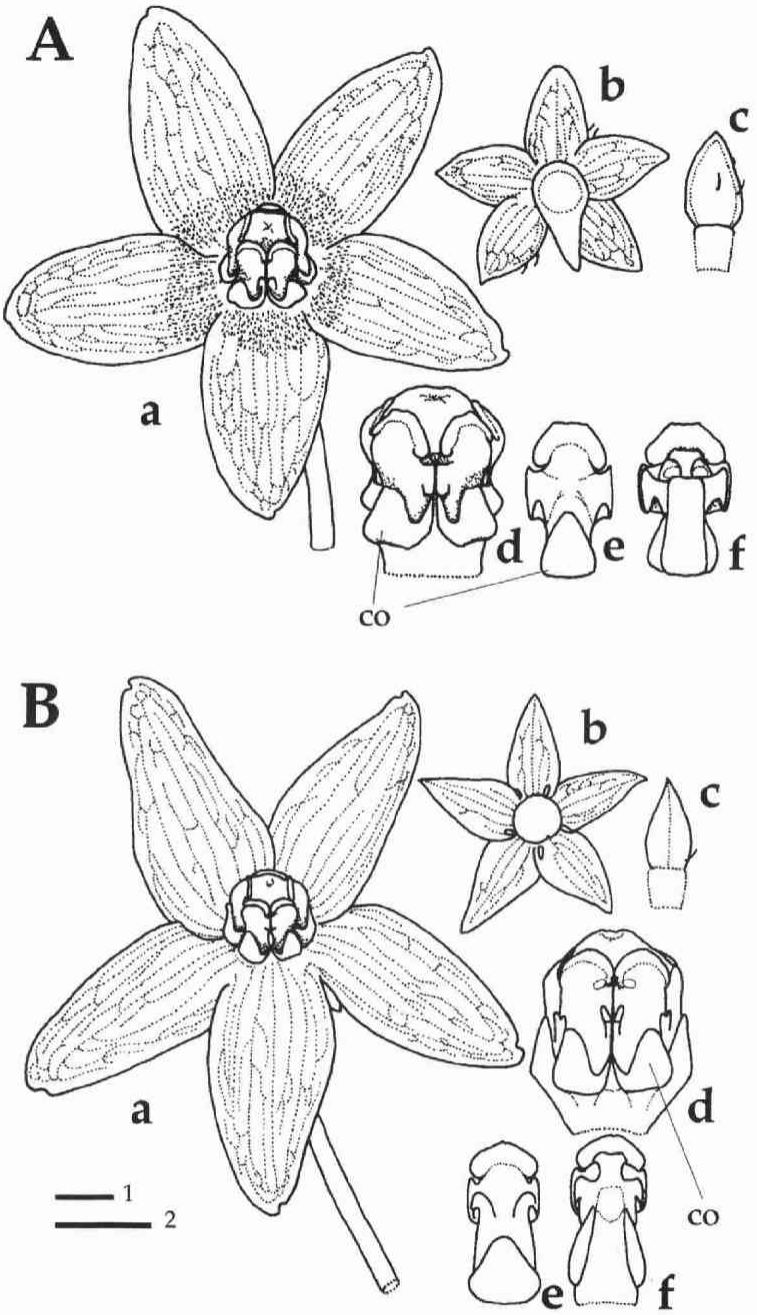

Fig. 7. Morphology of the flowers of *T. matsumurae* (A) and *T. tanakae* (B).
a. whole flower. b. adaxial side of calyx, pistil, corolla, corona and stamens are removed. c. abaxial side of caryx lobe. d. gynostegium and corona. e. abaxial side of stamen. f. adaxial side of stamen (co = corona lobe). Bar 1 indicates 1mm for a-c, and bar 2 indicates 1 mm for d-f.

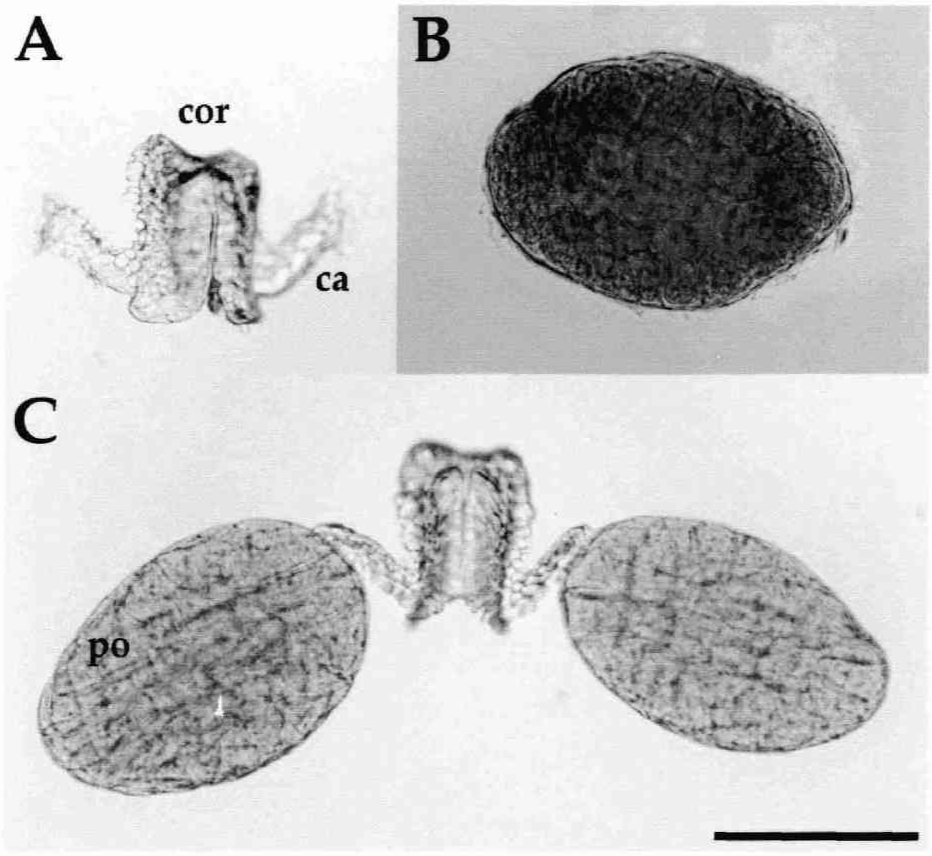

Fig. 8. Pollinaria of *T. matsumurae* (A-B) and *T. tanakae* (C).
A. Corpusculum and caudicle of *T. matsumurae*. B. Pollinium of *T. matsumurae*. C. Pollinarium of *T. tanakae* (cor = corpusculum, ca = caudicle, po = pollinium). Bar = 100 μm. In *T. matsumurae*, pollinarium could not be removed intactly from anther sac, because anther sac, dose not dehisce even in opened flower.

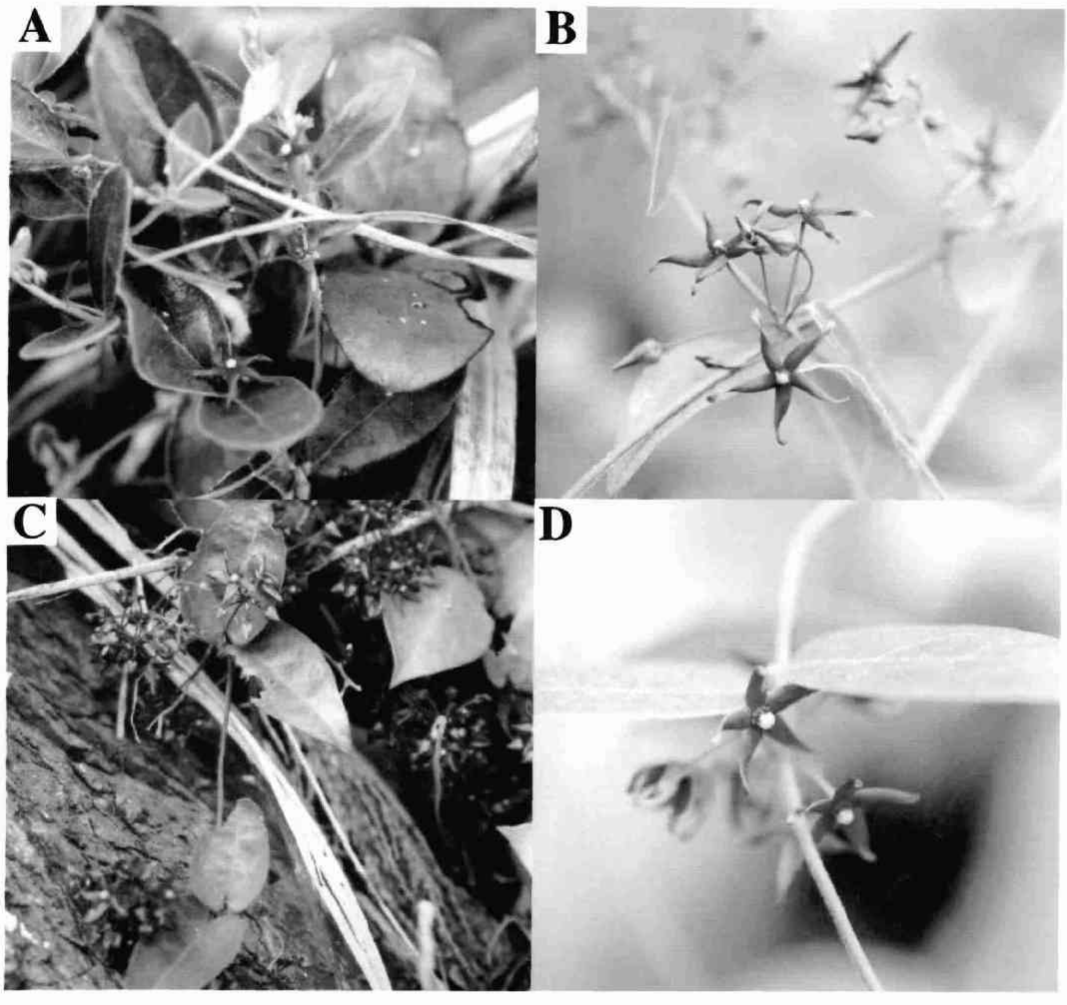

Fig. 9. Flowering plants of *Vincetoxicum izuense* (A, B) and *V. hoyoense* (C, D). Photographs from holotype (A, B for *T. Yamashiro 7435* and C, D for *T. Yamashiro & A. Yamashiro 7447*, respectively).

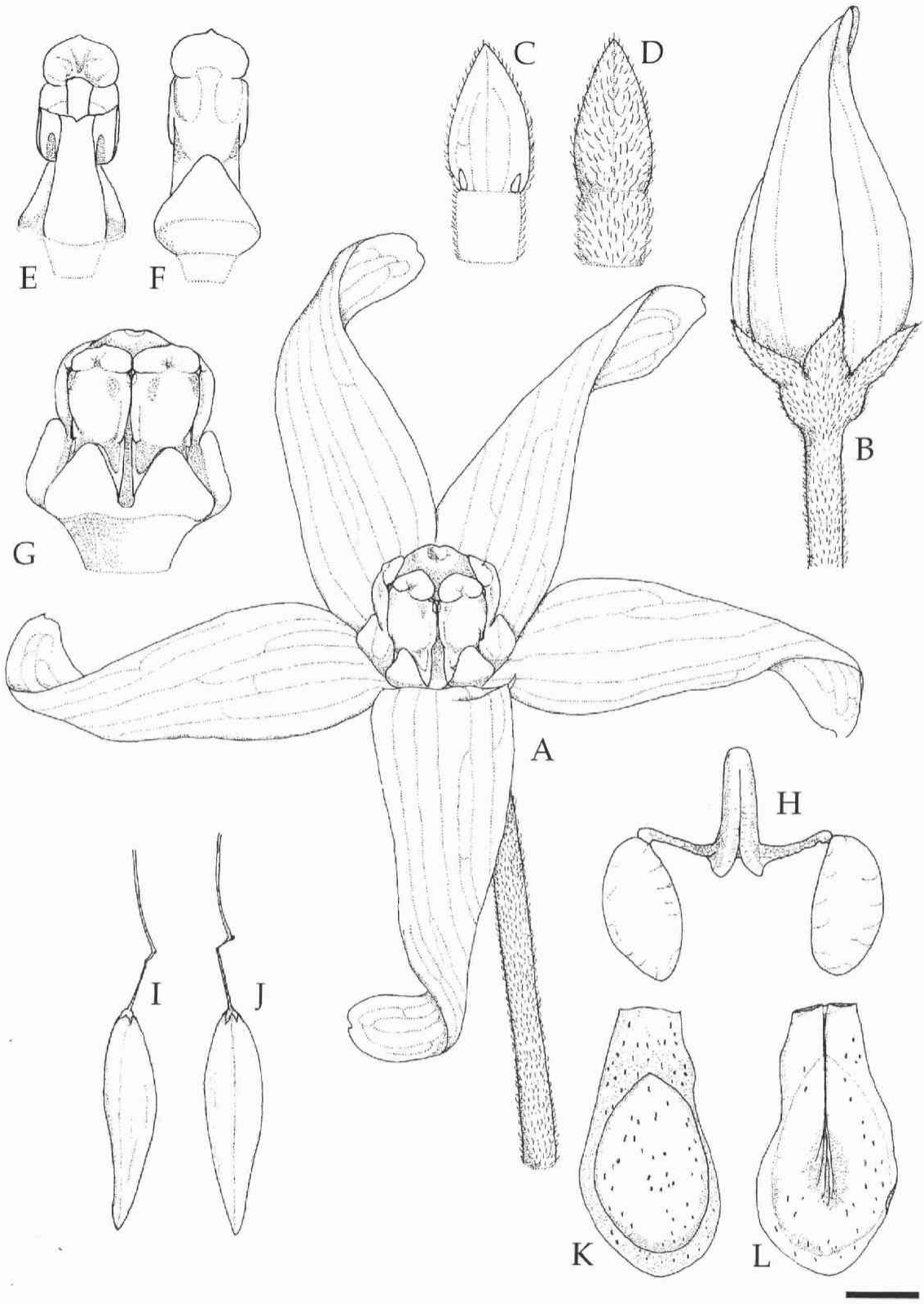

Fig. 10. Flower of *Vincetoxicum izuense*. A. whole flower. B. bud. C. adaxial view of calyx lobe D. abaxial view of calyx lobe. E. adaxial view of stamen. F. abaxial view of stamen. G. corona and gynostegium. H. pollinarium. I. adaxial view of follicle. J. lateral view of follicle. K. dorsal view of seed, coma is removed. L. ventral view of seed, coma is removed. Scale bar represents 1 mm for A-D, 0.5 mm for G-F, 0.15 mm for H, 1.5 cm for I, J and 2 mm for K, L. All figures were drawn from holotype (T. Yamashiro 7435).

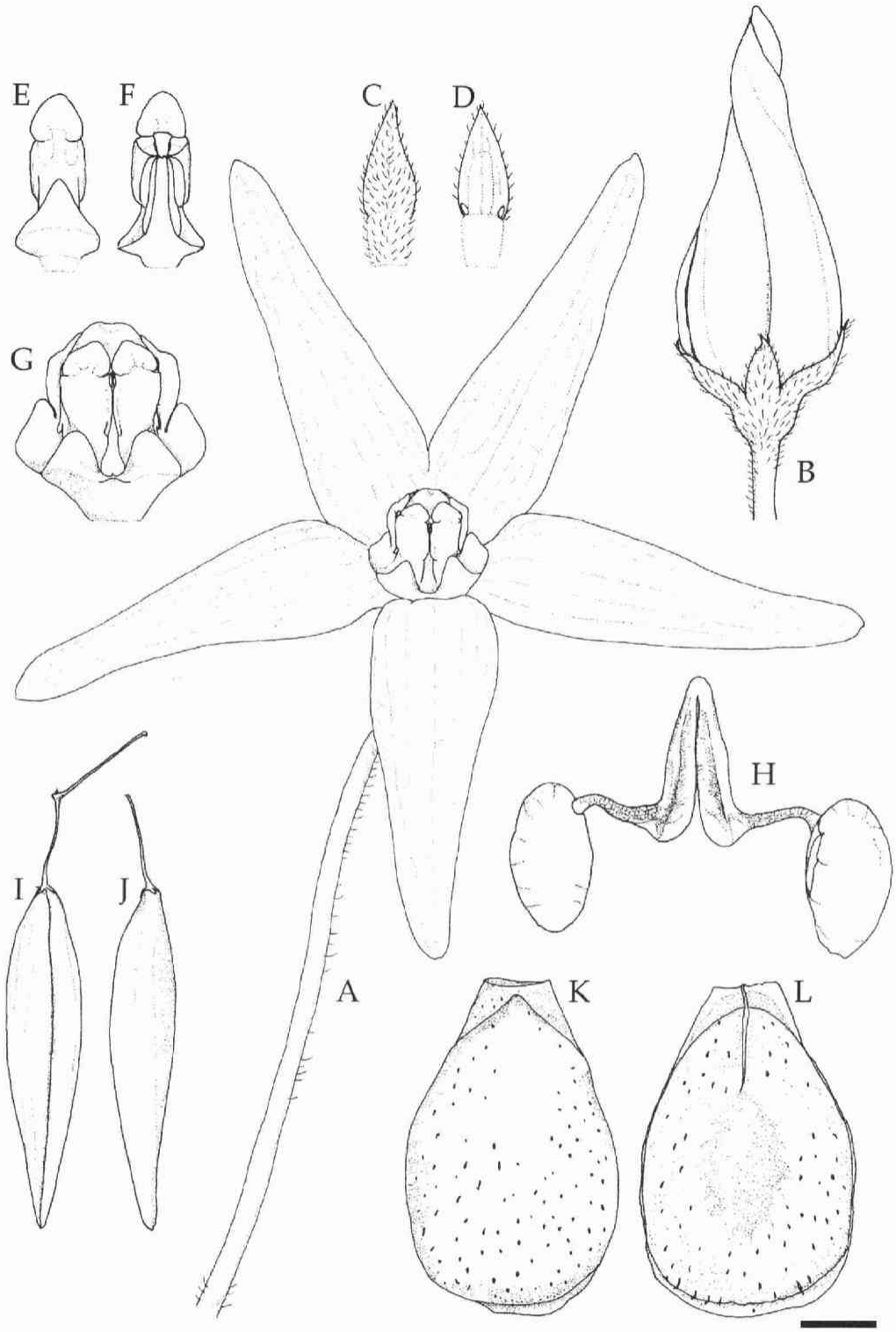

Fig. 11. Flower of *Vincetoxicum hoyoense*. A. whole flower. B. bud. C. adaxial view of calyx lobe D. abaxial view of calyx lobe. E. adaxial view of stamen. F. abaxial view of stamen. G. corona and gynostegium. H. pollinarium. I. adaxial view of follicle. J. lateral view of follicle. K. dorsal view of seed, coma is removed. L. ventral view of seed, coma is removed. Scale bar represents 1.5 mm for A, B, 1.25 mm for C, D, 1 mm for G-F, 0.15 mm for H, 1.5 cm for I, J and 2 mm for K, L. Voucher specimens. A-H: holotype (*T. Yamashiro & A. Yamashiro 7447*). J-L: *T. Yamashiro & A. Yamashiro 7798*.

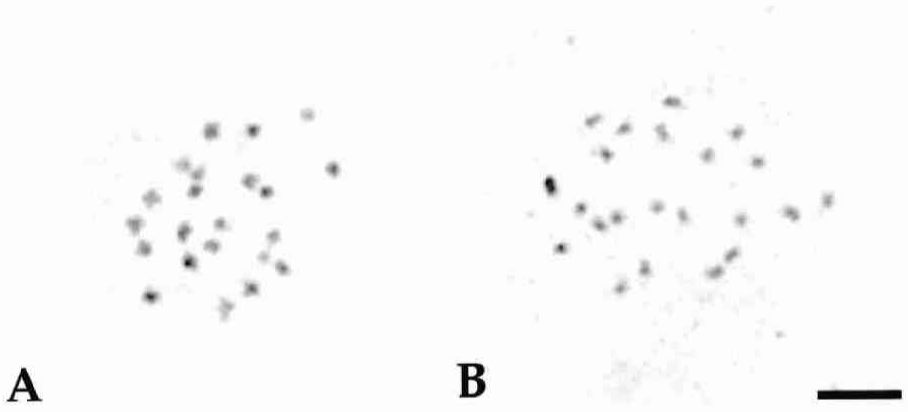

Fig. 12. Microphotographs of somatic chromosomes at metaphase of *V. izuense* (A) and *V. hoyoense* (B). Scale bar represents 4μm.

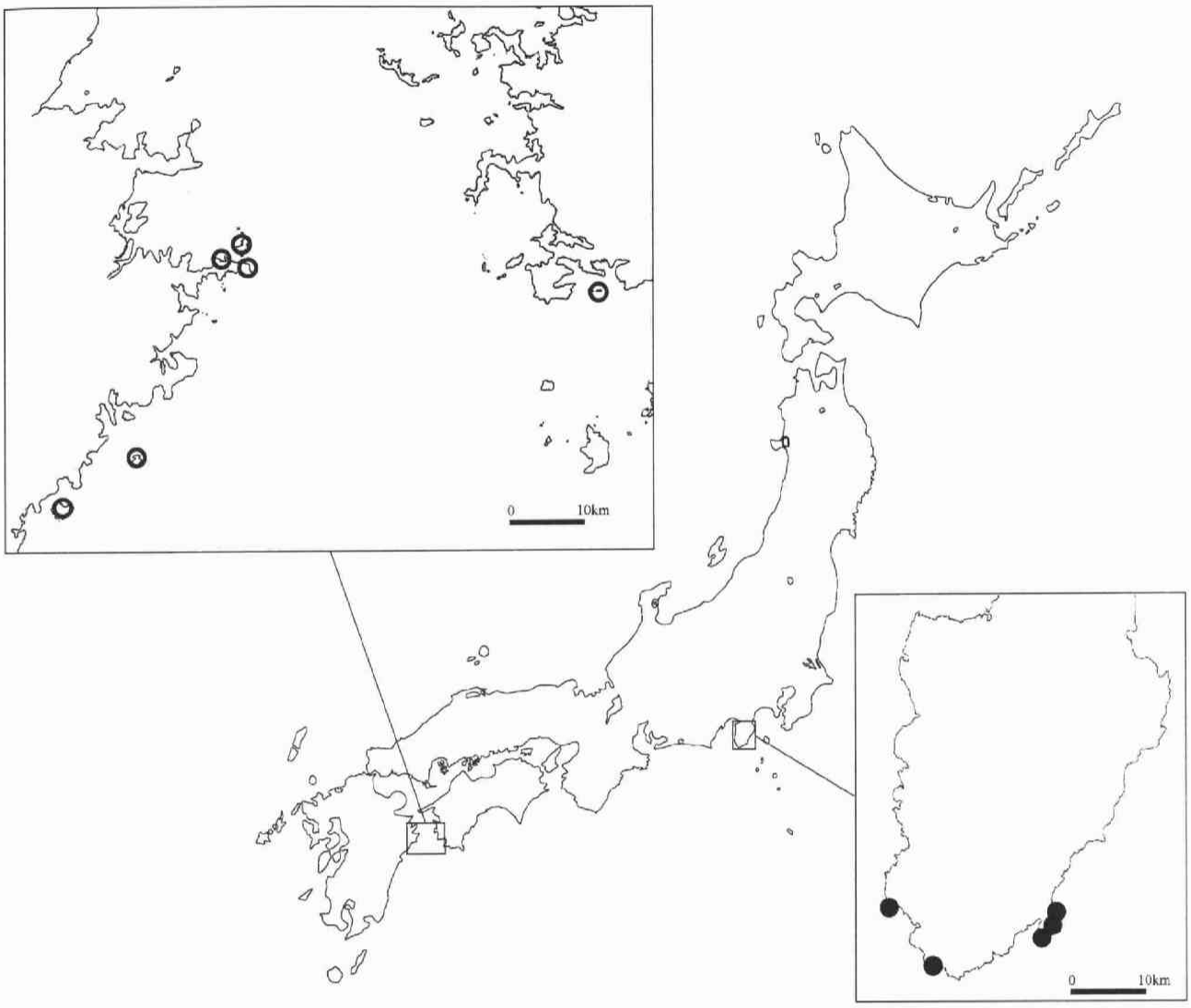

Fig. 13. Distribution map of *Vincetoxicum izuense* and *V. hoyoense*. Solid circles indicate the distribution of *V. izuense* and open circles indicate the distribution of *V. hoyoense*.

CHAPTER III. CHROMOSOME NUMBERS OF JAPANESE ASCLEPIADOIDEAE SPECIES

Although chromosome numbers of more than 300 species in subfamily Asclepiadoideae (Apocynaceae) have been counted so far, most of previous cytological studies focused on succulent species of the tribe Stapelieae (Albers, 1983). Excepting the tribe, relatively little information about chromosome number is available for the species of Asclepiadoideae (Albers, 1983). According to previous reports on chromosome counts, x = 11 is the most frequently observed basic chromosome number in Asclepiadoideae and is therefore considered to be an ancestral condition (Albers, 1983). In some genera, *Araujia* Bort., *Cynanchum* L., *Daemia* R. Br., *Heterostemma* Wight & Arn., *Microloma* R. Br., *Sarcostemma* R. Br., and *Vincetoxicum* Wolf, however, several basic chromosome numbers such as x = 8, 9, 10, 11 and 12 have been reported for some species (Albers et al., 1993).

In Japanese Asclepiadoideae, chromosome numbers were counted for 10 species (Yamazaki, 1993). However, only three species, *Cynanchum caudatum* (Miq.) Maxim. (Nishikawa, 1985), *Hoya carnosa* (L. f.) R. Br. (Nakamura, 1993), *Vincetoxicum japonicum* Morr. & Decne. (Jinno, 1956), have been studied for chromosome numbers using the materials collected in Japan.

The most wide-spread and distinctive cytogenetic process which has affected the evolution of higher plants is polyploidy (Stebbins, 1971). Thus, the cytological information is one of the most essential to examine phylogeny and speciation in a certain family. In this chapter, to provide additional cytological information about Japanese asclepiads, I report here chromosome numbers for 28 species and two varieties of seven genera in Asclepiadoideae collected in Japan.

MATERIALS AND METHODS

Twenty-eight species and two varieties belonging to seven genera were collected from a total of 50 localities in Japan (Table 2). The collected plants were cultivated in pots in the green house of University of the Ryukyus or the experimental gardens of Tohoku University.

Taxonomic treatments of the species examined follow Kitagawa (1959), Akasawa (1981), Kigawa (1989), Ohashi (1990), Yamazaki (1993), Li *et al.* (1995), and Endress and Bruyns (2000). Voucher specimens are deposited at TUS and URO.

For the observation of somatic chromosomes, root tips were pretreated in a 2 mM 8-hydroxyquinoline aqueous solution for 3.5 hours at room temperature. The root tips were

then fixed with a mixture of 100% ethanol and acetic acid (3:1) for 30 minutes and preserved at 5°C until the observations that followed after maceration with a mixture of 1N HCl and 45% acetic acid (2:1) at 60°C for 30 seconds and were staining in 2% aceto-orcein solution for an hour. The meristematic parts of the root-tips were squashed in 2% aceto-orcein on slide glasses and were observed using a microscope at 1500 magnification. Chromosome numbers of at least three cells for each individual were counted at somatic metaphase.

RESULTS AND DISCUSSION

The chromosome numbers of 28 species and two varieties observed in this study are presented in Table 2 and Figs. 14-17. Of these taxa, the chromosome number of 21 species and the two varieties were newly counted in this study (Table 2).

All of 30 taxa investigated in this study had chromosome number of 2n = 22 except for *Cynanchum boudieri* H. Lév. & Vaniot (2n = 24), *C. caudatum* var. *caudatum* (2n = 22, 44), *Vincetoxicum ambiguum* Maxim. (2n = 44), *V. sublanceolatum* (Miq.) Maxim. var. *sublanceolatum* (Miq.) Maxim. (2n = 44), and *V. sublanceolatum* (Miq.) Maxim. var. *macranthum* Maxim. (2n = 44). The chromosome numbers of 2n = 22 and 24 represent diploid of x = 11 and x = 12, respectively, while 2n = 44 represents tetraploid of x = 11. In *Cynanchum caudatum* var. *caudatum*, tetraploid was found only in the individual collected on Mt. Fuji, while diploids were found in other two localities. At this point, it is uncertain that the tetraploids observed in this study are autotetraploid or allotetraploid. Genetic studies aided with molecular makers are desirable to analyze the origin of these tetraploid taxa.

Chromosome counts in the present study are consistent with previous reports of 2n = 22 for *C. caudatum* (Nishikawa, 1985), *V. acuminatum* Decne. (Probatova and Sokolovskaya, 1983), *V. atratum* (Bunge) Morr. et Decne. (Ge et al., 1987; Probatova and Sokolovskaya, 1990), *V. japonicum* (Jinno, 1956) and *V. pycnostelma* Kitagawa (Ge et al., 1987). By contrast, our results disagreed with previous reports for some taxa. We counted the chromosome number of 2n = 22 for *Metaplexis japonica* (Thunb.) Makino from four localities in Japan, while 2n = 24 has been reported for the same species from Russia and China (Skolovskaya, 1966; Skolovskaya and Probatova, 1986; Ge et al., 1988). Similarly, although chromosome numbers of 2n = 16 and 2n = 24 have been reported in *V. amplexicaule* Sieb. et Zucc. from Korea (Lee, 1970) and *V. japonicum* from Russia (Gieszczykowna, 1934), respectively, we observed 2 n = 22 for these two species in Japan. Japanese populations of these species may be cytologically differentiated from the continental one. Otherwise, it is possible to explain these disagreements of the chromosome numbers by misidentification of the materials or

miscounting of chromosome numbers. Further cytological studies on continental materials are needed.

The chromosome number of *Cynanchum boudieri* was counted to be 2n = 24. Although *C. boudieri* is also distributed in Taiwan and south China, the chromosome number of this species has not been investigated in these areas (Li et al., 1995). In the genus *Cynanchum*, 2n = 24 has been reported for two species, *C. sibiricum* Willd. and *C. virens* Dietr. (Rostovtseva, 1977; Albers et al., 1993), and all other species have 22 or 44 chromosomes. The basic chromosome numbers of x = 12 is also found among several genera such as *Metaplexis* R. Br., *Pergularia* L. and *Heterostemma* (Skolovskaya, 1966; Skolovskaya and Probatova, 1986; Ge et al., 1988; Albers et al., 1993). Considering that these genera are taxonomically distinct from each other and that the basic chromosome number of x = 11 may be an ancestral condition in Asclepiadoideae, evolutionary changes from x=11 to x=12 seem to have occurred recurrently in this family. To test this hypothesis, phylogenetic studies using molecular information are needed.

Table 2. List of taxa, localities, voucher and chromosome numbers of Japanese Asclepiadoideae

* indicates new count for the species.

Taxa	Localities	2n	Voucher**
Marsdenieae			
Dischidia formosana Maxim.	Okinawa Pref., Uotsuri Isl. (Cultivated in University of the Ryukyus)	22*	YaT 7593
Jasminanthes mucronata (Blanco) W. D. Stevens & P. T. Li	Okinawa Pref., Onna-son	22*	Ta 51172
	Shizuoka Pref., Fukuroi	22*	YaT & YaA 7217
Marsdenia tinctoria R. Br. var. *tomentosa* Masam.	Okinawa Pref., Chinen	22*	YaT & Ta 3328
M. tomentosa Morr. & Decne.	Okinawa Pref., Ogimi-son	22*	YaT 3793
	Shizuoka Pref., Fukuroi	22*	YaT 4182.
Asclepiadeae			
Cynanchum boudieri H. Lév. & Vaniot	Kagoshima Pref., Amami Isl., Naon	24*	Ta & YaT 45546
C. caudatum (Miq.) Maxim.	Shizuoka Pref., Misakubo-cho	22	YaT 3984
var. *caudatum*	Nagano Pref., Karakemi Moor	22	Ta & YaT 55290
	Yamanashi Pref., N. E. foot of Mt. Fuji	44*	Ta 45367
var. *tanzawamontanum* Kigawa	Kanagawa Pref., Yamakita	22*	YaT 7581
C. liukiuense Warb.	Okinawa Pref., Ishigaki Isl.	22*	YaT 4069
C. wilfordii (Maxim.) Hemsl.	Shizuoka Pref., Mikkabi	22*	YaT 3830
Metaplexis japonica (Thunb.) Makino	Hokkaido Pref., Shiraoi	22	Yo s. n.
	Miyagi Pref., Sendai	22	YaT 7585
	Fukushima Pref., Sukagawa	22	YaT 7586
	Shizuoka Pref., Mikkabi-cho,	22	YaT 4184
	Nagano Pref., Hiyoshi	22	Ta, YaT & HC 55287
Tylophora aristolochioides Miq.	Miyagi Pref., Kakuda	22*	YaT & YaA 7580
T. floribunda Miq.	Aichi Pref., Shinshiro-shi	22*	YaT 3937
T. japonica Miq.	Okinawa Pref., Nakijin	22*	YaT 3737
T. matsumurae (Yamazaki) T. Yamashi & Tateishi	Okinawa Pref., Onna	22*	Ta & YaT 45144
T. tanakae Maxim.	Okinawa Pref., Itoman	22*	YaT 4003
	Okinawa Pref., Yonaguni Isl.	22*	YaT 4198

Table 2. continued

Vincetoxicum acuminatum Decne.	Gunma Pref., Mts. Haruna	22	YaT & YaA 7469
V. ambiguum Maxim.	Miyazaki Pref., Sadowara	44*	YaT & YaA 7320
V. amplexicaule Sieb. & Zucc.	Miyazaki Pref., Takaoka	22*	YaT & YaA 7234
	Nagasaki Pref., Fukue	22*	YaT & YaA 7454
V. atratum (Bunge) Morr. & Decne.	Yamanashi Pref., N. E. foot of Mt. Fuji	22	Ta 45370
V. austrokiusianum (Koidz.) Kitag.	Kagoshima Pref., Kaseda	22*	Ta, O & YaT 45499
V. calcareum (Ohashi) Akasawa	Kochi Pref., Mt. Ishidate	22*	YaT & YaA 7199
V. japonicum Morr. & Decne.	Aichi Pref., Irago Cape	22	YaT 3853
	Nagasaki Pref., Sanwa	22	YaT & YaA 7452
	Hyogo Pref., Mihara	22	YaT & YaA 7434
V. katoi (Ohwi) Kitag.	Shizuoka Pref., Fukuroi	22*	YaT & YaA 7181
	Aichi Pref., Shinshiro	22*	YaT & YaA 7421
	Chiba Pref., Tomisato-cho	22 *	YaT & YaA 7417
V. macrophyllum Sieb. & Zucc.	Nagasaki Pref., Mts. Tara	22*	YaT & YaA 7457
	Gunma pref., Niiharu	22*	M & HS s. n.
V. magnificum (Naki) Kitag.	Iwate Pref., Sumita	22*	Taz s. n.
V. nipponicum (Matsum.) Kitag.	Fukushima Pref., Shirakawa	22*	YaT & YaA 7571
	Hyogo Pref., Yashiro	22*	YaT & YaA 7458
V. pycnostelma Kitag.	Aichi Pref., Shinshiro	22	YaT 3911
V. sublanceolatum (Miq.) Maxim.	Aichi Pref., Shinshiro-shi	44*	YaT 4183
var. *sublanceolatum*	Shizuoka Pref., Iwata	44*	YaT & YaA 7436
	Gunma Pref., Haruna	44*	YaT & YaA 7577
var. *macranthum* Maxim.	Miyagi Pref., Mt. Izumigatake,	44*	YaT & YaA 7536
	Yamagata Pref., Mt. Gatussan	44*	YaT & YaA 7540
V. yamanakae (Ohwi & H. Ohashi)	Kochi Pref., Kagami	22*	YaT & YaA 7189
H. Ohashi	Kochi Pref., Tosashimizu	22*	YaT & YaA 7198

**: HC; Hokama, C., HS; Horie, S., M; Maki, M., O; Ogawa, H., Ta; Tateishi, Y., Taz; Tazawa, Y., YaT; Yamashiro, T., YaA; Yamashiro, A., Yo; Yokoyama, J.

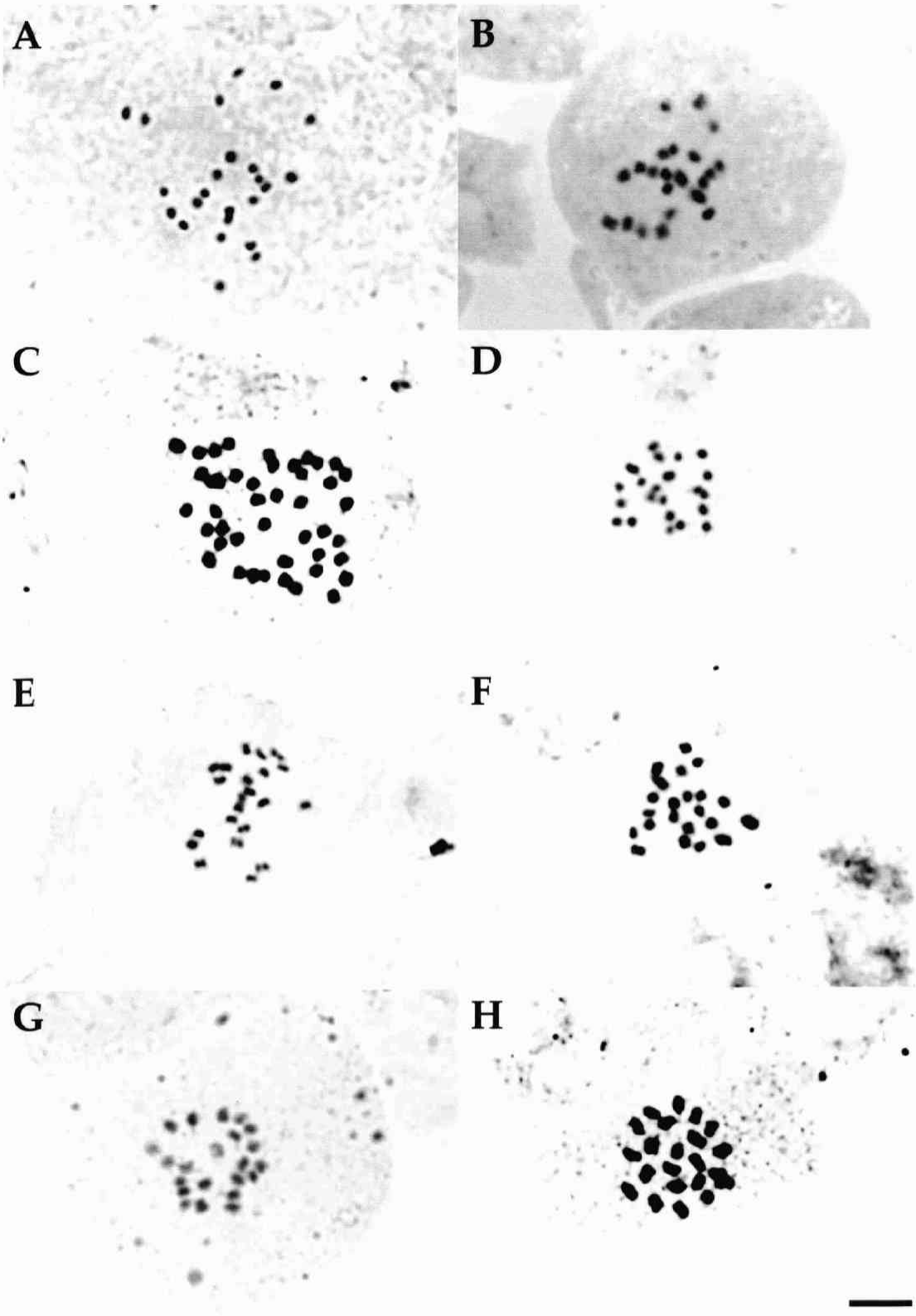

Fig. 14. Microphotographs of somatic chromosomes at metaphase of Japanese Asclepiadoideae (Apocynaceae). A. *Cynanchum boudieri* (2n = 24). B. *C. caudatum* var. *caudatum* (2n = 22). C. *C. caudatum* var. *caudatum* (2n = 44). D. *C. caudatum* var. *tanzawamontanum* (2n = 22). E. *C. liukiuense* (2n = 22). F. *C. wilfordii* (2n = 22). G. *Dischidia formosana* (2n = 22). H. *Jasminanthes mucronata* (2n = 22). Scale bar represents 4 mm.

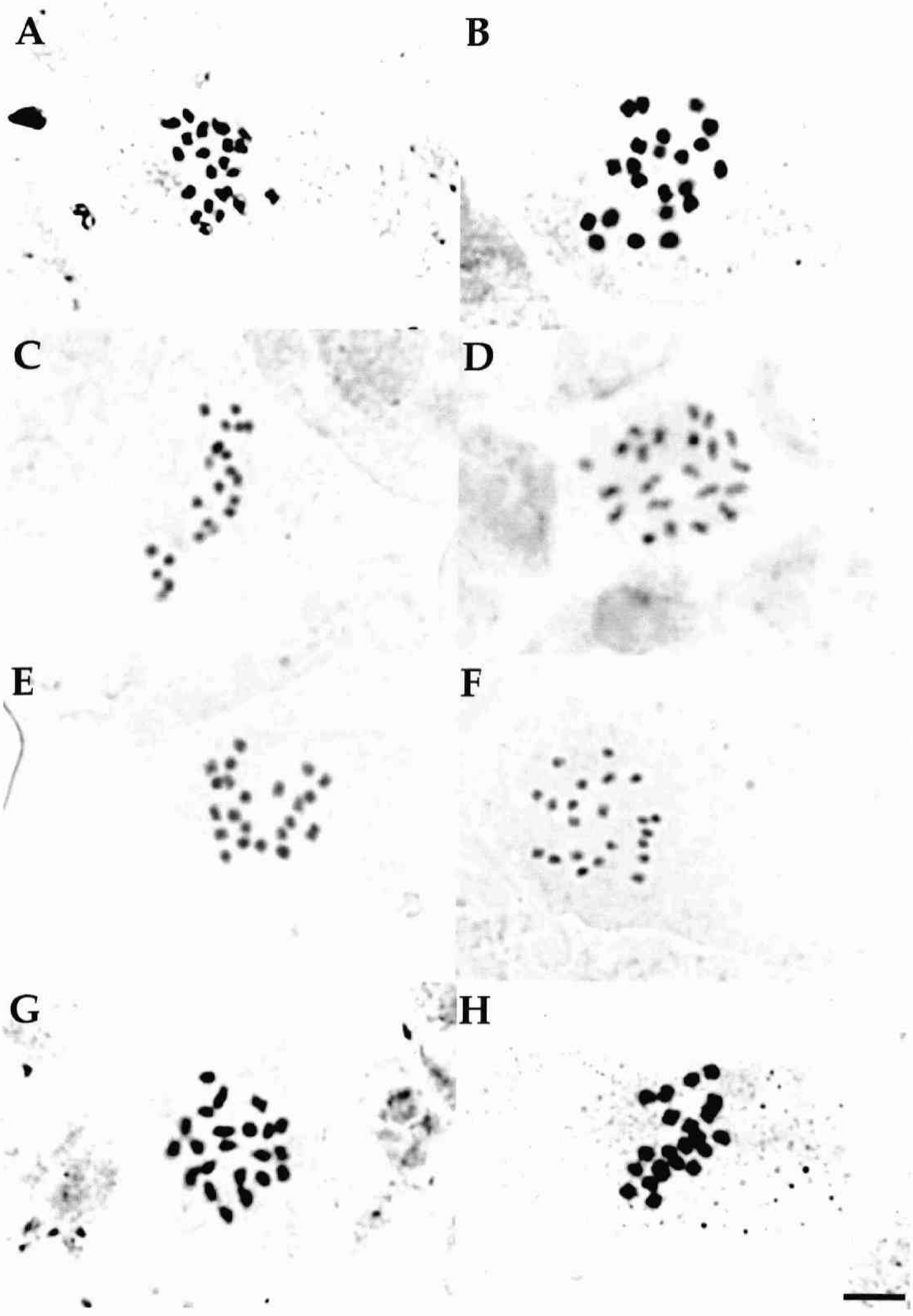

Fig. 15. Microphotographs of somatic chromosomes at metaphase of Japanese Asclepiadoideae (Apocynaceae). A. *Marsdenia tinctoria* var. *tomentosa* (2n = 22). B. *M. tomentosa* (2n = 22). C. *Metaplexis japonica* (2n = 22). D. *Tylophora aristolochioides* (2n = 22). E. *T. floribunda* (2n = 22). F. *T. japonica* (2n = 22). G. *T. matsumurae* (2n = 22). H. *T. tanakae* (2n = 22). Scale bar represents 4 mm.

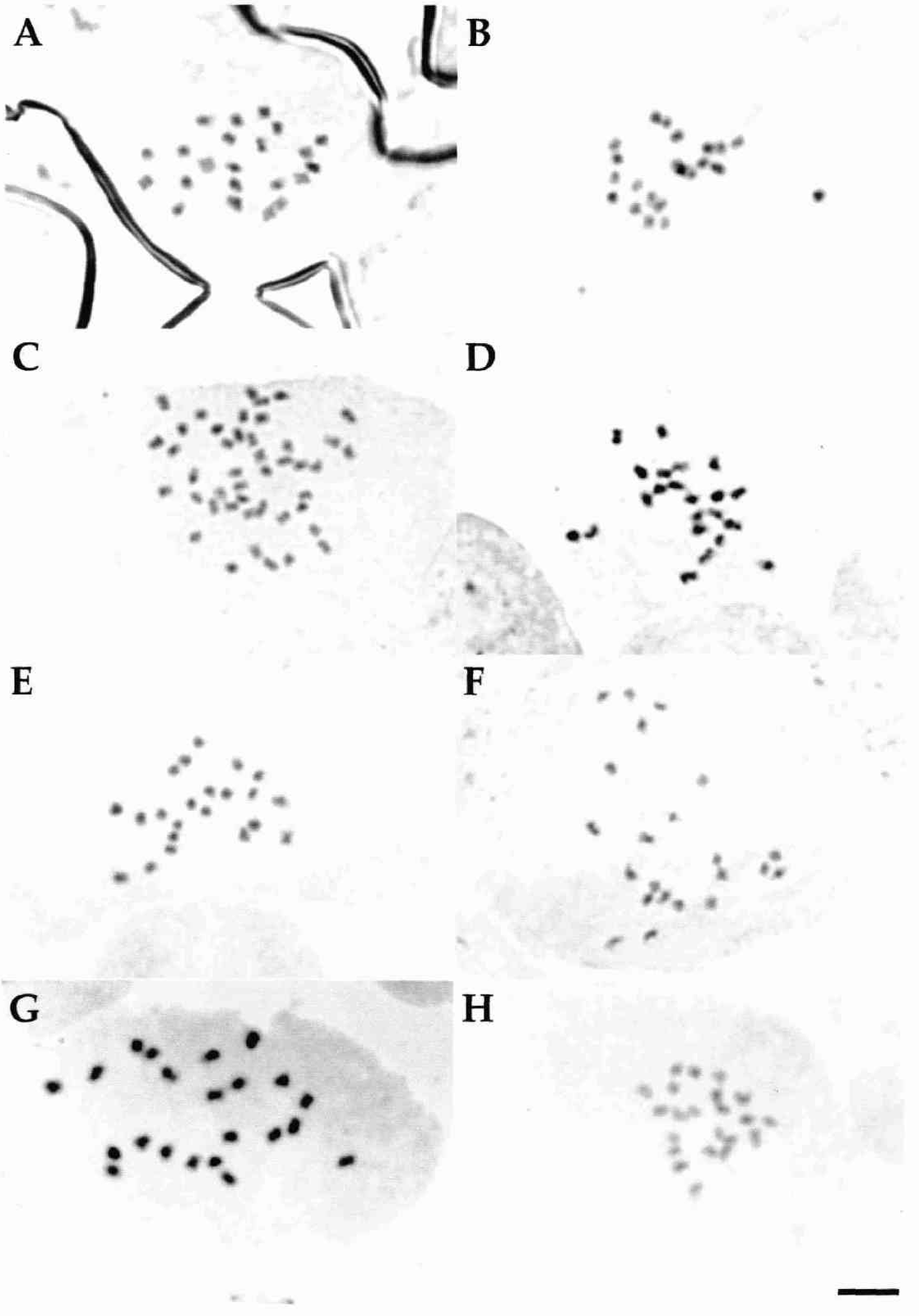

Fig. 16. Microphotographs of somatic chromosomes at metaphase of Japanese Asclepiadoideae (Apocynaceae). A. *Vincetoxicum acuminatum* (2n = 22). B. *Vincetoxicum calcareum* (2n = 22). C. *V. ambiguum* (2n = 44). D. *V. amplexicaule* (2n = 22). E. *V. atratum* (2n = 22). F. *V. austrokiusianum* (2n = 22). G. *V. japonicum* (2n = 22). H. *V. katoi* (2n = 22). Scale bar represents 4 mm.

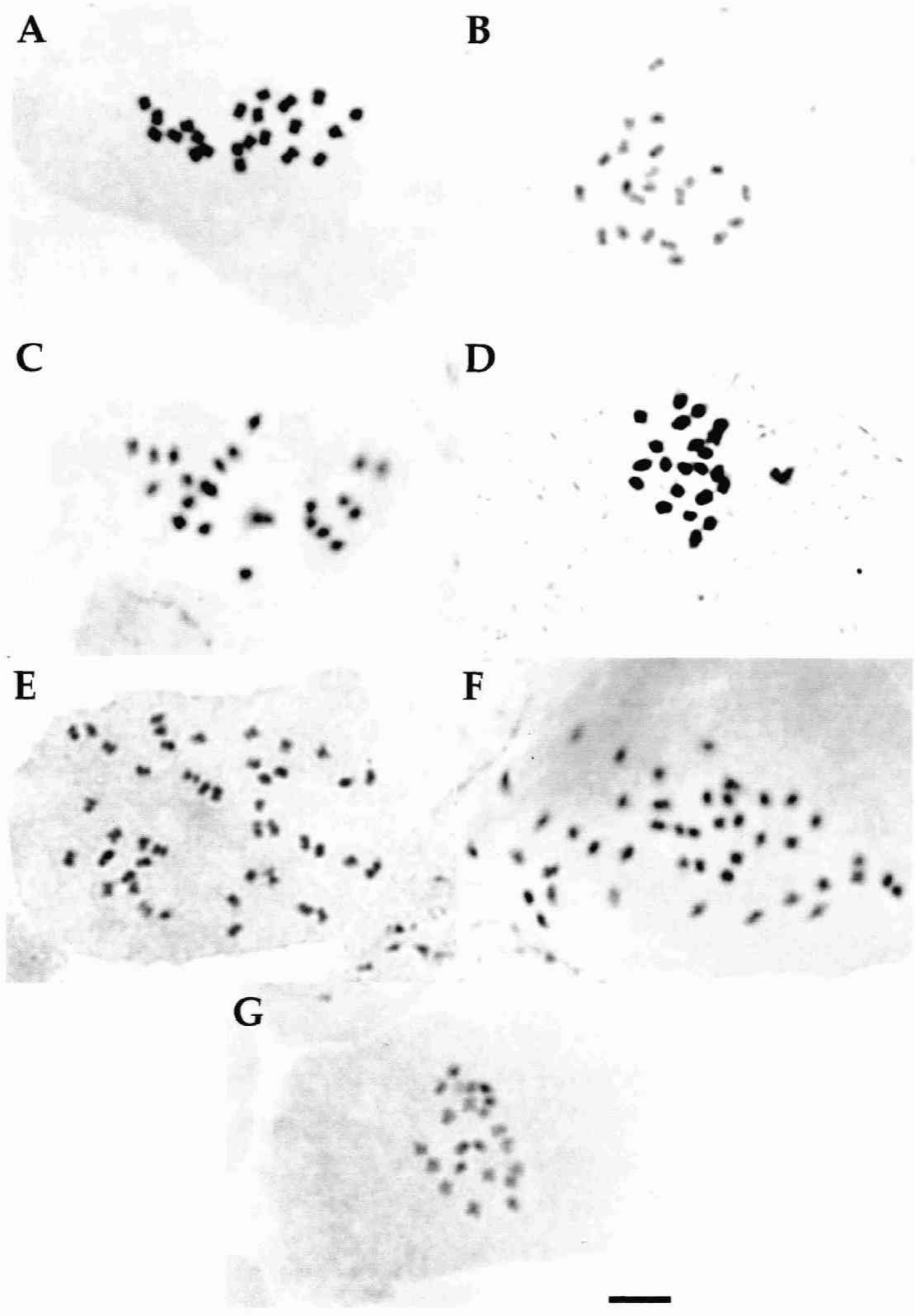

Fig. 17. Microphotographs of somatic chromosomes at metaphase of Japanese Asclepiadoideae (Apocynaceae). A. *V. macrophyllum* (2n = 22). B. *V. magnificum* (2n = 22). C. *V. nipponicum* (2n = 22). D. *V. pycnostelma* (2n = 22). E. *V. sublanceolatum* var. *sublanceolatum* (2n = 44). F. *V. sublanceolatum* var. *macranthum* (2n = 44). G. *V. yamanakae* (2n = 22). Scale bar represents 4 mm.

CHAPTER IV. POLLINATION BIOLOGY OF JAPANESE ASCLEPIADOIDEAE SPECIES

Although Asclepiadoideae has elaborate and complicated flowers, for animal pollination, studies on its pollination have been conducted only on restricted groups. While most of the studies on pollination ecology have been concentrated to the North American members of the genus *Asclepias* (reviewed by Wyatt & Broyles, 1994) and South African stapeliad (Meve and Liede, 1994), the other members of the subfamily remain poorly understood (Ollerton and Liede, 1996).

Although 37 species belonging nine genera are known to occur in Japan (Yamazaki, 1993; Chapter II), to my knowledge, no study has been conducted for their pollinator, levels of specialization and breeding system. Therefore, I address the following questions in this chapter. 1) Are these asclepiad species differed for their pollinator? 2) How ecologically specialized are these asclepiads in their pollinator requirements? 3) What kinds of floral structures are mechanically correlated with pollination modes?

MATERIALS AND METHODS

Flower morphologies, nectar volumes and sugar concentrations

Flower materials of 28 species belonging seven genera, i.e., two *Cynanchum* species, *Hoya carnosa*, *Jasminates mucronata*, two *Marsdenia* species, *Metaplexis japonica*, five *Tylophora* species, and sixteen *Vincetoxicum* species, for the morphological observation were collected from a total of 28 localities (Table 3). Taxonomic treatments of the species examined were made according to Li et al. (1994), Kitagawa (1959), Akasawa (1981), Ohashi (1990), Yamazaki (1993), and Chapter II of this thesis. Observations and measurements of flower characters were conducted for the FAA (formalin, acetic acid and 70% ethanol are mixed, 5: 5: 90 in volume, respectively) fixed materials. Nectar volumes of each species were measured using MICRCAPS microcapillarry tubes (Drummond Scientific Co., USA) in the fields and cultivated plants on the experimental garden of Tohoku University. Sugar concentration for each species was measured by portable sugar refractometer (KIKUCHI Co., Japan).

Pollinator observation

Pollinator observations were done on 28 species at a total of 38 populations (Table 3). The observations were conducted for both of diurnal and nocturnal insect visitors.

Observation duration at each population is listed in Table 3.

During the observation, insect visitors were captured with a net and were checked for whether the pollinarium is attached or not. The insect visitors with pollinarium were recognized as pollinators. The position of attached pollinarium on the insect bodies was investigated and the number of pollinarium was counted under a dissecting microscope.

RESULTS

Flower morphologies of study species

The morphological, phenological, and nectar characters in 28 species were summarized in Table 4. The 28 species represent seven genera belonging to two tribes of the asclepiads, the Marsdenieae (*Hoya, Jasminathes* and *Marsdenia*) and the Asclepiadeae (*Cynanchum, Metaplexis, Tylophora* and *Vincetoxicum*). These species considerably varied in their flower size, color, and other floral traits such as morphology, scent and nectar characteristics.

Marsdenieae

The flower morphologies of three genera of Marsdenieae are quite different from each other. In *Hoya carnosa*, many white rotate flowers clustered and formed round shape inflorescence (Fig. 19A). *Jasminathes mucronata* bears 2-4 whitish and relatively large salverform flowers on the axial (Fig. 19B). *Marsdenia tinctoria* also has round inflorescence, although each flower is small and urceolate (Fig. 19E). *Marsdenia tomentosa* has yellowish white campanulate corolla approximately 5 mm in length.

Asclepiadeae

Most of the species belonging to Asclepiadeae have rotate corolla and its lobes are reflex or patent at anthesis, although *C. wilfordii* have closed corolla lobes and *Metaplexis japonica* have half opened corolla lobes (Fig. 19G). The flowers of most of all species observed opened both day times and night. Whereas, the flowers of three species, i.e., *Tylophora floribunda, Vincetoxicum ambiguum* and *V. pycnostelma*, opened during night and cloudy days (Table 4). A corona of *Cynanchum* and *Metaplexis japonica* is different from those of *Vincetoxicum* and *Tylophora*. Two *Cynanchum* species and *Metaplexis japonica* have menbranaceous corona. In contrast, *Vincetoxicum* and *Tylophora* have five fleshy staminal corona lobes completely separated form or partly connected by much thinner interstaminal parts.

The coronas of 21 taxa of *Vincetoxicum* and *Tylophora* can be classified into three types based on the interstaminal parts. The first is that the interstaminal parts were completely absent and consisted of only stamainal corona lobes (Fig. 18A). This type of corona was observed for five species of *Tylophora* and *Vincetoxicum pycnostelama*. The second is that the interstaminal parts were present but short and lack a tube-like structure (Fig. 18B). This type of corona is observed for *T. japonica* and five species of *Vincetoxicum*. The third is that the interstaminal parts developed and consisted of a cup-shape (Fig. 18C). This type of corona was observed for remaining species of *Vincetoxicum*.

Pollinator observations

The flower visitors observed for each species are listed in Table 5. The average number of the species of flower visitor per plant species was 7.8 species, although the majorities of these visitors were uncommon and usually did not pick up pollinaria (Table 5).

Marsdenieae

Three genera belonging to Marsdenieae were pollinated by Hymenoptera and Lepidoptera. The flowers of *Hoya carnosa* emitted strong scent at night and were pollinated by aganaid and noctuid moths (Fig. 19A). The flowers of *Jasminathes mucronata* were visited by both of diurnal and nocturnal insects (Fig. 19B). The two of four bumble bee (*Bombus ardens ardens* and *B. diversus diversus*) individuals carried pollinaria on their glossa (Fig. 19C). *Sypnoides hercules* (Noctidiae) was most frequently observed and carried pollonarium of *J. mucronata* on its mouth part (Fig. 19C, D). Two *Marsdenia* species were visited by scolid wasps, vespid wasps and anthophorid bee in daytime.

Asclepiadeae

Four genera belonging to Asclepiadeae were pollinated by four insect orders, i.e. Coleoptera, Diptera, Hymenoptera and Lepidoptera. Flowers of two *Cynanchum* species were visited by three insect orders, i.e., Coleoptera, Diptera and Hymenoptera, in daytime. The flowers of *C. caudatum* were mainly visited by apoid bees and vespid wasps, whereas, *C. wilfordii* are visited by wasp belonging to Scolidae and Vespidae. The pollinaria of *Metaplexis japonica* were carried by both diurnal and nocturnal insects belonging to four orders, i.e., Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Fig. 19G).

Four *Tylophora* species were visited by small insects belonging to Diptera and most of these insects were belonging to the family Cecidomyiidae and Sciaridae (Fig. 20B, D). All

pollinarium were attached to a maxillary palp of the insects. No insect visitor was observed for the flowers of *T. matsumurae*.

The flowers of fifteen *Vincetoxicum* species were visited by insects belonging to four orders, although dipteran and lepidopteran pollination were dominated in this genus. The pollinaria of the eight *Vincetoxicum* species were carried only by Diptera, and these visitors were differed at family level (Table 5). Especially, Sciaridae species were observed carrying pollinaria of *V. japonicum*, *V. katoi* and *V. sublanceolatum* var. *sublanceolatum* (Fig. 20E, F). Moth pollination was observed in four *Vincetoxicum* species, i.e. *Vincetoxicum ambiguum*, *V. amplexicaule*, *V. austrokiusinaum* and *V. yonakuniense* (Fig. 20G, H). Pollinaria of *V. pycnostelma* were carried by Tipulidae species and moths. Flower of *V. acuminatum* are the largest flower in 15 *Vincetoxicum* species and visited by four insect orders, i.e., Coleoptera, Diptera, Hymenoptera and Lepidoptera, in daytime.

DISCUSSION

Pollination system of the Japanese asclepiads

From the results of the observations, 28 asclepiad species were pollinated by insect belonging to four orders. Although *Metaplexis japonica* and *Vincetoxicum acuminatum* were pollinated by insects belonging three or four orders, most of other species were pollinated by one or two insect orders. Among the examined 28 species, I found following pollination systems. i) moth pollination: *Hoya carnosa*, *Jasminathes mucronata*, *Vincetoxicum ambiguum*, *Vincetoxicum austrokiusianum* and *Vincetoxicum yonakuniense*, ii) generalized insect pollination, *Cynanchum caudatum*, *Metaplexis japonica* and *Vincetoxicum acuminatum*, iii) wasp pollination: *Cynanchum wilfordii*, and two *Marsdenia* species, iv) dipteran pollination: three *Tylophora* species, *Vincetoxicum atratum*, *V. inamoenum*, *V. japonicum*, *V. katoi*, *V. nipponicum* and *V. sublanceolatum*, v) dipteran and lepidopteran pollination: *Vincetoxicum pycnostelma*. Furthermore, pollination does not require pollinators found. Autogamy via *in situ* pollen tube germination in anther sac (Kunze 1991) was observed on *Tylophora matsumurae*. This will be examined in more detail in the next chapter.

Moth pollination of two Marsdenieae species, i.e., *Hoya carnosa* and *Jasminathes mucronata*, has been expected form its floral morphology and scents (Martile and Altenburger, 1988; Ollerton and Liede, 1997). This expectation was supported by my observation, although *Jasminathes mucronata* were also pollinated by bumble bees in daytime.

Although fly and moth pollination were thought to be predominant in *Marsdenia*

(Ollerton and Liede 1997), two *Marsdenia* species in Japan were pollinated by scolid and vespid wasps. Wasp pollination is one of the most frequently observed pollination modes in Asclepiadeae (Ollerton and Liede, 1997) and has been documented for the number of North American *Asclepias* species (e.g. Robertson 1895) as generalized pollination systems.

Cynanchum wilfordii was also pollinated by eumenid, scolid, and vespid wasps. The size and structure of the flower of *Cynanchum wilfordii* is similar to those of *Cynanchum caudatum*. However, the former has closed corolla and pollinated by wasp and the latter has patent or reflex corolla lobes and pollinated by generalized insects. Floral traits shared by wasps-pollinated three species, i. e., *Cynanchum wilfordii, Marsdenia tinctoria* and M. *tomentosa* are whitish yellow corolla, cupshape, campanulate or urceolate corolla with narrow opening. It seems that these floral traits might have convergently evolved through specialization for wasp pollination.

In the tribe Asclepiadeae, Hymenoptera and Lepidoptera are thought to be predominant pollinators. However, flies are observed as important pollinator for some groups of the tribe (Ollerton and Liede, 1996). The flowers of *Tylophora* and *Vincetoxicum* are characterized by patent corolla lobes and have been considered fly pollination (Ollerton and Liede, 1996). The pollinaria of four *Tylophora* species, i.e., *T. japonica, T. aristolochioides, T. floribunda* and *T. tanakae* and three *Vincetoxicum* species, i.e., *V. japonicum, V. katoi,* and *V. sublanceolatum* var. *sublanceolatum* were carried by small dipteran insects belonging to the families Cecidomyiidae or Sciaridae (Table 5). These two dipteran families are known to serve as pollinators of Aristolochiaceae, Araceae, and Orchidaceae which deceit insects by fungus gnat flowers (Endress 1994). Although flowers of *Tylophora japonica, Vincetoxicum japonicum, V. katoi,* and *V. sublanceolatum* var. *sublanceolatum* secreted approximately 0.5-0.7μl of nectars, the flowers of the other three species, i.e., *T. aristolochioides, T. floribunda* and *T. tanakae,* lack visible nectars (Table 4). Thus, it is possible that *T. aristolochioides, T. floribunda,* and *T. tanakae* deceptively attracted these small insects by odor. Further investigation for volatile chemical component analyses of flower odor are needed for these species.

Moth pollination has never been recorded for *Vincetoxicum* in the compiled data of asclepiads pollinators by Ollerton and Liede (1996). However, I observed moths species as the visitor of seven *Vincetoxicum* species, i.e., *Vincetoxicum ambiguum, V. amplexicaule, V. austrokiusianum, V. nipponicum, V. pycnostelma, V. sublanceolatum var. sublanceolatum* and *V. yonakuniense.* Although *Vincetoxicum ambiguum, V. austrokiusianum* and *V. yonakuniense* scented at night and exclusively visited by moth, *V. pycnostelma, V. sublanceolatum* var. *sublanceolatum* and *V. nipponicum* were visited by not only moth but also Diptera such as Culicidae, Sciaridae and Tipulidae (Table 5). These results suggested that pollinator mode

transition from Diptera to Lepidoptera may be occurring in this genus. To examine this hypothesis, combining phylogenetic analysis with field surveys of pollination systems is needed. For further discussion, the result of combined phylogenetic and pollination system will be conducted in the Chapter VI.

Floral traits correlated pollinator systems in Vincetoxicum

The flowers of 16 *Vincetoxicum* species have rotate corolla and rather simple corona structure than those of other genera of Asclepiadoideae. However, these species were pollinated by combination of two distinct insect orders. Here I discuss functional aspects of detailed floral morphology in this genus.

In the flower morphologies of 16 *Vincetoxicum* species examined, I found three important organs for adapting various pollinators. The first is relatively long guide rails and these are shared with eight species. Guide rails play a crucial role in trapping the legs or proboscis of insect and in guiding these body parts to extract pollinarium (Fig. 18) (Liede 1996b). If the insect is too small to remove the pollinarium, it will be stuck in the guide rails and dies (Liede 1996b). Thus, this organ also plays an important role to exclude unimportant pollinators. Relatively long guide rails, ranging from 0.66 to 0.90 mm in length of stamen, were observed for *V. atratum*, *V. austrokiusianum*, *V. pycnostelma* and *V. yonakuniense*, and many small insects are captured and died there. The pollinarium of these four *Vincetoxicum* species were carried by Lepidoptera or relatively large Diptera such as Calliphorid flies or Muscid flies (Table 5).

Furthermore, relatively large gynostegium also appears to be effective for preventing small insects. The flower morphology of *V. katoi* is very similar to that of *V. yamanakae*. However *V. katoi* have rather smaller gynostegium and pollinarium than *V. yamanakae* does. Although the pollinarium of the former were carried by the small mosquitoes belonging to Sciaridae, the insect could not take out the pollinarium from the flower of the latter and were captured. Thus, size of flower is also important to exclude the inadequate pollinators.

The corona functions the optical attractant for pollinators and nectar reservoir (Endress, 1994). The complicate canal system constructed corona lobes and gynostegium helping to lead insect bodies to guide rail (Endress, 1994). In the 16 species examined, I found three types in corona structures based on the interstaminal part (Fig. 18). The interstaminal parts of corona on eleven *Vincetoxicum* species are well developed and have various shapes. Interstaminal parts of corona aid to construct canal system between two corona lobes which develop backs of the stamens. A well-developed interstaminal part conceals stigmatic

chambers and limits the insect accession to nectar only from upper direction of the flower. Thus, well developed interstaminal parts of corona restrict only long-tongued insects as adequate pollinators.

I found bowl shape corollas which enclosed 2/3-1/3 part of gynostegium and were rather deep nectar reservoir. This structure is observed in six *Vincetoxicum* species. The above mentioned three characters seem to sift pollinator of sixteen *Vincetoxicum* species from dipterian to long tongued insects.

Table 3. List of localities of population and number of hours observed pollinator

Taxa	Hours observed		Locality of population
	Day	Night	
Marsdenieae			
Hoya carnosa (L. fil.) R. Br.	5	12	Okinawa Pref., Urasoe
Jasminanthes mucronata (Blanco) W. D. Stevens & P. T. Li	4	4	Shizuoka Pref., Mt. Ogasa
Marsdenia tinctoria R. Br. var. *tomentosa* Masam.	3	0	Okinawa Pref., Itoman
Marsdenia tomentosa Morr. & Decne.	3	1	Shizuoka Pref., Mt. Ogasa
Asclepiadeae			
Metaplexis japonica (Thunb.) Makoino	4	2	Miyagi Pref., Shibata
Cynanchum caudatum (Miq.) Maxim	2.5	1	Miyagi Pref., Mt. Tokura
Cynanchum wilfordii (Maxim.) Hemsl.	3	0.5	Miyagi Pref., Ogatsu
Tylophora aristolochioides Miq.	3	2	Miyagi Pref., Mt. Taihaku-san
	2	0	Aichi Pref., Mt. Horaiji-san
T. floribunda Miq.	0.5	1.5	Aichi Pref., Mt. Kichisho-yama
T. japonica Miq.	2	2	Okinawa Pref., Aha
T. matsumurae (T. Yamaz.) T. Yamash. & Tateishi	2	1	Okinawa Pref., Onnna
T. tanakae Maxim.	2	1.5	Okinawa Pref., Onnna
	2	0	Okinawa Pref., Itoman
Vincetoxicum acuminatum Decne.	3	0.5	Gunma Pref., Mt. Akagi-san
	2	0	Shiga Pref., Mt. Ibuki-yama
V. ambiguum Maxim.	2	2	Miyazaki Pref., Sadowara
V. amplexicaule Sieb. & Zucc.	2	0.5	Kagoshima Pref., Sumiyoshi Pond
V. atratum (Bunge) Morr. & Decne.	2	0	Iwate Pref., Tanesashi coast
	1.5	0	Nagasaki Pref., Hirado-shi
V. austrokiusianum (Koidz.) Kitag.	2	2	Miyazaki Pref., Toimisaki Cape
V. calcareum (H. Ohashi) Akasawa	0.5	0	Kochi Pref., Mt. Ishidate-yama
V. inamoeanum Maixm.	0.5	0	Hokkaido Pref., Bihoro
V. japonicum Morr. & Decne.	3	0	Miyagi Pref., Onagawa

Table 3. continued

	2	0	Aichi Pref., Irego Cape
	1	1.5	Mie Pref., Arashima
V. katoi (Ohwi) Kitag.	3	0.5	Shizuoka Pref., Fukuroi
	2	0	Aichi Pref., Mt. Kishisho-san
V. macrophyllum Sieb. & Zucc.	3	2	Tochigi Pref., Nikko
V. magnificum (Nak.) Kitag.	3	2	Miyagi Pref., Mt. Sasakura-yama
V. nipponicum (Matsum.) Kitag.	3	2	Fukushima Pref., Shirakawa
V. pycnostelma Kitag.	1	4	Miyagi Pref., Sendai
	2	2	Aichi Pref., Mt. Kichisho-san
V. sublanceolatum (Miq.) Maxim. var. *macranthum* Maxim.	3	0.5	Miyagi Pref., Mt. Izumigatake
V. *sublanceolatum* (Miq.) Maxim. var. *sublanceolatum*	3.5	2.5	Miyagi Pref., Sendai
	1	1	Shizuoka Pref., Fukuroi
V. yamanakae (Ohwi & H. Ohashi) H. Ohashi	2	0	Kochi Pref., Kagami
V. yonakuniense (Hats.) T. Yamash. & Y. Tateishi	0.5	0.75	Okinawa Pref., Yonaguni Isl.

Table 4. Comparison of the flower morphologies of 28 asclepiads.

Taxa	phenology	corolla color	hair of corolla	corolla diameter (mm)	corona type	length of stamen (mm)	length of guid rail (mm)	corpusculum length x width (mm)	pollinia length x width (mm)	nectar volume (μl) / flower	sugar concentratio (%)
Marsdenieae											
Hoya carnosa	day-night	white	pubescent	10	b	2	1	0.9 x 04	1.65 x 0.6	-	-
Jasminates mucronata	day-night	white	glabrous***	22	absent	5.1	2.6	0.7 x 0.36	0.6 x 0.5	-	-
Marsdenia tinctoria var. *tomentosa*	day-night	yellowish	glabrous***	3.5	absent	1.3	0.7	0.17 x 0.07	0.45x 0.09	-	-
Marsdenia tomentosa	day-night	yellowish	glabrous***	4	absent	1.5	0.78	0.35 x 0.15	0.48 x 0.18	-	-
Asclepiadeae											
C. caudatum	day-night	greenish	pubescent	7.5-8	b	2	0.9	0.32 x 0.2	0.31 x 0.18	3-4	22-24
C. wilfordii	day-night	greenish	pubescent	12-13*	b	2.65	0.9	0.34 x 0.16	0.42x 0.2	3-4	25
Metaplexis japonica	day-night	pinkish	pubescent	11	absent	2	1.3	0.4 x 0.26	0.46 x 0.27	-	-
T. aristolochioides	day-night	purplish	pubescent	5.5	b	0.9	0.2	0.06 x 0.04	0.08 x 0.08	-**	-**
T. floribunda	night	purplish	pubescent	6	b	0.75	0.16	0.08 x 0.05	0.12 x 0.08	-**	-**
T. japonica	day-night	purplish	pubescent	11.4	b	2.25	1	0.21 x 0.1	0.16 x 0.1	0.5	13
T. matsumurae	day-night	pale green	glabrous	9.5	b	1	0.25	0.07 x 0.05	0.17 x 0.1	-**	-**
T. tanakae	day-night	pale green	glabrous	10.5	b	1	0.24	0.07 x 0.05	0.17 x 0.1	-**	-**
V. acuminatum	day-night	white	glabrous	20	c	1.7	1.0	0.34 x 0.22	0.36 x 0.24	2	14
V. ambiguum	night	white	pubescent	9	c	1.45	0.83	0.22 x 0.1	0.24 x 0.14	-	-

Table 4. continued

V. amplexicaule	day-night	yellowish	pubescent	9-11	c	0.94	0.5	0.22 x 0.14	0.29 x 0.12	-	-
V. atratum	day-night	purplish	glabrous	18	c	2.45	1.44	0.35 x 0.16	0.32 x 0.2	8-10	19-22
V. austrokiusianum	night	white	pubescent	17-18	c	1.3	0.95	0.2 x 0.12	0.35 x 0.22	-	-
V. calcareum	day-night	white	glabrous	18	c	1.35	0.89	0.3 x 0.18	0.32 x 0.21	0.9-1	22
V. inamoenum	day-night	yellowish	glabrous	11	c	1.5	0.59	0.28 x 0.14	0.22 x 0.14	-	-
V. japonicum	day-night	yellowish	glabrous	12-14	c	1.75	0.5	0.22 x 0.08	0.24 x 0.1	0.5	13
V. katoi	day-night	yellowish & purplish	glabrous	8.5-9.5	a	1.5	0.51	0.29 x 0.12	0.25 x 0.17	0.72	22
V. macrophyllum	day-night	purplish	pubescent	5-7	c	0.8	0.2	0.12 x 0.06	0.08 x 0.05	-	-
V. magnificum	day-night	brownish	glabrous	16	c	0.8	0.15	0.14 x 0.03	0.06 x 0.04	0.2	-
V. nipponicum	day-night	purplish & brownish	glabrous	12.5	a	2.2	0.87	0.45 x 0.24	0.32 x 0.22	4	16
V. pycnostelma	night	yellowish	glabrous	12	b	1.56	1.03	0.22 x 0.12	0.4 x 0.14	12	14
V. sublanceolatum											
var. *macranthum*	day-night	white	pubescent	19-20	c	2	0.74	0.35 x 0.18	0.21 x 0.1	-	-
var. *sublanceolatum*	day-night	purplish	glabrous	16	a	1.5	0.4	0.22 x 0.1	0.24 x 0.12	-	-
V. yamanakae	day-night	purplish	glabrous	14	a	2	0.74	0.28 x 0.14	0.34 x 0.2	10	13
V. yonakuniense	day-night	white	glabrous	18-21	a	2.1	1.9	0.24 x 0.1	0.3 x 0.16	-	-

*corolla lobes dose not patent or reflect at anthesis, **indicates nectars are not visible *** inside of corolla tube is pubescent

Table 5. List of flower visitors of *Vincetoxicum-Tylophora* complex and *Cynanchum*.

Plant species	Flower visitor		Number of individuals observed	Numbers of pollinia attached	*Organ of pollinia attached
	Order Family	Scientific name			
Marsdenieae					
Hoya carnosa	Lepidoptera				
	Aganaidae	*Asota egens confinif*	4	4	L
	Aganaidae	*Asota heloconia riukiuana*	3	4	L
	Lymantridae	*Lymantria dispar japonica*	1	0	-
	Noctuidae	*Eredus crepuscularis*	3	4	L
	Noctuidae	*Pindra illibata*	5	0	-
	Noctuidae	*Parallelia fulvotaenia*	1	0	-
Jasminathes mucronata	Hymenoptera				
	Apidae	*Bombus ardens ardens*	3	1	M
		Bombus diversus diversus	1	1	M
	Lepidoptera				
	Geometridae	*Ourapteryx maculicaudaria*	1	1	M
	Noctuidae	*Sypnoides hercules*	13	6	M
Marsdenica tinctoria var. *tomentosa*	Hymenoptera				
	Vespidae	*Polistes jadwigae okinawensis*	4	20	M
	Anthophoridae	*Amegilla senahai subflavescens*	1	0	-
Marsdenia tomentosa	Hymenoptera				
	Scoliinae	*Megacampsomeris prismatica*	2	3	M
Asclepiadeae					
C. caudatum	Coleoptera				
	Cerambycidae	*Leptura ochraceofasciata*	2	-	-
	Hymenoptera				
	Anthophoridae	*Xylocopa appendiculata circumvolans*	1	1	M
	Apidae	*Apis cerana*	1	10	L, M
	Vespidae	*Polistes snelleni*	2	13	L, M
		Vespa tropica pulchra	1	1	M
		Vespa simillima xanthoptera	1	1	M
C. wilfordii	Hymenoptera				
	Eumenidae	*Anterhynchium* sp.	1	1	M
	Scoliidae	*Carinoscolia melanosoma fascinata*	1	1	M
		Scolia oculata	1	3	M
	Vespidae	*Polistes snelleni*	1	-	-
Metaplexis japonica	Coleoptera				
	Scarabaeidae	*Protaetia orientalis submarumorea*	4	2	M
	Diptera				
	Tachinidae	*Tachina nupta*	1	1	M
	Hymenoptera				
	Apidae	*Aips cerna*	8	7	M
	Anthophoridae	*Xylocopa appendiculata circumvolans*	2	1	M
	Scoliinae	*Megacampsomeris grossa matsumurai*	2	7	L, M
	Scoliinae	*Megacampsomeris prismatica*	2	4	M
	Vespidae	*Polistes chinensis antennalis*	2	6	L, M
	Lepidoptera				
	Hesperiidae	*Polytremis pellucida pellucida*	1	0	-
	Noctuide	*Abrostola paucifica*	5	0	-
		Autographa amurica	3	1	M
		Macdunnoughia purissima	4	0	-
		Trichoplusia intermixta	2	0	-
	Nymphalidae	*Cynthis cardui*	1	1	M
	Pyralidae	*Hymenia recurvalis*	2	0	-
		Pyrausta panopealis	1	0	-
T. aristolochioides	Diptera				
	Cecidomyiidae	Cecidomyiidae sp. 1	6	0	-
		Cecidomyiidae sp. 2	1	1	-
	Chironomideae	Chironomideae sp.	1	-	-
T. floribunda	Diptera				
	Cecidomyiidae sp.		2	-	-

Table 5. continued

T. japonica	Diptera				
	Sciaridae sp.		13	5	M
T. tanakae	Diptera				
	Cecidomyiidae sp.		2	-	-
	Chloropidae	*Chlorops oryzae*	1	-	-
	Sciaridae	Sciaridae sp.	4	2	M
V. acuminatum	Coleoptera				
	Cerambycidae sp.		8	6	M
	Diptera				
	Anthomyiidae	*Delia platura*	1	-	-
		Anthomyiidae sp.	1	-	-
	Empididae	Empididae sp.	1	-	-
	Syrphidae	*Eristalis tenax*	4	-	-
		Helophilus virgatus	2	1	M
	Hemenoptera				
	Tenthredinidae sp.		2	1	L, M
V. ambiguum	Lepidoptera				
	Arctiidae	*Manoba rectilinea chinesica*	1	-	-
	Geometridae	*Pogonopygia nigralbata*	1	1	M
	Noctuidae	*Spodoptera exigua*	1	-	-
		Spodoptera litura	1	-	-
	Pyralidae	*Cnaphalocrocis medinalis*	1	-	-
		Hedylepta misera	2	-	-
		Hymenia recurvalis	1	-	-
		Crambinae sp.	1	-	-
V. amplexicaule	Lepidoptera				
	Noctuidae	*Amyna octo*	1	-	-
		Lophoruza pulcherrina	1	-	-
	Pyralidae	Nacoleia chrysorycta	1	-	-
V. atratum	Diptera				
	Anthomyiidae sp.		5	-	-
	Calliphoridae	*Onesia hokkaoidensis*	2	-	-
		Paradichosia itoi	4	1	M
	Muscidae sp.		3	1	M
V. austrokiusianum	Lepidptera				
	Pyralidae	*Endotricha icelusalis*	1	-	-
		Bradina sp.	3	-	-
		Palpita nigropunctalis	1	-	-
		Pyraustinae sp.	1	-	-
	Geometridae	*Hypomecis punctinalis conferenda*	1	1	M
	Nocutidae	*Hydrillodes repugnalis*	1	1	M
		Herminia innocens	2	-	-
	Choreutidae	*Choreutis hyligenes*	1	-	-
V. calcareum	Diptera				
	Anthomyiidae sp.		4	-	-
	Empididae	*Empis kyushuensis*	3	-	-
		Empididae sp.	1	-	-
	Sepsidae	*Sepsis monostigma*	1	-	-
	Sciaridae	Sciaridae sp.	4	-	-
	Tipulidae	*Elephantomyia dietziana*	1	-	-
V. inamoeanum	Diptera				
	Empididae	*Empis flavobasalis*	6	-	-
		Empididae sp. 1	1	-	-
		Empididae sp. 2	1	-	-
	Pachyneuridae	Pachyneuridae sp.	1	-	-
V. japonicum	Diptera				
	Bibionidae	*Bibio flavihalter*	3	-	-
		Bibionidae sp.	5	-	-
	Calliphoridae	*Lucilia illustris*	1	-	-
		Polleniopsis mongolica	1	1	M
	Chloropidae	*Chlorops oryzae*	1	-	-
	Drosophilidae	*Drosophila melanogaster*	1	-	-
		Drosophila sp.	1	-	-
	Muscidae	*Eudasyhora cyanicolor*	1	-	-
	Sciaridae	Sciaridae sp. 1	2	3	M
		Sciaridae sp. 2	18	17	L, M
		Sciaridae sp. 3	1	1	M
		Sciaridae sp. 4	3	1	M

Table 5. continued

	Sepsidae	*Sepsis monostigma*	23	-	-
V. katoi	Diptera				
	Cecidomyiidae	*Contarinia inouyei*	1	-	-
	Chironomidae	Orthrocladiini sp.	1	-	-
	Drosophilidae	*Drosophila immigrans*	2	-	C
		Drosophila coracina	1	-	C
	Empididae	*Empis* sp.	1	-	C
	Sciaridae	Sciaridae sp.1	1	1	M
		Sciaridae sp. 2	2	-	C
		Sciaridae sp. 3	10	6	M
V. magnificum	Diptera				
	Tipulidae	Tipulidae sp.	3	-	-
	Sciaridae	Sciaridae sp.	1	-	-
V. nipponicum.-1	Diptera				
	Anthomyiidae	Anthomyiidae sp.	1	-	-
	Chloropinae	*Chlorops oryzae*	2	-	C
	Culicinae	Culicinae sp.	3	-	C
	Drosophilidae	*Drosophila* sp.	3	-	C
	Sarcophagidae	*Blaesoxipha japonensis*	1	-	-
		Scathophagidae sp.	1	-	C
	Tipulidae	*Limonia bifasciata flavoabdominalis*	1	1	M
	Lepidptera				
	Geometridae	*Agathia carissima*	2	-	-
	Pyralidae	*Bradina geminalis*	1	-	-
		Glyphodes quadrimaculalis	1	-	-
		Hedylepta misera	2	-	-
	Noctuidae	Micreremites pyraloides	1	-	-
V. pycnostelma	Diptera				
	Culicidae	*Culex pipens pallens*	1	1	L
	Chironomidae	Chironomidae sp.	3	-	C
	Muscidae	*Lispe orientalis*	1	-	C
	Sciaridae	Sciaridae sp.	3	-	C
	Tipulidae	*Limonia nohirrai*	2	2	M
		Limonia pulchra	5	1	L
		Limonia quadrimaculat?	6	5	L, M
	Lepidoptera				
	Pyralidae	*Acropentias aurea*	1	-	-
		Bradina admixtalis	2	1	L
		Bradina geminalis	5	-	-
		Endotrica kuznetzovi	1	-	-
		Pleuroptya chlorophanta	2	1	M
		Uresiphita gracilis	2	-	-
	Noctuidae	*Anachrostis nigripunctalis*	2	-	-
		Parallelia dulcis	1	-	-
		Stenhypena nigripuncta	1	-	-
		Zanclognatha leechi	1	-	-
		Noctuidae sp.	1	1	M
V. sublanceolatum					
var. *macranthum*	Diptera				
	Anthomyiidae	Anthomyiidae sp.1	28	1	M
		Anthomyiidae sp.2	13	-	-
	Calliphoridae	Calliphoridae sp.	1	-	-
	Dolichopodidae	Dolichopodidae sp.	5	-	-
	Ephydridae	Ephydridae sp.	20	-	-
var. sublanceolatum	Diptera				
	Culicidae	Culicidae sp.	1	-	-
	Sciaridae	Sciaridae sp.1	4	3	M
	Sciaridae	Sciaridae sp .2	1	-	C
	Lepidoptera				
	Pyralidae	*Eurrhyparodes accessalis*	3	-	-
		Chabula onychinalis	4	-	-
		Glyphodes pryeri	1	-	-
		Hedylepta miscra	3	-	-
		Uresiphita tricolor	1	-	-
		Crambus argyrophorus	1	-	-
	Torticidae	Olethreutinae sp.	1	-	-
V. yonakuniense	Diptera				
	Anthomyiidae	Anthomyiidae sp.	1	-	-

Table 5. continued

Lauxaniidae	Lauxaniidae sp.	5	-	-
Muscidae	Muscidae sp.	1	-	-
Sciaridae	Sciaridae sp.	1	-	C
Lepidptera				
Hypsidae	*Asota heliconia riukiuana*	1	-	-
Noctuidae	*Metaemene atriguttata maculata*	1	-	-
	Spodoptera pecten	1	-	-
	Simplicia ryukyuensis	1	-	-

*: M. mouth parts; L, M. legs and mouths; C. captured.

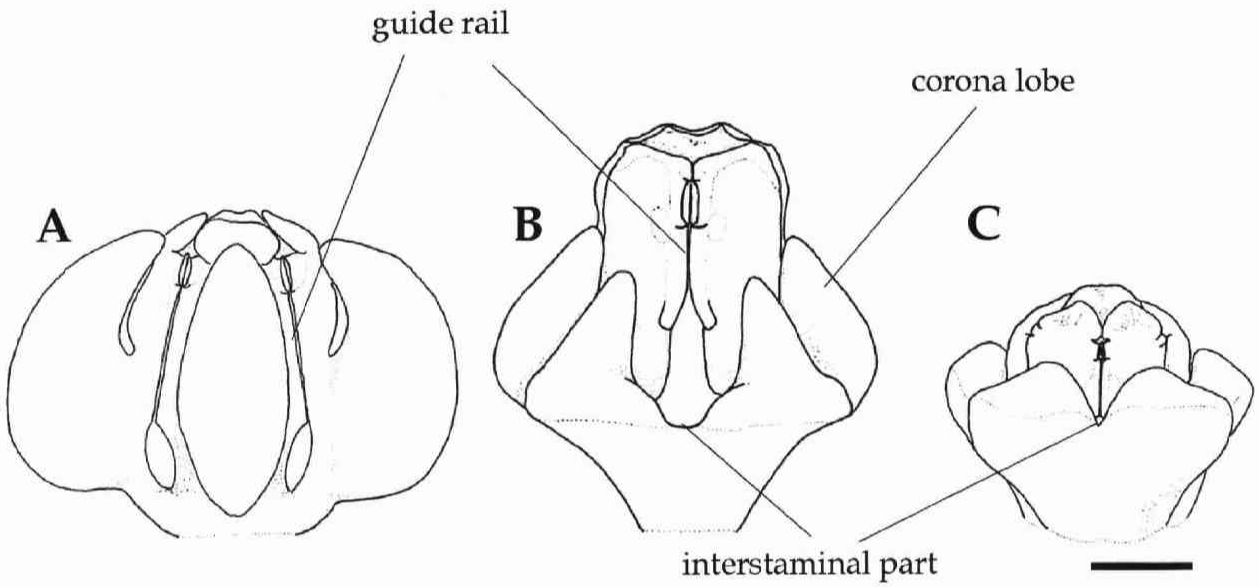

Fig. 18. Three corona types found in *Vincetoxicum*. A. *V. pycnostelma*. B. *V. nipponicum*. C. *V. japonicum*. Bar = 1.5 mm for A, =1 mm for B, C.

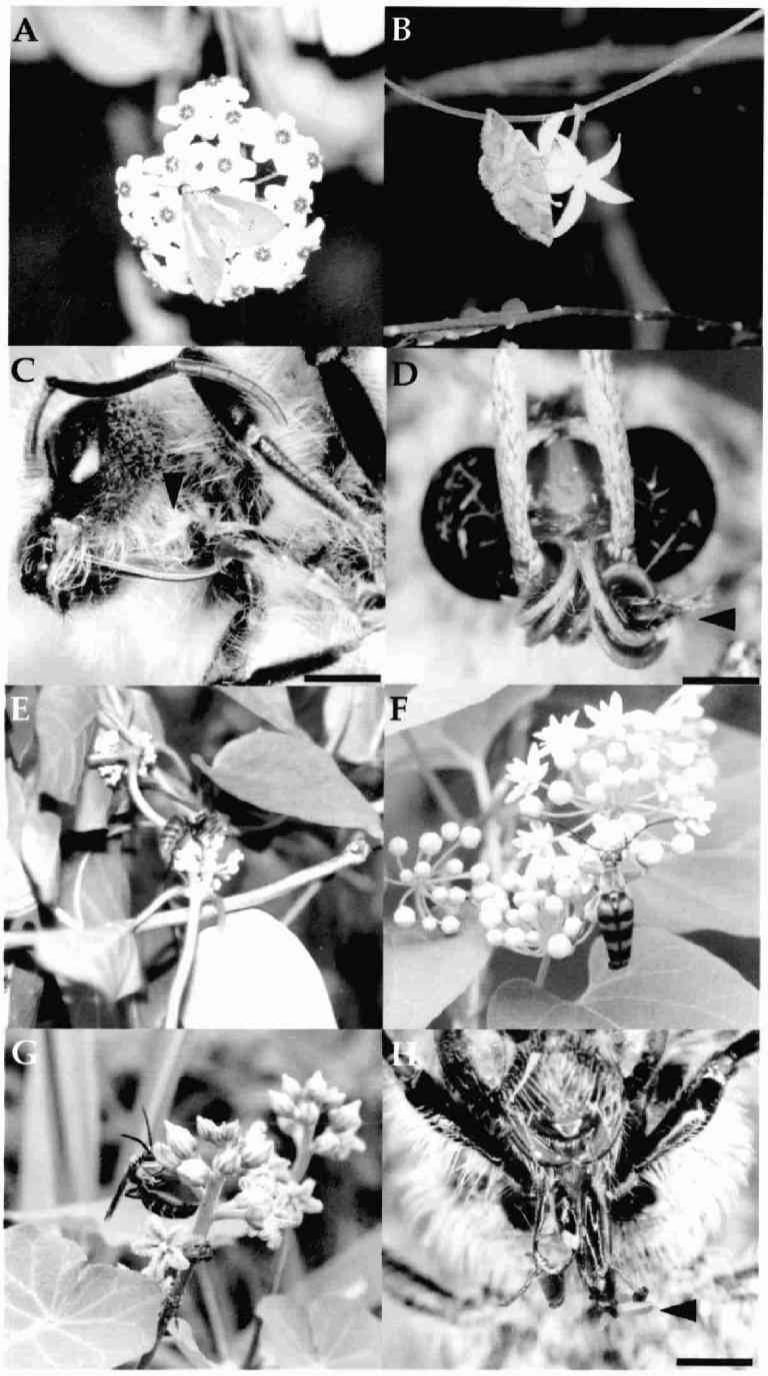

Fig. 19. Pollinator of asclepiads (three Marsdenieae, *Metaplexis japonica*, and two *Cynanchum*). A. *Asota heloconia riukiuana* (Aganaidae) visiting flowers of *Hoya carnosa*. B. *Sypnoides hercules* (Noctuidae) visiting a flower of *Jasminathes mucronata*. C. Mouth part of *Bombus ardens ardens* showing the attachement of pollinarium of *Jasminanthes mucronata* to glossa. Scale bar =1.3 mm. D. Head of *Sypnoides hercules* (Noctuidae) showing the attachement of corpusculum of *Jasminathes mucronata* to proboscis. Scale bar = 0.8 mm. E. *Polistes jadwigae okinawens*is (Vespidae) visiting flowers of *Marsdenia tinctoria* var. *tomentosa*. F. *Leptura ochraceofasciata* (Cerambycidae) visiting flowers of *Cynanchum caudatum*.
G. *Megacampsomeris grossa matsumurai* visiting the flowers of *Metaplexis japonica*.
H. Mouth part of *Megacampsomeris grossa matsumurai* showing the attachment of the pollinarium of *Metaplexis japonica* to a maxillary palp. Scale bar = 1 mm. Arrow heads indicate pollinarium and corpusculum.

ig. 20. Pollinators of asclepiads (*Tylophora* and *Vincetoxicum*). A. Cecidomidae sp. visiting a flower f *Tylophora aristolochioides*. B. Cecidomidae sp. showing attachement of pollinarium of *T. ristolochioides* to the mouth part. Scale bar = 0.2 mm. C. *Empis flavobasalis* (Empididae) visiting lowers of *Vincetoxicum inamoenum*. D. Sciaridae sp. showing attachement of pollinarium of *ylophora tanakae* to the mouth part. Scale bar = 0.4 mm. E. Sciaridae sp. visiting flowers of *incetoxicum japonicum*. F. Sciaridae sp. showing attachement of pollinarium of *Vincetoxicum iponicum* to the mouth part. Scale bar = 0.7 mm. G. *Herminia innocens* (Nocutidae) visiting flowers f *Vincetoxicum austrokiusianum*. H. Head of *Pogonopygia nigralbata* (Geometridae) showing the ttachement of pollinarium of *Vincetoxicum ambiguum* to proboscis. Scale bar = 0.7 mm. Arrow eads indicate pollinarioum

CHAPTER V. A COMPARATIVE STUDY OF REPRODUCTIVE CHARACTERISTICS AND GENETIC DIVERSITIES ON AN AUTOGAMOUS DERIVATIVE *TYLOPHORA MATSUMURAE* AND ITS PROGENITOR *T. TANAKAE*

The evolution of selfing taxa from outcrossing progenitor is one of the well documented evolutionary pathway in angiosperms (Stebbins, 1970). Flowers of selfing taxa have typically smaller corollas, reduced herkogamy, and reduced dicogamy compared to flowers of related outcrossing taxa (Ornduff, 1969). Even if highly elaborate flowers prevent self-pollination in most of taxa, the breakdown of such systems have been reported in many plant families (e.g. Boraginaceae: Schoen et al., 1997; Orchidaceae: Pedersen and Ehlers, 2000). Furthermore, not only in floral traits, self-pollinating taxa also often differ from outcrossing ones in life history and ecology (Ornduff, 1969; Hill et al., 1992). These associations suggested that the frequently observed evolutionary transition from outcrossing to selfing involves adaptation to local pollination environments (Fishman and Wyatt, 1999).

The members of the subfamily Asclepiadoideae has complicate structures for adapting animal-mediated pollination (see chapters I, IV). Thus, in this subfamily, outcrossing is considered to be a dominant mode of pollination, although the occurrence of autogamy via *in situ* pollen tube germination has been rarely reported in a few species (Chaturvedi and Pant, 1986; Kuntz, 1991; Lumer and Yost, 1995). In the course of the morphological re-examination (Chapter II) and investigation of the pollination biology for 28 Japanese Asclepiadoideae (Chapter IV), I found an autogamous species in the genus *Tylophora.*

Tylophora matsumurae (T. Yamaz.) T. Yamash. & Tateishi is a perennial herb growing on glass lands of windy headlands or exposed lime stones near seashore, and is endemic to Amami, Okinoerabu, Yoron, and Okinawa Islands in the Ryukyu Archipelago, Japan (Chapter II; Fig. 22). This species is listed as endangered in the Red List of Japanese vascular plants (Environment Agency of Japan, 2000).

Tylophora matsumurae is characterized by short erecting stems, almost absent peduncles, and a few inconspicuous flowers, and is thought to be related closely to *T. tanakae* Maxim, which is distributed from the South Kyushu to the Ryukyu Archipelago (Chapter II; Fig. 22). Moreover, although no insect visitors have been observed in the field investigation (Chapter IV), this species has very high fruit-flower ratio (14.2-41.6%) compared to other species of Asclepiadoideae. These morphological and reproductive features seem to be consistent with a syndrome of autogamous species (Ornduff, 1969; Wyatt, 1988). Thus it is possible that *T. matsumurae* has autogamous mode of reproduction and has been derived from the

relative *T. tanakae*.

In the Asclepiadoideae, autogamous species that accompanied with morphological modifications have never been documented. Thus *T. matsumurae* is an ideal species to investigate how elaborate floral traits preventing self-pollination are lost and how the morphological modifications occur accompanying with autogamy in Asclepiadoideae.

Allozyme studies provide much useful information on genetic structures within and among populations (Loveless and Harmick, 1984). Some studies have demonstrated that the multiple and single occurrences of selfing species or population by examining isozyme variations (e.g. Inoue and Kawahara, 1990; Wyatt et al., 1992).

The purposes of the present chapter are to examine the differences of reproductive traits of *T. matsumurae* and its progenitor of *T. tanakae* and to estimate the level of genetic divergences of both species.

MATERIALS AND METHODS

Study species

Tylophora matsumurae is growing on glass lands of windy headlands or exposed lime stones near seashore, and is endemic to Amami, Okinoerabu, Yoron, and Okinawa Islands in the Ryukyu Archipelago (Yamazaki, 1968; Chapter II; Fig. 21A, 22). This species is morphologically resembles *Tylophora tanakae*, which is distributed from Kyushu Island to the Ryukyu Archipelago (Fig. 21B, 22). *Tylophora matsumurae* has short and erect stems up to 40 cm long, although *T. tanakae* has many branched and twining stems up to 2 m long. The most conspicuous difference in reproductive organs is length of peduncles. *Tylophora matsumurae* has absent or very short peduncles and only 2-4 flowers are directly attached on axial and inconspicuous. On the other hand, *T. tanakae* has rather long peduncle, 4-18 mm in length and has more than the 10 flowers per inflorescence. Furthermore, *Tylophora matsumurae* can be also distinguished from *T. tanakae* by much narrower follicles and smaller seeds (Chapter II).

Androecium differentiation and pollination biology

In situ pollen tube germination and pollen tube growth in ovary

In Asclepiadoideae autogamy has been reported to occur via *in situ* pollen tube germination in anther sacs (Kunze, 1991). Thus, to examine how autogamy occurs on *T.*

matsumurae, I observed androecium differentiation of *T. matsumurae* and *T. tanakae*.

Flowers and buds of both species were collected at consecutive stages of development and fixed in FAA. Fixed materials were dehydrated through an alcohol series, embedded in paraffin, and sectioned at thickness of 6-8 μm with a rotary microtome. The sections were stained with hematoxylin and fast-green (Sass, 1958) and mounted with Entellan. Prepared slides were observed with a light microscope.

The pollen tubes growth in pistil was also observed by using fluorescence microscopy for flowers of *T. matsumurae*. I used the flowers of *T. matsumurae* collected from M1, M2, and M6 populations (Fig. 22, Table 6). Five flowers that were not inserted pollinia of other individuals were selected for observation from each of three populations. The pistils of the flowers were cut at the base with a raiser. Thereafter, the pistils were washed by distilled water and then softened in 1N NaOH for 30 minutes at 60°C. The softened pistils were placed on a slide glass and stained with 0.01% aniline blue solution, and squashed with the cover glass. Prepared slides were examined for stained callose plugs and pollen tubes using Olympus BH microscope equipped with a fluorescence apparatus of Olympus BH-RFL.

Pollination biology

In order to estimate the levels of pollinator activity in natural populations, I randomly collected 20 flower (a flower per individual) from five populations of M1, M3, M4, M5, and M6 of *T. matsumurae* and from four populations of T12, T13, T14, and T15 of *T. tanakae* (Table 6). The flowers collected were fixed in FAA and preserved until use. These flowers were dissected under the microscope, and I counted the number of both inserted pollinia to stigmatic chambers and removed pollinia from anther sacs.

To examine autogamy, bagging treatments were conducted for four, five, and 11 individuals (a total of 442 flowers) of *T. mastumurae* at M3, M4, and M6 population, respectively and five, three, two, and four individuals (a total of 210 flowers) of *T. tanakae* at T9, T11, T13, and T14 population (Fig. 22). The flower buds were bagged by nylon bags and the numbers of mature fruits were counted.

Allozyme electrophoresis

Population sampling

I sampled a total of 169 individuals form eight populations of *Tylophora matsumurae* and 361 individuals form 18 populations of *T. tanakae*. Furthermore, to estimate outcrossing rate of *T. tanakae*, I collected mature follicles from four populations, T9, T14, T15, and T18 of *T.*

tanakae. Due to the lack of isozyme variation on *T. matsumurae* (shown in the results), the mating system analyses were conducted only for the populations of *T. tanakae*.

Allozyme electrophoresis

For mature plants, one hundred mg of a fresh leaf sample was homogenized in 1000 μl of an extract buffer. The extract buffer was made up of 93 mmol/L Tris HCl (pH 7.5), 23.4% glycerol, 0.6% (v/v) Tween 80, 2.8 mmol/L dithiothreitol, and 0.5% 2-mercaptoethanol (Uchida et al., 1991). The homogenates were centrifuged at 20,000 x g at 4°C for 15 min. The resulting supernatant was used for crude extract of the enzymes. Polyacrylamide vertical slab gel electrophoresis was carried out according to the procedures described by Davis (1964) and Ornstein (1964). Ten microlitres of the crude extract of enzyme were used for polyacrylamide gel electrophoresis. The following 11 enzyme systems were examined: asparate amino transferase (AAT; EC 2.6.1.1), glcose-6-phosphate isomerase (GPI; EC 5.3.1.9), glyceraldehyde-3-phosphate dehydrogenase (G3PDH; EC 1.2.1.12), leucine aminopeptidase (LAP; EC 3.4.11.1), malate dehydrogenase (MDH; EC 1.1.1.40), menadion reductase (MNR; EC 1.6.99.2), phosphoglucomutase (PGM; EC 5.4.2.2), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), shikimate dehydrogenase (SKDH; EC 1.1.1.25), superoxide dismutase (SOD; EC 1.15.1.1), and triose-phosphate isomerase (TPI; EC 5.3.1.1). Staining protocols followed those of Tsumura et al. (1990), except that for G3PDH, which followed Wendel and Weeden (1989).

Statistical analysis

Based on the results of allozyme electrophoresis, allele frequencies in each population of *T. matsumurae* and *T. tanakae* were calculated for the loci encoding the 11 enzyme systems. The following indices were used to quantify the amount of genetic diversity within each population examined: the proportion of polymorphic loci (P) at the 95% criterion, the number of alleles per locus (A), number of alleles per polymorphic loci (AP), and the expected heterozygosity (h). Genetic diversity parameters (P, A, AP and h) were also calculated at the species level. As in Harmick and Godt (1989), I treated the loci polymorphic in at least one population as polymorphic at the species level.

Genetic differentiation among populations of *T. matsumurae* and *T. tanakae* was estimated by gene diversity statistics corrected for errors due to small sample sizes (Nei and Chesser, 1983). The population genetic structure was analyzed by calculating: total genetic diversity (H_T), genetic diversity within populations (H_S), and proportion of the total diversity among populations (G_{ST}) for polimorphic loci.

Unbiased genetic identity ($\hat{I}$), and unbiased genetic distance ($\hat{}D$) (Nei, 1978) were calculated for each pair-wise comparison of all populations examined. A phenogram based on the unbiased genetic distance was obtained using the neighbor-joining method (Saitou and Nei, 1987) with PHYLIP version 3.5c (Felsenstein, 1993).

Mating system analysis

The follicles collected from the four populations, T9, T14, T15 and T18 (Table 6) of *T. tanakae* were dried at room temperature and seeds were extracted from them after dehiscence. Seeds were sown in petri dishes. Six seedlings per a follicle were used for mating system analysis. For the seedlings, 10-20 mg of a flesh leaf sample was homogenized in 150 μl of a same extract buffer used for mature plants and two isozyme loci, i.e. *Lap* and *Skdh*, were examined.

Selfing rates and their standard errors for *T. tanakae* were estimated from allozyme data using multi-locus estimators (Ritland and Jain, 1981), which were computed using the mating-system program developed by Ritland (1990).

RESULTS

Differentiation of anther

No difference was observed for the anther differentiation between *T. matsumurae* and *T. tanakae* until the buds at 1-2 mm in diameter. The pollinia of both species were enclosed with anther sac (Fig. 23A-D). In *T. tanakae*, abaxial side of the anther sacs began to reduce the buds at approximately 2.3 mm in diameter and completely shrink at approximately 3.2 mm in diameter (Fig. 23E). By contrast, in *T. matsumurae*, anther sacs did not fully shrunk even at opened flower (Fig. 23H).

Pollinia removal and insertion in natural populations

In *T. tanakae*, the pollinarium removal and insertion rate at T12 population were 43% and 12%, respectively, and the values were highest among seven populations. The number of removed or inserted pollinia was lowest at T11 in seven populations (Table 7).

In contrast, all pollinia were not removed from the anther sacs in *T. matsumurae*. *In situ* pollen tube germination in anther sacs are observed in all localities, and anther sacs of these flowers did not dehisce. These pollen tubes were entered into ovaries and reached to

ovules (Fig. 24).

Bagging experiments

Mature fruits were produced by 22.4 - 27.2% of bagged flowers in *V. matsumurae,* while in *T. tanakae,* no fruit were produced from bagged flowers (Table 8).

Genetic diversity

A total of 14 putative loci were scored: *Aat, G3pdh, Lap, Mdh, Mnr, Pgi-1, Pgi-2, Pgm-1, Pgm-2, 6pg, Skdh, Sod, Tpi-1,* and *Tpi-2* for both species. Although all populations of *T. matsumurae* were monomorphic in all of 14 loci, *T. tanakae* were polymorphic for five loci, i.e., *Aat-1, Lap, Mnr, Skdh* and*Tpi-2,* at least one population and the others were monomorphic.

Table 9 summarizes values of *P, A, AP* and *h* for each population and at species level of *T. matsumurae* and *T. tanakae.* At the population level, the mean values of *P, A, AP,* and *h* in *T. tanakae* were 16.7, 1.21, 2.28, and 0.074, respectively. At the species level, the values of *P, A, AP,* and *h* in *T. tanakae* were 42.0, 1.35, 2.71, and 0.126, respectively. In contrast, seven populations of *T. matsumurae* had no isozyme variation.

Population genetic structure and Population differentiation

The total genetic diversity (H_T) and the coefficient of gene differentiation (G_{ST}) overall for all populations of *T. tanakae* were 0.126 and 0.409, respectively (Table 10). The latter value indicates that 59% of the total genetic variation was shared in all 19 populations of *T. tanakae,* and the remaining 41% was partitioned for each population.

The mean values of $\hat{I}$ and $\hat{}D$ between *T. matsumurae* and *T. tanakae* were 0.86 (with range, 0.79-0.94), and 0.14 (with range, 0.06-0.23), respectively. The mean value of $\hat{I}$ and $\hat{}D$ among the populations of *T. tanakae* were 0.94 (with range 0.79-1.00) and 0.06 (with range 0-0.24), respectively. In most pair-wise $\hat{I}$ value between all populations of *T. tanakae,* the values were over 0.9, although genetic differentiation between the population of Minami-daito Island and those of the other islands were relatively large (0.78-0.87). The phenogram constructed using the neighbor-joining method (Saitou and Nei, 1987) based on Nei's (1978) unbiased genetic distance is shown Fig. 25. Due to complete lack of allozyme porimorphism in all populations of *T. matsumurae,* the relationships cannot be resolved among these populations.

Outcrossing rate of T. tanakae

The results of multilocus estimates of outcrossing rates and their standard errors (Ritland and Jain, 1981) for four populations of *T. tanakae* were shown in Table 11. The values of outcrossing rates of T14, T15, and T18 were 0.59, 0.36, 0.37, respectively, indicating that these populations had mixed mating system, whereas that of at the T9 population was 0.18, indicating that the population was highly self-pollinationg.

DISCUSSION

Reproductive characteristics of Tylophora matsumurae and T. tanakae

From the result of high percentage of fruit sets on bagged flowers in *T. matsumurae* suggested that this species exhibits autogamous self-pollination (Table 8). In Asclepiadeae, autogamy via *in situ* pollen tube germination has been reported on *Vincetoxicum nigrum*, *Tylophora hirsuta* and *Tylophora* sp. (Chaturvedi & Pant, 1986; Kuntz, 1991; Lumer & Yost, 1995). The autogamy of *T. matsumurae* is also occurred via *in situ* pollen tube germination, and this is because the morphology of stamen has a unique mechanism for autogamy. The anther sac of the species did not dehisce even in opened flower. Furthermore many pollen tubes germinated *in situ* anther sac and these pollen tubes reached ovules. This feature was also supported from the observations that only the corpusculums were removed from the flowers, and no pollinia were removed from anther sacs by insects in M1, M3, and M5 populations of *T. matsumurae*. These floral characters indicate that the flowers of *T. matsumurae* are extremely adapted for autogamy.

In contrast, four populations examined of *T. tanakae* showed wide variation in outcrossing rate from high selfing to mixed mating. Although self-fertilization of *T. matsumurae* are caused by autogamy, that of *T. tanakae* are probably caused by geitonogamy. Because no fruit was obtained on bagged flowers of *T. tanakae*, the fertilization of the species would depend on an insect visitation. The flowers of *T. tanakae* lack visible nectar and the small mosquitoes belonging to the family Sciaridae are observed as a pollinator of *T. tanakae* in the Okinawa Island (Chapter IV). The low frequency of pollinia removal and insertion in the fields suggests that the frequencies of the insect visitations are low. The evolutionary advantage of autogamy is fertility assurance when cross-fertilization is inadequate or unreliable (Baker, 1955; Allard, 1965). In *T. tanakae*, self-compatibility might have been

advantageous when the species colonize into new islands. Autogamy in *T. matsumurae* would be most advantageous for colonizing on their habitat, limestone rocks facing sea and exposing winds, because, on the habitat, it would have been difficult to receive a pollinator visitation.

Genetic diversity of *T. tanakae and origin of T. matsumurae*

At the species level, total allozyme genetic diversity in *T. tanakae* (H_T =0.126) is two times lower than that of the mean value of the compiled data of 52 endemic plant species (Hamrick and Godt, 1989). The values of H_T of endemic plants for the Ryukyu Archipelago have been reported between 0.134 and 0.232 (Hiramatsu et al., 1999; Maki, 1999; 2001; Maki et al., 2003) and that of *T. tanakae* was slightly lower than the those values. The mean allozymic diversity within populations of *T. tanakae* (H_S = 0.074) was comparable to the mean values previously recorded for insular endemic plants (Hamrick and Godt, 1989). However, the values of genetic differentiation (G_{ST} = 0.407) was found to be 1.6 times higher than the other endemic species (Hamrick and Godt, 1989). In general, the breeding system of flowering plant species greatly affects their G_{ST} values (Hamrick and Godt, 1989). The high G_{ST} value of *T. tanakae* indicates conspicuously limited gene flow between islands. The pollen mediated gene flow between islands seem to be unlikely, because main pollinator observed for *T. tanakae* is quite small mosquitoes such as Sciaridae. Thus, seed dispersal via wind may rarely cause gene flow between islands.

The values of non biased genetic identities and genetic distances indicates that population of *T. tanakae* are only slightly genetically differentiated excepting for T19 and T20 populations and no tendency between geographic structure were detected (Fig. 25). This result suggests that *T. tanakae* recently derived from its ancestral species and expanded its distribution, and that genetic drift works at each of islands.

On the other hand, a total of 169 individuals of *T. matsumurae* had no genetic variation at the 14 isozyme loci examined in this study (Table 9). In particular, Okinoerabu Island population of *T. tanakae* has all alleles seen in *T. matsumurae* and clustered with *T. matsumurae* in the phenogram constructed using the neighbor-joining method (Saitou and Nei, 1987) based on Nei's (1978) unbiased genetic distance (Fig. 25). Thus, *T. matsumurae* might have derived from the central Ryukyu Islands population of *T. tanakae* quite recently, and it has rapidly enlarged distribution. The autogamous nature of *T. matsumurae* has played a major role in this quick expansion.

Table 6. Population codes, locality, sample sizes (N) for examined populations of *Tylophora matsumurae* and *T. tanakae*.

Population code	Locality	N
T. matsumurae		
M1	Okinoerabu Isl.	40
M2	Yoron Isl.	24
M3	Okinawa Isl., Hedo Cape	13
M4	Okinawa Isl., Onna-1	24
M5	Okinawa Isl., Onna-2	26
M6	Okinawa Isl., Onna-3	17
M7	Okinawa Isl., Yonashiro	25
T. tanakae		
T1	Kagoshima, Sata Cape	20
T2	Yaku Isl., Isso	25
T3	Kuchinoshima Isl.	24
T4	Akuseki Isl.	15
T5	Takara Isl.	13
T6	Amami Isl., Kasari	19
T7	Amami Isl., Honohoshi	8
T8	Tokunoshima Isl. Mushiroze	19
T9	Okinoerabu Isl.	20
T10	Okinawa Isl. Ogimi	22
T11	Ie Isl.	9
T12	Okinawa Isl., Onnna	13
T13	Okinawa Isl., Naha	6
T14	Aka Isl.	20
T15	Kume Isl.	24
T16	Miyako Isl.	24
T17	Ishigaki Isl.	14
T18	Kuroshima Isl.	24
T19	Yonaguni Isl.	13
T20	Minamidaito Isl.	29

Table 7. Pollinarium removal, insertion and in situ pollen tube germination observed on 20 flowers of *T. matsumurae* and *T. tanakae*.

Species Population code	Number of pollinia removed (%)	Number of pollinia inserted (%)	Number of pollinia germinated in anther sacs (%)
T. matsumurae			
M1	4.0*	0.0	78.0
M3	6.0*	0.0	92.5
M4	0.0	0.0	91.0
M5	1.0*	1.0*	80.0
M6	0.0	0.0	90.0
T. tanakae			
T11	9.0	0.0	1.0
T12	43	12.0	0.0
T13	28	6.2	0.0
T14	28	1.0	0.0

*indicates number of corpusculums removed of inserted (%).

Table 8. Fruit sets of bagging treatment on flowers of *T. matsumurae* and *T. tanakae*

Species Population code	Number of flower bagged	Number of fruit sets	Fruit set per flower (%)	Number of individual
T. matsumurae				
M3	44	12	27.2	4
M4	153	35	22.9	5
M6	245	55	22.4	11
T. tanakae				
T9	72	0	0	5
T11	65	0	0	3
T13	61	0	0	2
T14	84	0	0	4

Table 9. Proportion of polymorphic loci (*P*), mean number of polymorphic loci (*A*), the number of alleles per polymorphic loci (*AP*), and gene diversity within a population (*h*) at 14 loci for examined populations of *Tylophora matsumurae* and *T. tanakae*.

Species Population code	*P*	*A*	*AP*	*h*
T. matsumurae				
M1	0.0	1.00	-	0.000
M2	0.0	1.00	-	0.000
M3	0.0	1.00	-	0.000
M4	0.0	1.00	-	0.000
M5	0.0	1.00	-	0.000
M6	0.0	1.00	-	0.000
M7	0.0	1.00	-	0.000
Mean of *T. matsumurae*	0.0	1.00	-	0.000
T. matsumurae at the species level	0.0	1.00	-	0.000
T. tanakae				
T1	14.2	1.21	2.50	0.066
T2	14.2	1.21	2.50	0.068
T3	0.00	1.06	2.00	0.005
T4	14.2	1.13	2.00	0.072
T5	7.10	1.06	2.00	0.037
T6	28.5	1.35	2.25	0.119
T7	28.5	1.35	2.25	0.126
T8	21.4	1.33	2.66	0.114
T9	21.4	1.35	2.66	0.116
T10	21.4	1.26	2.33	0.078
T11	14.2	1.13	2.00	0.063
T12	21.4	1.26	2.33	0.108
T13	7.10	1.06	2.00	0.012
T14	14.2	1.26	3.00	0.062
T15	14.2	1.26	3.00	0.088
T16	14.2	1.13	2.00	0.066
T17	28.5	1.28	2.00	0.096
T18	14.2	1.13	2.00	0.066
T19	28.5	1.35	2.25	0.107
T19	7.10	1.07	2.00	0.017
Mean of *T. tanakae*	16.7	1.21	2.28	0.074
T. tanakae at the species level	42.0	1.35	2.71	0.126

Table 10. Genetic diversity statistics based on Nei (1973) for polymorphic loci from the population examined of *T. matsumurae* and *T. tanakae*. H_S = genetic diversity within populations, H_T = total genetic diversity, and G_{ST} = proportion of the total diversity among populations.

Species	H_S	H_T	G_{ST}
T. matsumurae	0.000	0.000	-
T. tanakae	0.074	0.126	0.409

Table 11. Out crossing rate (t) and their standard dieviation (SD) for four populations of *T. tanakae* estimated from *Lap* and *Skdh*.

Population code	Number of family analyzed	Number of seedlings analyzed	t ± SD
T9	17	101	0.18 ± 0.05
T14	21	126	0.59 ± 0.11
T15	24	136	0.36 ± 0.05
T18	22	128	0.37 ± 0.09

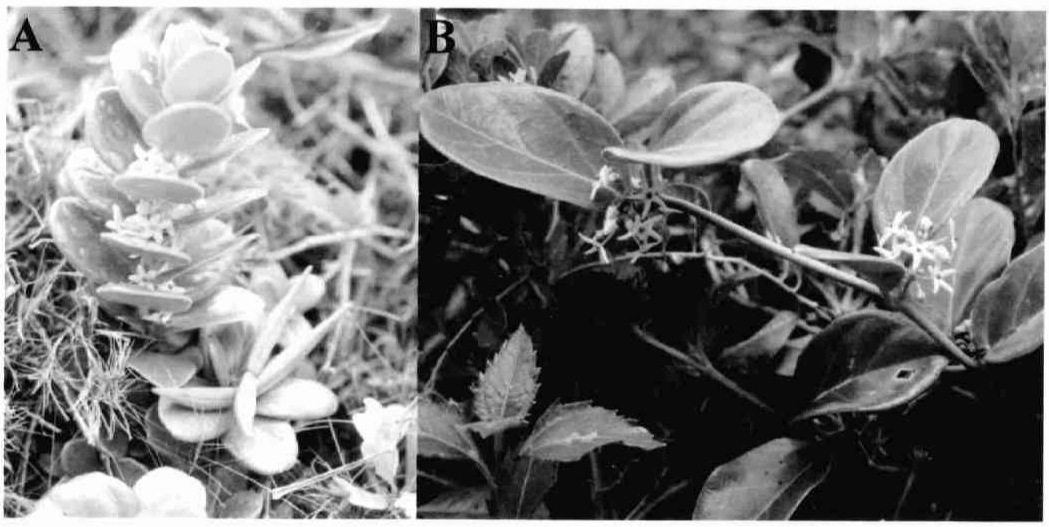

Fig. 21. Flowering individual of *Tylophora matsumurae* (A) and *T. tanakae* (B).

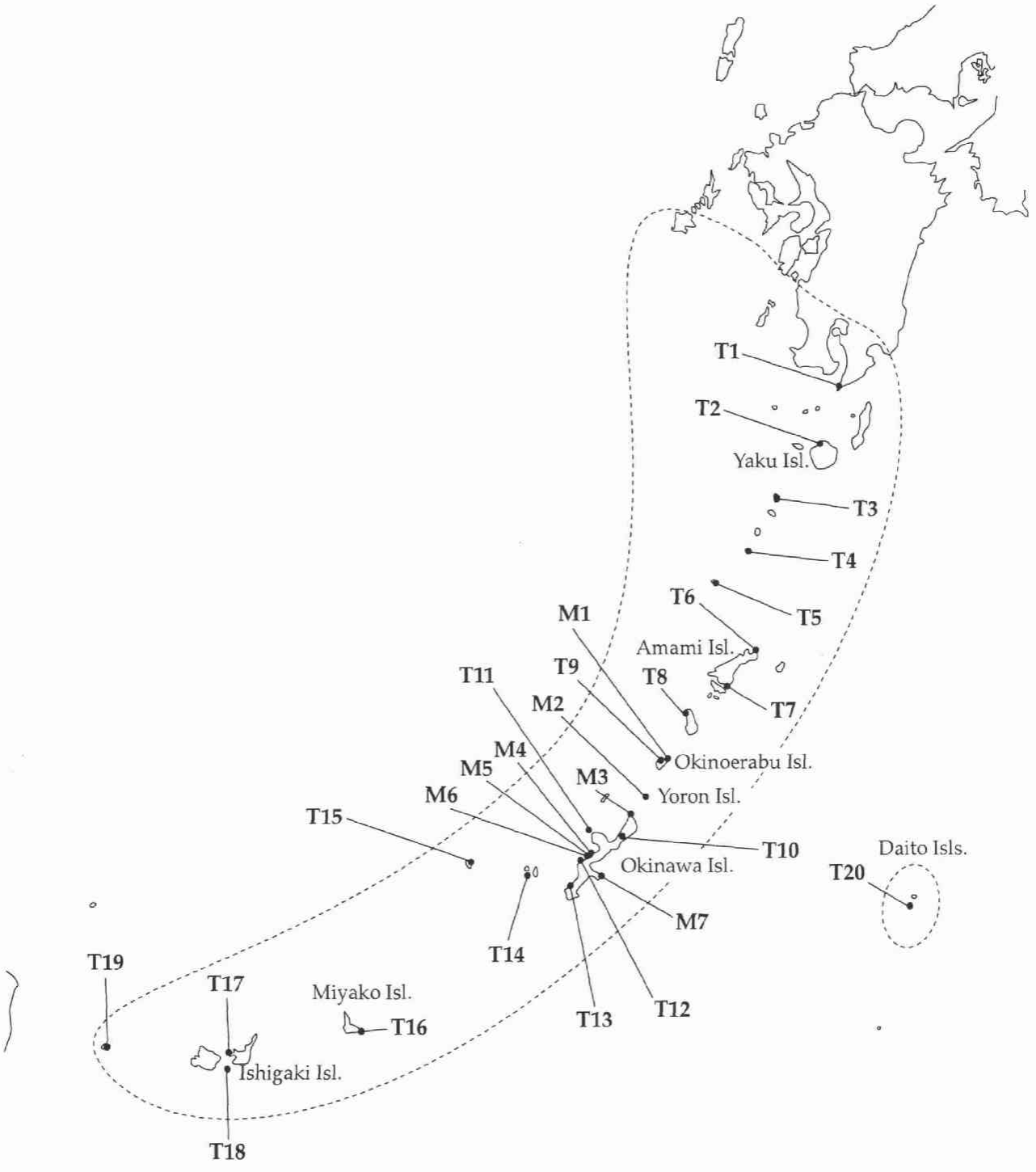

Fig. 22. Distribution of the populations examined. Population codes and localities are summarized in Table 1. Dashed lines indicate distribution area of *T. tanakae*.

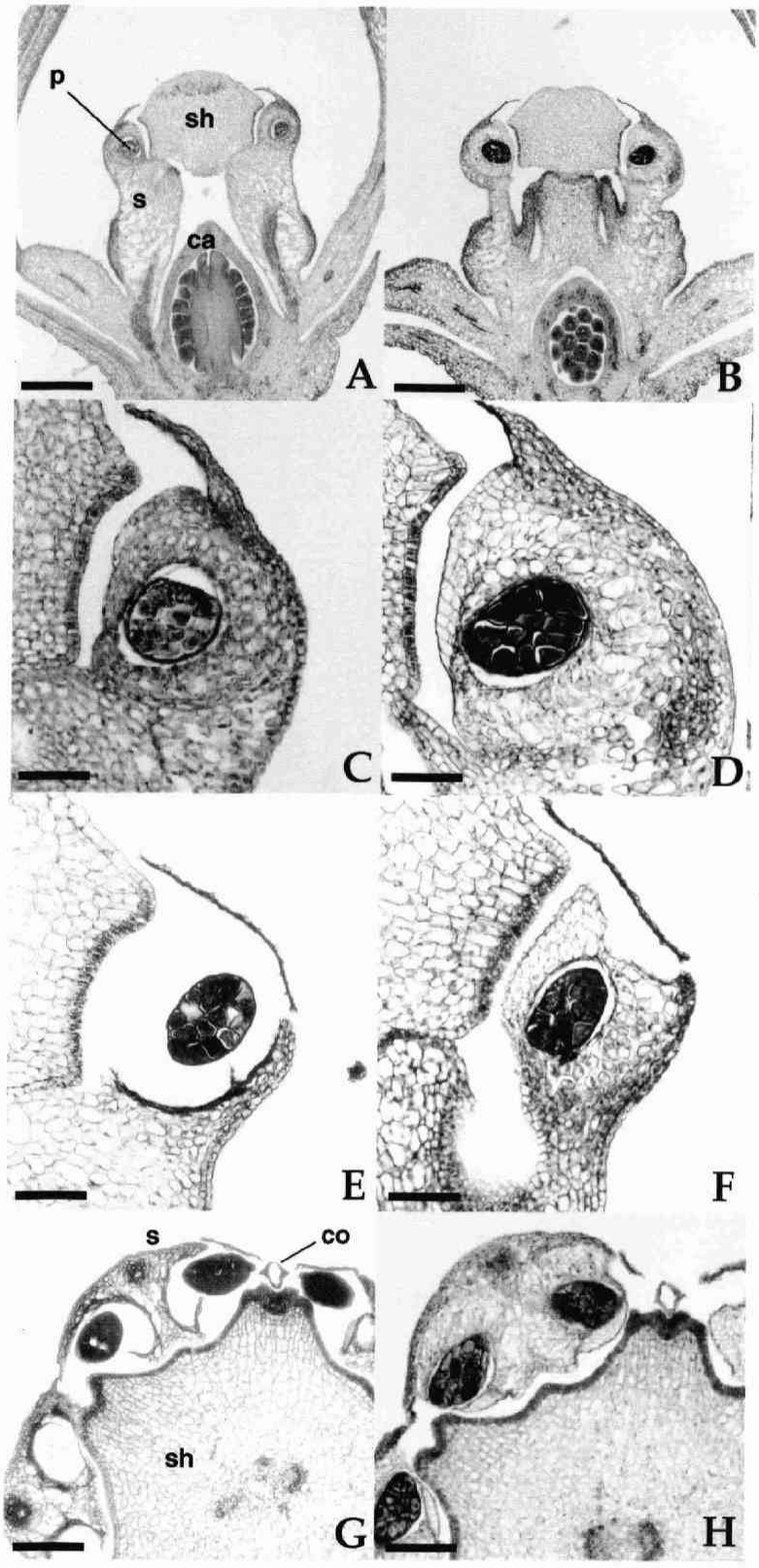

Fig. 23. Vertical (A-F) and transverse (G and H) sections showing anther development of *T. tanakae* (A, C, E and G) and *T. matsumurae* (B, D, F, H). Bars indicate 700μm for A, B, 140μm for C-D and 280μm for G, H. ca: carpel; co: corpusculum; p: pollinia; s: stamen; sh: style head.

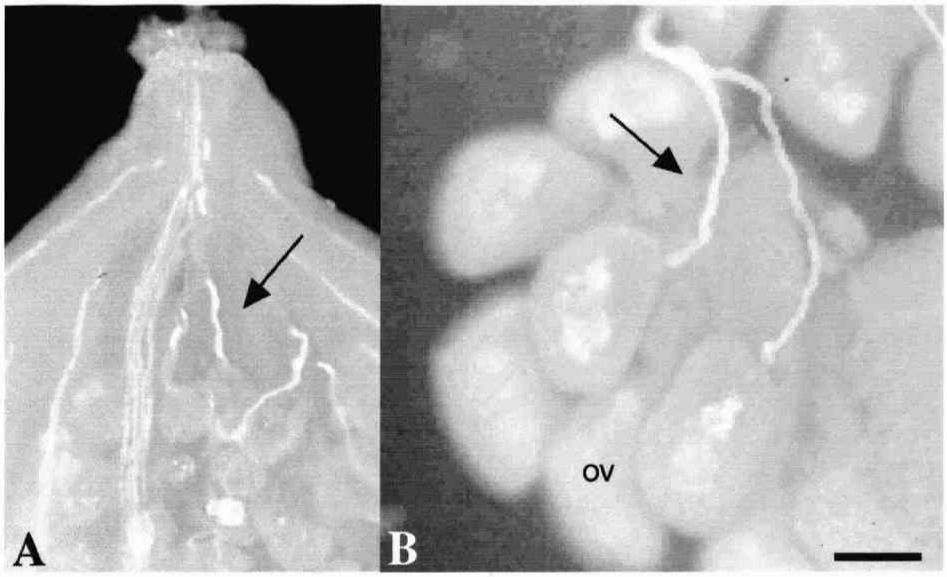

Fig. 24. Fluorescence micrographs of pistils of *T. matsumurae*. A. Pollen tubes grow through the ovary and reached ovules. B. Ovules are penetrated by pollen tubes. Arrows indicate pollen tubes. Bar indicates 80 μm for A and 40μm for B. ov: ovule.

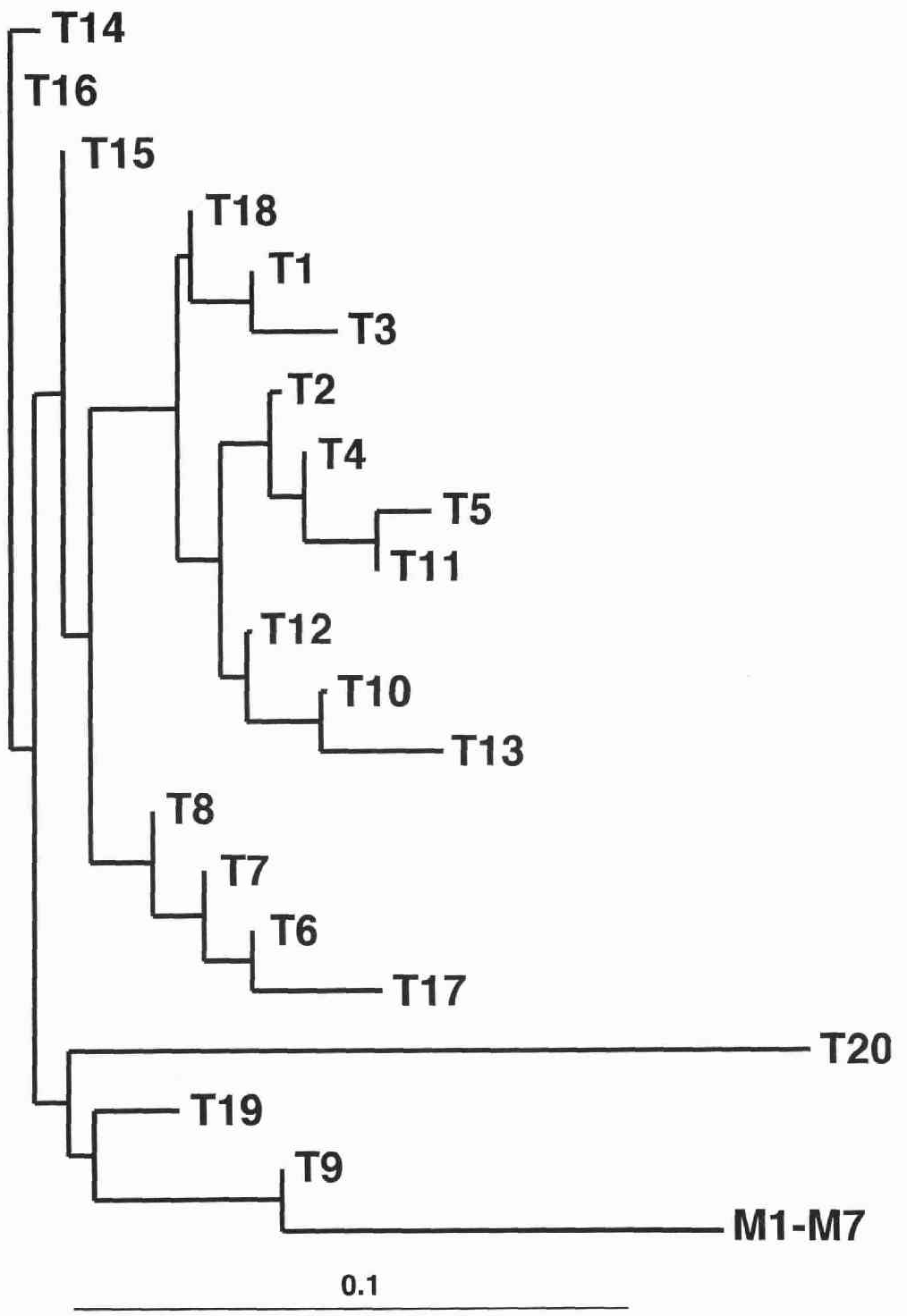

Fig. 25. Phenogram for seven population of *T. matsumurae* and twenty populations of *T. tanakae* using the neighbor-joining method on Nei's (1972) standard genetic distance.

CHAPTER VI. MOLECULAR PHYLOGENY OF *VINCETOXICUM* AND ITS ALLIED GENERA

The genus *Vincetoxicum* Wolf comprises approximately 100 species distributed in Asia, especially in mountain ranges. Most of the known species occur in China and Japan (Liede, 1996a). The morphological characteristics of this genus are as follows: erect or twining stems, fascicled roots, membranaceous or chartaceous leaves in its vegetative organs, a rotate corolla, and five fleshy staminal corona lobes completely separate or partly connected by much thinner interstaminal parts in its reproductive organs (Liede, 1996a).

There are two taxonomic interpretations of the relationships of *Vincetoxicum* and its allied genera have been conducted so far. In one, the genus *Vincetoxicum* was grouped with the closely related genus *Cynanchum* (e.g. Hooker, 1883; Tsiang and Li, 1977; Yamazaki, 1993; Gilbert et al., 1995). In the other, these two genera were considered to be distinct (e.g. Kitagawa, 1959; Markgarf, 1972; Qiu et al., 1989; Ohashi, 1990). The former interpretation was based on the continuity of corona structure between *Vincetoxicum* and *Cynanchum* s. s. (e.g. Hooker, 1883; Gilbert et al., 1995), in the latter, *Vincetoxicum* and *Cynanchum* were considered to be independent genera based on the characteristics of the corona, as well as on chemical substances such as alkanoids and aglycones (Qiu et al., 1989). Based on cladistic analysis of morphological and chemical characters, Liede (1996a) proposed an alternative hypothesis, namely, that *Vincetoxicum* is more similar to *Tylophora* than to *Cynanchum*.

Recent molecular phylogenetic studies have revealed new aspects of the relationships between *Vincetoxicum* and it close relatives. Based on *rbcL* sequence data, Civeyrel et al. (1998) indicated that *Vincetoxicum* and *Tylophora* comprise of a monophyletic group. More recently, Liede (2001) conducted a molecular phylogenetic analysis of the subtribe Astephaninae, including four species of *Vincetoxicum*, based on the sequences of the *trnL* (UAA) intron and two intergenic spacers, i.e., *trnT* (UGU)-*trnL* (UAA) and *trnL* (UAA)-*trnF* (GAA). This result indicated that the four species of *Vincetoxicum* formed a monophyletic group together with included *Tylophora indica*, and *Biondia henryi*. In addition it was shown that there is no close relationship between *Cynanchum* and *Vincetoxicum* (Liede, 2001). However, the monophyly of *Vincetoxicum* was unclear because the support value of this monophyletic group was so weak, and sampled species of *Vincetoxicum* were too few. Liede et al. (2002) conducted a further phylogentic analysis of the subtribe Tylophoriniae, including the same four species of *Vincetoxicum*, based on the above-mentioned regions of cpDNA and the ITS region of nrDNA, as in the previous study. Although monophyly of the subtribe Tylophoriniae was strongly supported, intra-subtribe relationships were poorly

resolved and the monophyly of *Vincetoxicum* still remains obscure (Liede et al., 2002).

In order to reconstruct a more reliable phylogeny of *Vincetoxicum*, it is necessary to use additional samples as well as more informative DNA regions. In my study, I attempted to solve this problem using two approaches. One approach was to employ the regions in which a high rate of nucleotide substitutions were observed, and the other was to combine multiple data sets to estimate phylogenetic relationships more precisely (e.g. Mort et al., 2002). Recently, some genomic regions have been reported to have sufficiently high nucleotide substitution rates to clarify the relationships among closely related species (Soltis and Soltis, 1998). Therefore, I employed a total of seven regions, two intergenic spacers, i.e., *trnL-trnF* and *psbA- trnH*, three introns, i.e., *trn L, trnG* and *atpF* in cpDNA, and internal and external transcribed spacers (ITS and ETS) in nrDNA, to reconstruct phylogenetic relationships of *Vincetoxicum*. The utility of non-coding cpDNA regions and the ITS region in nrDNA at the specific- and generic-level phylogeny has been demonstrated in many taxonomic groups (non-coding cp DNA regions: reviewed by Kelchner, 2000; ITS region: reviewed by Baldwin et al., 1995). The ETS region in nrDNA has been recently shown to be more phylogenetically informative than the ITS region (Baldwin and Markos, 1998). The substitution rate of the ETS region has been estimated to be approximately 1.4 times as fast as that of the ITS region (Baldwin and Markos, 1998). Therefore, the ETS region was expected to be useful in the study of infra-generic relationships, even when the ITS region lacks sufficient variation.

In this chapter, I address the following questions: i) Is *Vincetoxicum* a monophyletic group? ii) What are the phylogenetic relationships within *Vincetoxicum*? iii) Can evolutionary histories be reconstructed in *Vincetoxicum*? To address these questions, I determined the sequences of some regions in cpDNA (two intergenic spacers, i.e., *trnL-trnF* and *psbA-trnH* and three introns, i.e., *atpF, trnG* and *trnL*) and nrDNA (ITS and ETS regions) using samples not employed in previous studies, and analyzed them phylogenetically.

MATERIALS AND METHODS

Plant materials

Twenty-one samples of *Vincetoxicum* and eight samples of *Tylophora* were examined in this study. Three taxa of *Cynanchum* were selected as an outgroup following the results of the analyses of Liede (2001) and Liede et al. (2002). A total of 32 samples were examined and these have not been included in previous phylogenetic analysis except for *Vincetoxicum*

atratum (Table 12). Taxonomic treatments of the species examined were done according to Kitagawa (1959), Akasawa (1981), Ohashi (1990), Yamazaki (1993), and Chapter II in thesis study. Sources and voucher specimens of the materials are listed in Table 12.

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from 200 to 300 μg of fresh leaf tissues based on the procedure of Doyle and Doyle (1987). The isolated DNA was resuspended in 100 to 200 μL TE. Double-stranded DNA was amplified after incubation at 94°C for 2 min, by 30 cycles of incubation at 94°C for 1.5 min, 48°C for 2 min, and 60°C for 3 min, with a final extension at 72°C for 15 min. I amplified the *trnL* (UAA)-*trnF* (GAA) intergenic spacer and the *trnL* (UAA) intron with primers designed by Taberlet et al. (1991), the *psbA-trnH* intergenic spacer with primers designed by Sang et al. (1997), *trnG* (UCC) and *atpF* introns with primers designed by Nishizawa and Watano (2000), and two regions, i.e., ITS 1 and ITS 2, with primers designed by White et al. (1990). The ETS region was amplified using primers newly designed in this study, AsETS-F (5'-CAAGCATATGACTACTGGCA-3') and As ETS-R (5'-TCAGTTGTGGCTCAAGTG-3'). After amplification, reaction mixtures were subjected to electrophoresis in 1% low-melting-temperature agarose gel for purification of amplified products. I sequenced the purified PCR products using a DYEnamic ET-Terminator Cycle Sequencing Kit (Amersham Pharmacia) and a Model 373A automated sequencer (Applied BioSystems) following the manufacturer's instructions. For sequencing, I used the same primers as those used for amplification. All sequences have been deposited in DDBJ/EMBL/GenBank data bases (Table 13).

Data analysis

Sequences for each region were prealigned with ClustalW (Higgins et al., 1992) and then ambiguously aligned regions were manually corrected. Alignment for all DNA regions required the inclusion of the several indels. These indels were coded as binary (0 or 1) and treated as the fifth character.

The five cpDNA regions sequenced were combined into a single data set because the chloroplast genome is not subject to recombination. Separate phylogenetic analyses were conducted for the ITS, ETS and cpDNA data sets. The results of alignment for each region are available from the first author upon request.

Phylogenetic analysis and a test of clade support were conducted using a PAUP*

(version 4.0b10; Swofford, 2002). Maximum parsimony analyses were carried out through a heuristic search with TBR branch swapping and MULPARS option. Multiple islands of equally most parsimonious trees (Maddison, 1991) were searched for using the heuristic option with 100 random sequence additions. To estimate confidence levels of monophyletic groups, the bootstrap method with 1000 replications was employed (Felsenstein, 1985).

To examine constancy of evolutionary rate among each lineage of *Vincetoxicum* and *Tylophora*, a lineage relative-rate test (Wu and Li, 1985) was employed to assess the homogeneity of substitution rates. I conducted all pair-wise comparisons of *Vincetoxicum* and *Tylophora* using three *Cynanchum* species as reference taxa.

Further, to investigate the phylogenetic positions of the species examined in this study among other allied species, I conducted phylogenetic analysis including the sequences data of *trnL* intron, *trnL-trnF* intergenic spacer and the ITS region in Liede et al. (2002). The sequences data of *trnL* intron, *trnL-trnF* intergenic spacer and the ITS region of *Blyttia fruticulosum, Cynanchum ellipticum, Diplostigma canescens, Gomphocarpus physocarpus, Goydera somaliense,* two species of *Pentatropis, Pleurostelma cernuum, Schizostephanus alatus,* 18 species of *Tylophora* and four species of *Vincetoxicum* were obtained from DDBJ/EMBL/GenBank data bases. Phylogenetic analyses were conducted for two cpDNA, the ITS, and combined cpDNA and nrDNA data sets as same method as above. The results of alignment for each region are available from the first author upon request.

RESULTS

Variations among sequences in Vincetoxicum, Tylophora *and* Cynanchum

I determined sequences of five regions of cpDNA and three regions of nrDNA from nineteen taxa of *Vincetoxicum,* eight of *Tylophora,* and three of *Cynanchum.* The variations found among the sequences of all samples examined in the present study are summarized in Table 14.

In five regions in cpDNA, the length of the *atpF* intron was 188 base pairs (bp), except for the sequences of *Cynanchum boudieri* and *C. caudatum* (200 bp). I found one informative indel in this region from the result of multiple alignment. In the *trnG* intron, the length of all taxa examined varied from 161 to 164 bp. This variation was caused by the poly T tracts in this region. In the *trn L* intron, although the length was 481 bp in all samples of *Vincetoxicum* and *Tylophora,* that in *Cynanchum* varied from 475 to 477, and one informative indel was present in this region. In the *trnL-trnF* intergenic spacer, although all sequences

of *Vincetoxicum* were 306 bp long, the length of sequences of *Tylophora* and *Cynanchum* examined in this study varied from 297 to 306 bp. Two informative indels were present in this region. In the *psbA-trnH* intergenic spacer, the length of all sequences of *Cynanchum* varied from 450 to 457 bp, and the length in *Tylophora* and *Vincetoxicum* varied from 321 to 355 bp. From the result of multiple alignment of this region, there were relatively large indels in *Tylophora* and *Vincetoxicum*; *Tylophora japonica* had an 103 bp deletion, *T. floribunda* had an 114 bp deletion, and the other species of *Tylophora* and all species of *Vincetoxicum* had an 121 bp deletion compared with outgroup taxa *Cynanchum* spp. Moreover, ambiguously aligned regions from positions 76-87, 134-144, 380-412, and 424 -461 were found (Fig. 26).

In the ITS1 region in nrDNA, the length varied from 248 to 269 bp in all taxa examined. I found relatively many large indels in this region. Eight informative indels were found in this region and additional complicated indels from position 213 to 224 and from position 261 to 293 were as shown in Fig. 27. In the ITS 2 region, the length in all species of *Cynanchum* were 339 bp, and those in *Tylophora* and *Vincetoxicum* varied from 333 to 341 bp. Seven informative indels were found and ambiguously aligned regions from positions 113-118 and from positions 164-167 were as shown in Fig. 27. In the ETS region, the lengths of all examined taxa varied from 300 to 322 bp. Eight informative indels were found as was an ambiguously aligned region from position 222 to 236 shown in Fig. 27.

Phylogentic analysis

I used two data sets for phylogenetic analyses based on cpDNA sequences. First, the data set of 1410 characters from a combination of the five cpDNA regions, excluding all indels and ambiguously aligned regions, was analyzed. I obtained 10 most parsimonious trees of 68 steps with a consistency index (CI) of 0.870, excluding autapomorphies, and a retention index (RI) of 0.951. Second, I added the binary code for four informative indels found. Phylogentic analysis using 1414 characters also yielded 10 most parsimonious trees having 72 steps with a CI of 0.888, excluding autapomorphies, and an RI of 0.875. A strict consensus tree of these, taking the four informative indels into consideration, has the same topology as that obtained by the first analysis and is shown in Fig. 28A.

Tylophora japonica was as sister to all other species of *Tylophora* and *Vincetoxicum*, and which were separated into two clades, which I call Clade I and Clade II hereafter (Fig. 28A). Each of these two clades was supported by a relatively high bootstrap value (Clade I: 81 % and Clade II: 86 %). Clade I consisted of seven taxa of *Tylophora*, i.e., *T. aristolochioides*, *T. brownii*, *T. floribunda*, *T. matsumurae*, *T. ovata* var. *brownii*, *T. ovata* var. *ovata* and *T. tanakae* and

three taxa of *Vincetoxicum*, i.e., *V. magnificum*, *V. macrophyllum* and *V. inamoenum*. Clade I had two subclades; one consisted of *Tylophora brownii*, *T. ovata* var. *brownii*, *T. ovata* var. *ovata*, *T. tanakae* and *T. matsumurae* and was well supported by a high bootstrap value of 98%, and the other consisted of *Vincetoxicum magnificum* and *V. macrophyllum* and was also well supported by a bootstrap value of 95%. Clade II consisted of 16 taxa of *Vincetoxicum*, i.e., *V. katoi*, *V. yamanakae*, *V. yonakuniense*, *V. austrokiusianum*, *V. pycnostelma*, *V. ambiguum*, *V. nipponicum*, *V. sublanceolatum* var. *sublanceolatum*, *V. sublanceolatum* var. *macranthum*, *V. izuense*, *V. hoyoense*, *V. japonicum*, *V. acuminatum*, *V. calcareum*, *V. amplexicaule* and *V. atratum*.

I used two data sets for phylogenetic analyses based on the ITS region. First, a data set of 539 characters resulting from a combination of ITS 1 and ITS 2, excluding all indels and ambiguously aligned regions, was analyzed. I obtained one most parsimonious tree with 84 steps. The CI, excluding autapomorphies, was 0.849 and the RI was 0.952. Second, I added 15 informative indels and obtained seven equally parsimonious trees having 102 steps with a CI of 0.845, excluding autapomorphies, and an RI of 0.953. The strict consensus tree resulting from both analyses is shown in Fig. 28B. As in the cpDNA tree, *Tylophora japonica* was sister to all other taxa of *Tylophora* and *Vincetoxicum*, but this was not supported when indel information was included (Fig. 28B). Although the monophyly of Clade II was also supported by ITS analyses, Clade I in the cpDNA tree was not retrieved in the ITS tree when indels were both added and excluded (Fig. 28B).

I also used two data sets for the analyses based on ETS regions. First, an aligned data matrix with 311 characters of the ETS region, excluding all indels and ambiguously aligned regions, was used for the analysis. I obtained 201 most parsimonious trees having 72 steps with a CI of 0.884, excluding autapomorphies, and an RI of 0.966. Second, I added the eight informative indels. I obtained 145 equally parsimonious trees having 80 steps with a CI of 0.902, excluding autapomorphies, and an RI of 0.969. The strict consensus tree resulting from both analyses is shown in Fig. 28C. The strict consensus tree of the second analysis has almost the same topology as that of the first analysis except for the relationship of *V. magnificum* and *V. macrophyllum*-1 supported by a 2-bp insertion (Fig. 28C). As with the cpDNA and ITS regions, the monophyly of Clade II was also supported (Fig. 28C). However, the position of *Tylophora japonica* could not be determined, although the number of informative characters in the ETS region was very large (Table 14). Moreover, as with the ITS region, the monophyly of Clade I was also not supported (Fig. 28B).

Combined analysis of nrDNA and cpDNA data

To assess the congruence between the trees based on different data sets, the Mickevich–Farris incongruence index (IMF; Mickevich and Farris, 1981) was calculated for each data set. The length of the most parsimonious trees for the ITS, the ETS and the combined with data sets, excluding indels, were 84, 72 and 156, respectively, and those including indels were 102, 80 and 183, respectively. The IMF value of the former data set was 0 and that of the latter was 0.0055, indicating that 0.5% of this total character incongruence originated from combining the ITS and ETS data sets. Because of the low IMF value, I conducted phylogentic analysis using the combined data sets. The combined data of the ITS and ETS regions had 850 characters when indels were excluded. I obtained the four most parsimonious trees with a CI excluding autapomorphies of 0.856 and an RI of 0.955. The result of the analysis including indels yielded eight equally parsimonious trees with a CI, excluding autapomorphies, of 0.862 and an RI of 0.957. The strict consensus tree resulting from both analyses is shown in Fig. 29A. Although the positions of *T. japonica* and the clade consisting of *Tylophora brownii, T. ovata* var. *brownii, T. ovata* var. *ovata, T. tanakae* and *T. matsumurae* were not supported when indels were added, the sister position of *V. yonakuniense* in Clade II was supported by the 2bp insertion (Fig. 29A).

The lengths of the most parsimonious trees for the combined nrDNA, the five combined cpDNA regions, and the combined nrDNA and cpDNA, excluding indels, were 156, 68, and 229, respectively, and those including indels were 183, 72, and 258, respectively. The IMF value of the former data set was 0.0218 and that of the latter was 0.0116. As with the above-mentioned ITS+ETS tree, these values were also low. The combined data of nrDNA and cpDNA had 2260 characters when indels were excluded. I obtained 52 most parsimonious trees with a consistency index CI, excluding autapomorphies, of 0.837 and an RI of 0.945. The analysis including indels provided the results of 13 most parsimonious trees with a CI excluding autapomorphies of 0.852 and RI of 0.951. The strict consensus tree resulting from both analyses is shown in Fig. 29B. In both analyses, *Tylophora japonica* was located sister to all species of *Tylophora* and *Vincetoxicum*, which were split into two clades (Fig. 29B).

Although the evolutionary rates of the five taxa of *Tylophora*, i.e., *T. brownii, T. matsumurae, T. ovata* var. *brownii, T. ovata* var. *ovata* and *T. tanakae*, were estimated to be higher than those of the other samples in the relative rate test based on the cpDNA and nrDNA, there were no significant differences in evolutionary rates between Clade I and Clade II (data not shown).

Phylogenetic analysis of* Vincetoxicum *and its allied genera

In cpDNA, the aligned matrix of combined of *trnL* intron and *trnL-trnF* intergenic spacer comprised of 64 samples and 793 characters. In the matrix, four informative indels and eight ambiguously aligned poly-nucleotide repeats were found. The formers were binary coded and the latters were excluded from the matrix. Thus, the aligned matrix combined of *trnL* intron and *trnL-trnF* intergenic spacer included a total of 784 characters. From the result of phylogenetic analysis, I obtained 3194 most parsimonious trees of 85 steps with a consistency index (CI) of 0.704, excluding autapomorphies, and a retention index (RI) of 0.753.

In nrDNA, the aligned matrix of the ITS region comprised of 64 samples had 764 characters. In this region twenty-five ambiguously aligned regions were found and these were all excluded from the matrix. I used a total of 542 characters for the phylogenetic analysis. Parsimony analysis of the ITS region resulted in more than 28000 equally parsimonious trees of 366 steps with a consistency index (CI) of 0.496, excluding autapomorphies, and a retention index (RI) of 0.445.

The length of the most parsimonious trees for the cpDNA, the ITS and the combined data sets, were 85, 366 and 558, respectively. The IMF value of the data set was 0.19, indicating that 19% of this total character are incongruence originated from combining the cpDNA and ITS data sets. I used 1326 characters for the combined analysis of the cpDNA and ITS regions. Parsimony analysis of the combined cpDNA and the ITS region resulted in more than 28000 equally parsimonious trees with a consistency index (CI) of 0.391, excluding autapomorphies, and a retention index (RI) of 0.561. The strict consensus tree resulting from the analysis is shown in Fig. 30. The monophyly of nine taxa of *Tylophora*, i.e, *T. brownii*, *T. hirsuta*, *T. indica*, *T. matsumurae*, *T. ovata* var. *brownii*, *T. ovata* var. *ovata*, *T. tanakae*, *T. villosa* and *T. yunnanensis* was recognized (Fig. 30). For the other samples of *Tylophora* and *Vincetoxicum*, although several monophyletic groups were recognized in my analysis, these were almost identical with the result of Liede et al. (2002).

DISCUSSION

Comparisons of nucleotide substitutions in the five cpDNA regions, and in the ITS and ETS regions

A comparison of the number of informative sites in the five regions in cpDNA indicates that the *psbA-trnH* intergenic spacer has the most informative sites (Table 14). Although the

percentage of informative sites in cpDNA indicated that the *trnG* intron has a higher value than the others, the length of this region was very short (Table 14). Therefore, the *psbA-trnH* intergenic spacer appears to be the most informative among the regions examined in my study. Other studies have also concluded that the *psbA-trnH* intergenic spacer is one of the most useful regions in cpDNA (e.g. Chanderbali et al., 2001; Utelli et al., 2000).

Although the percentage of phylogenetically informative sites among three regions in nrDNA was similar, the ETS region was the most informative of the three (Table 14). In my result, however, the ETS region also contained more autapomorphic characters than the ITS region. Although the ITS region has been frequently used in nrDNA (Baldwin et al., 1995), my result indicates that the ETS region may be more useful than the ITS region in phylogenetic analyses as pointed out by Baldwin and Markos (1998) and Clevinger and Panero (2000).

Re-examination of the taxonomic treatment of Vincetoxicum

All phylogenetic trees indicated that sixteen taxa of *Vincetoxicum* form a cluster (Clade II), strongly suggesting that Clade II is monophyletic (Fig. 28, 29). In contrast, four taxa of this genus and some taxa of *Tylophora* constituted a monophyletic group in cpDNA (Fig. 28A) but comprised a paraphyletic assemblage in nrDNA (Fig. 28B, C). *Vincetoxicum* is morphologically distinguished from *Tylophora* based on corona morphology and orientation of pollinia (Li et al., 1995). While the corona lobes of *Vincetoxicum* are inserted at the base of gynostegium and its pollinia are in a pendulous position, corona lobes of *Tylophora* are inserted on the backs of anthers, and the position of its pollinia ranges from horizontal to erect (Li et al., 1995). However, the delimitation of these two genera based on the differences of pollinia orientation and corona structure was not supported by my results. My results support the suggestion of Liede et al. (2002) that *Vincetoxicum* and *Tylophora* be lumped together.

Phylogenetic position of East Asian Vincetoxicum *and its allied genera*

In the result of phylogentic re-analysis including the sequences of Liede et al. (2002), the tree topologies were almost identical with their result, except for the position of *T. apiculata*. Three African *Tylophora* clades and two Asian *Tylophora* clades were also recognized. At least, two lineages with low support are recognized in *Vincetoxicum*. One is five *Vincetoxicum* species and these were consisted of monophyletic clade with eleven

Asian taxa of *Tylophora* (Fig. 30: Clade A). The Clade I recognized from my analysis (Fig. 28) was included in this clade. The other is remaining sixteen species of *Vincetoxicum* and most of them might have been diversified in Asia. Although the monophyly of fifteen East Asian *Vincetoxicum* species were strongly supported from my cpDNA, nrDNA and combined analysis (Fig. 28, 29), I could not solve the relationships between other genera in this additional analysis (Fig. 30: Clade B). This was due to insufficient variations in *trnL* intron and *trnL-F* intergenic spacer and many ambiguously aligned regions in ITS. To elucidate phylogenetic positions of remaining sixteen species of *Vincetoxicum*, further phylogenetic information is needed.

Liede et al. (2002) concluded that the most of these monophyletic units are composed largely of species of close geographic distribution and these geographic groups very rapidly evolved from a common, possibly widely distributed, ancestor. My result also supports their conclusion for the evolution of *Tylophora-Vincetoxicum* complex, because monophyly of the fifteen *Vincetoxicum* species were strongly suggested from my results.

Evolutionary histories of Vincetoxicum

My phylogenetic results showed a division of seven taxa of *Tylophora* and nineteen taxa of *Vincetoxicum* into two groups, i.e. Clade I and Clade II. The species in Clade II are morphologically diverse and easily distinguished from each other by morphological characters such as flower color, leaf shape, corona shape and length of inflorescence (Yamazaki, 1993; Chapter IV). However, the intra-clade genetic differentiation of Clade II was small, whereas relatively many nucleotide substitutions were observed in Clade I. The following two causes are possible for this discrepancy. One is that after the ancestral species of Clade II diverged from their close relatives, the lineage may have been undergone a rapid radiation. The other is that a slowdown in the accumulation of mutations occurred in the lineage leading to these species (Hodges and Arnold, 1994). As mentioned above, however, there was no significant difference in evolutionary rates between Clade I and Clade II in the relative rate test based on both cpDNA and nrDNA, except for five taxa of *Tylophora*, i.e., *T. brownii*, *T. matsumurae*, *T. ovata* var. *brownii*, *T. ovata* var. *ovata*, and *T. tanakae*. Therefore, the mutation slowdown hypothesis is not a valid argument in this lineage.

Most examples of rapid radiation in plant groups have been reported on oceanic islands such as the Hawaii Islands or the Canary Islands (e.g. Baldwin, 1997; Okada et al., 1997; Francisco-Ortega et al., 1997). However, there are a few reports of rapid radiation for continental plant species (Hodges and Arnold, 1994; Utelli et al., 2000; Soliva et al., 2001;

Malcomber, 2002). In the case of plants on oceanic islands, rapid radiation was hypothesized to have been driven by low levels of competition in new habitats (Liem, 1990). The competition based hypothesis can also apply to explain the rapid radiation in continental plants in Europe and North America which expanded rapidly after glacial era (Hewitt, 2000). The most species consist of CladeII, however, might have been diversified in south to middle China and Japan and these area is thought to have not covered by Ice sheets (Kamei and Research Group for the Biogeography from Würm Glacial, 1988). Thus it is difficult to apply competition based hypothesis for these plants. The other hypothesis is the concept of key innovations that has been employed to explain the rapid radiation of a lineage (Liem, 1990; Hodges and Arnold, 1994; Malcomber, 2002). Although most species in Clade I have tiny flowers, the species of Clade II have larger and more nectariferous ones. Moreover, pollinators in Clade I were considerably different from those of Clade II; whereas the species in Clade I were pollinated by small diptera such as Empidae and Sciaridae, those of Clade II were pollinated by various insects such as Diptera, Hymenoptera and Lepidoptera (Chapter IV). These observations suggest that the interactions of pollinators and the plants may have accelerated morphological radiation in the species of Clade II. A similar situation was reported for *Aquilegia* (Ranunculaceae), in which rapid radiation was found to have been driven by the evolution of a nectar spur, which enabled a lineage to attract specific pollinators and promoted reproductive isolation (Hodges and Arnold, 1994, 1995).

Table 12. List of taxa and sources of plant materials. Herbarium acronyms follow Index Herbariorum part I (Holmgren *et al.* 1990).

Taxon	Localities	Collectors, fild no. & vouchers*	
Cynanchum boudieri H. Lév. & Vaniot	Japan: Kagoshima Pref., Amami Isl.	Y. T. & T. Y. 45546	URO
C. caudatum (Miq.) Maxim.	Japan: Miyagi Pref., Kakuda	T. Y. & A. Y. 7578	TUS
C. wilfordii (Maxim.) Hemsl.	Japan: Miyagi Pref., Ogatsu	T. Y. & A. Y. 7228	TUS
Tylophora aristolochioides Miq.	Japan: Miyagi Pref., Kakuda	T. Y. & A. Y. 7580	TUS
T. brownii Hayata	Taiwan: Ludao Isl.	S. M. s. n.	URO
T. floribunda Miq.	Japan: Aichi Pref., Shinshiro	T. Y. 3937	URO
T. japonica Miq.	Japan: Okinawa Pref., Nakijin	T. Y. 3737	URO
T. matsumurae (T. Yamaz.) T. Yamash. & Y. Tateishi	Japan: Okinawa Pref., Onna	Y. T. & T. Y. 45144	URO
T. ovata (Lindl.) Hook. ex Steud.			
var. *brownii* (Hayata) Tsiang & P. T. Li	Taiwan: Taipei Co.	T. Y. 4092	URO
var. *ovata*	China: Hong Kong.	T. Y. 4118	TUS
T. tanakae Maxim.	Japan: Okinawa Pref., Itoman	T. Y. 4003	URO
Vincetoxicum acuminatum Decne.	Japan: Gunma Pref., Mts. Haruna-san	T. Y. & A. Y. 7469	TUS
V. ambiguum Maxim.	Japan: Miyazaki Pref., Miyazaki	T. Y. & A. Y. 7320	TUS
V. amplexicaule Sieb. & Zucc.	Japan: Miyazaki Pref., Takaoka	T. Y. & A. Y. 7234	TUS
V. atratum (Bunge) Morr. & Decne.	Japan: Nagasaki Pref., Hirado	T. Y. & A. Y. 7453	TUS
V. austrokiusianum (Koidz.) Kitag.	Japan: Miyazaki Pref., Toi Cape	T. Y. & A. Y. 7786	TUS
V. calcareum (H. Ohashi) Akasawa	Japan: Kochi Pref., Mt. Ishidate-yama	T. Y. & A. Y. 7199	TUS
V. inamoenum Maxim.	Japan: Hokkaido Pref., Bihoro	T. Y. 8227	TUS
V. izuense T. Yamash.	Japan: Shizuoka Pref., Shimoda	T. Y. & A. Y. 7435	TUS
V. hoyoense T. Yamash.	Japan: Ohita Pref., Tsurumi Cape	T. Y. & A. Y. 7447	TUS
V. japonicum Morr. & Decne.	Japan: Aichi Pref., Irago Cape	T. Y. 3853	URO
V. katoi (Ohwi) Kitag.	Japan: Shizuoka Pref., Fukuroi	T. Y. & A. Y. 7181	TUS
V. macrophyllum Sieb. & Zucc.-1	Japan: Nagasaki Pref., Mts. Tara-dake	T. Y. & A. Y. 7457	TUS
V. macrophyllum Sieb. & Zucc.-2	Japan: Tochigi Pref., Nikko	T. Y. & A. Y. 7216	TUS
V. magnificum (Naki) Kitag.	Japan: Ibaragi Pref., Kitaibaragi	T. Y. & A. Y. 8239	TUS
V. nipponicum (Matsum.) Kitag.-1	Japan: Fukushima Pref., Shirakawa	T. Y. & A. Y. 7571	TUS
V. nipponicum (Matsum.) Kitag.-2	Japan: Hyogo Pref., Yashiro	T. Y. & A. Y. 7458	TUS
V. pycnostelma Kitag.	Japan: Aichi Pref., Shinshiro	T. Y. 3911	URO
V. sublanceolatum (Miq.) Maxim.			
var. *sublanceolatum*	Japan: Aichi Pref., Shinshiro	T. Y. 4183	URO
var. *macranthum* Maxim.	Japan: Miyagi Pref., Mt. Izumigatake.	T. Y. & A. Y. 7536	TUS
V. yamanakae (Ohwi & H. Ohashi) H.Ohashi	Japan: Kochi Pref., Kagami	T. Y. & A. Y. 7189	TUS

Table 12. continued

V. yonakuniense (Hats.) T. Yamash. & Y. Tateishi	Japan: Okinawa Pref., Yonaguni Isl.	T. Y. & S. U. 4188	URO

*: S. M.; Matsumura, S., S. U.; Ujiie, S., T. Y.; Yamashiro, T., Y. A.; Yamashiro, A.; Y. T.; Tateishi, Y.

Table 13. List of GenBank accession numbers of nucleotide sequences for the taxa examined.

Taxon	*atpF*	*trnG*	*trnL* intron	*trnL-trnF*	*psbA-trnH*	ITS	ETS
Cynanchum boudieri H. Lév. & Vaniot	AB109044	AB109075	AB109166	AB109941	AB109134	AB109972	AB110035
C. caudatum (Miq.) Maxim.	AB109045	AB109076	AB109910	AB109942	AB109135	AB109973	AB110036
C. wilfordii (Maxim.) Hemsl.	AB109046	AB109077	AB109911	AB109943	AB109136	AB109974	AB110037
Tylophora aristolochioides Miq.	AB109047	AB109078	AB109912	AB109944	AB109137	AB109975	AB110038
T. brownii	AB109048	AB109079	AB109913	AB109945	AB109138	AB109976	AB110039
T. floribunda Miq.	AB109049	AB109080	AB109914	AB109946	AB109139	AB109977	AB110040
T. japonica Miq.	AB109050	AB109081	AB109915	AB109947	AB109140	AB109978	AB110041
T. matsumurae (T. Yamaz.) T. Yamash. & Y. Tateishi	AB109051	AB109082	AB109916	AB109948	AB109143	AB109979	AB110042
T. ovata (Lindl.) Hook. ex Steud.							
var. *brownii* (Hayata) Tsiang & P. T. Li	AB109052	AB109083	AB109917	AB109949	AB109141	AB109980	AB110043
var. *ovata*	AB109053	AB109084	AB109918	AB109950	AB109142	AB109981	AB110044
T. tanakae Maxim.	AB109054	AB109085	AB109919	AB109951	AB109144	AB109982	AB110045
Vincetoxicum acuminatum Decne.	AB109055	AB109086	AB109924	AB110215	AB109145	AB109983	AB110046
V. ambiguum Maxim.	AB109056	AB109087	AB109928	AB109956	AB109146	AB109984	AB110047
V. amplexicaule Sieb. & Zucc.	AB109057	AB109088	AB109925	AB109957	AB109147	AB109985	AB110048
V. atratum (Bunge) Morr. & Decne.	AB109058	AB109089	AB109926	AB109958	AB109148	AB109986	AB110049
V. austrokiusianum (Koidz.) Kitag.	AB109059	AB109117	AB109927	AB109959	AB109149	AB109987	AB110050
V. calcareum (Ohashi) Akasawa	AB109060	AB109118	AB109929	AB109960	AB109150	AB109988	AB110051
V. inamoenum Maxim.	AB110067	AB109133	AB109920	AB109952	AB109165	AB109989	AB110066

Table 13 continued

V. izuense T. Yamash.	AB109073	AB109131	AB109939	AB109970	AB109163	AB110002	AB110064
V. hoyoense T. Yamash.	AB109074	AB109132	AB109940	AB109971	AB109164	AB110003	AB110065
V. japonicum Morr. et Decne.	AB109061	AB109119	AB109930	AB109961	AB109151	AB109990	AB110052
V. katoi (Ohwi) Kitag.	AB109062	AB109120	AB109931	AB109962	AB109152	AB109991	AB110053
V. macrophyllum Sieb. & Zucc. - 1	AB109063	AB109121	AB109921	AB109953	AB109154	AB109992	AB110054
V. macrophyllum Sieb. & Zucc. - 2	AB109064	AB109122	AB109922	AB109954	AB109155	AB109993	AB110055
V. magnificum (Naki) Kitag.	AB109065	AB109123	AB109923	AB109955	AB109156	AB109994	AB110056
V. nipponicum (Matsum.) Kitag. -1	AB109066	AB109124	AB109932	AB109963	AB109157	AB109995	AB110057
V. nipponicum (Matsum.) Kitag. -2	AB109067	AB109125	AB109933	AB109964	AB109158	AB109996	AB110058
V. pycnostelma Kitag.	AB109068	AB109126	AB109934	AB109965	AB109161	AB109997	AB110059
V. sublanceolatum (Miq.) Maxim.							
var. *sublanceolatum*	AB109069	AB109127	AB109935	AB109966	AB109159	AB109998	AB110060
var. *macranthum* Maxim.	AB109070	AB109128	AB109936	AB109967	AB109160	AB109999	AB110061
V. yamanakae (Ohwi & H. Ohashi) H.Ohashi	AB109071	AB109129	AB109937	AB109968	AB109153	AB110000	AB110062
V. yonakuniense (Hatus.) T. Yamash. & Y. Tateishi	AB109072	AB109130	AB109938	AB109969	AB109162	AB110001	AB110063

Table 14. Comparison of phylogenetic information from variable sites for three introns, i.e., *atpF*, *trnG* and *trnL*, two intergenic spacers, i.e., *trnL* - *trnF* and *psbA* - *trnH* in cpDNA, and ITS and ETS in nrDNA.

Regions	Length (bp)	Number of variable sites	Number of informative sites	Percentage of informative sites	Number of informative indels
atpF intron	188 - 200	4	2	50.0	1
trnG intron	161 - 163	8	6	75.0	0
trnL intron	475 - 481	8	5	62.5	1
trnL-trnF	267 – 307	15	8	53.3	2
psbA -trnH	322 - 457	25	17	68.0	0
ITS1	248 - 269	36	22	61.1	8
ITS2	334 - 341	38	23	60.5	7
ETS	314 - 322	51	30	58.8	8

Samples	7 6	8 7	1 3 4	1 4 4	3 8 0	4 1 2	4 2 4	4 6 2
Cynanchum boudieri	GAAAATAAA	--	CTTTATTTT	--	TAAAAATAAA	----------	TTAATAAAT	TAAATATAAAAATAAATAGAAATAGAAATAGAGCCA-G
C. caudatum	GAAAATAAA	--	CTTTATTTT	--	TGAAAATAA	----------	TTTAATAAAT	TAAATAG------ATATAGAAATAGAAATAGAGCCA-G
C. wilfordii	GAAAATAAA	--	CTTTATTTT	--	TAAAAATAA	----------	TTTAATAAAT	TAAATAG------AAATAGAAATA------GAGCCA-G
Tylophora japonica	GAAAAAAAAAA		CTTTTTTTTTT		TAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
T. floribunda	GAAAAAAAAA	-	CTTTTTTT	---	TAAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
T. aristolochioides	GAAAAAAAAA	-	CTTTTTTTT	--	TAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAA---------- GAGCCA-G
T. ovata var. *bwownii*	GAAAAAAAAA	-	CTTTTTTTT	--	TCAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
T. ovata var. *ovata*	GAAAAAAAA	--	CTTTTTTTTT	-	TCAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
T. tanakae	GAAAAAAAAA	-	CTTTTTTTT	--	TCAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
T. matsumurae	GAAAAAAAAA	-	CTTTTTTTT	--	TCAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
T. brownii	GAAAAAAAAA	-	CTTTTTTTT	--	TCAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
Vincetoxicum magnificum	GAAAAAAAAA	-	CTTTTTTTT	--	TAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. macrophyllum-1	GAAAAAAAAA	-	CTTTTTTTT	--	TAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. macrophyllum-2	GAAAAAAAAA	-	CTTTTTTTT	--	TAAAAAAAA	----------	T	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. inamoenum	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAAAAGGAAAAAAAA	-	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. katoi	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAAA	----------	TTAATAAAT	------GAAA--TAAA---------- GATACAAG
V. yamanakae	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAAA	----------	TTAATAAAT	------GAAA--TAAA---------- GATACAAG
V. yonakuniense	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. austrokiusianum	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. pycnostelma	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. ambiguun	GAAAAAAAA	--	CTTTTTTTTT	-	TAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. nipponicum-1	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. nipponicum-2	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. sublanceolatum var. *sublanceolatum*	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. sublanceolatum var. *macranthum*	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. izuense	GAAAAAAA	---	CTTTTTTTT	--	TAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. hoyoense	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. japonicum	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. calcareum	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. acuminatum	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. amplexicaule	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G
V. atratum	GAAAAAAAA	--	CTTTTTTTT	--	TAAAAAAA	----------	TTAATAAAT	TAAATAGAAAAATAAATAGAAATA------GAGCCA-G

Fig. 26. Ambiguously aligned regions on intergenic spacer of *psbA-trnH* in cpDNA. The numbers indicate the relative positions determined from the result of multiple alignments.

Samples	ITS1				ITS2				ETS	
	2 1 3	2 3 6	2 6 1	2 9 6	1 1 3	1 8	1 6 4	1 6 7	2 2 2	2 3 6
Cynuncum boudieri	GTATCCTC	----GCCGCGGGGATT	CAATCTT	----------ATACG	-------	ACGA	A-CCCC	G---	CCCAA-GCATGC	---A
C. caudatum	GTATCCTC	----GCCGCGGGGATT	CAATCTT	----------ATACG	-------	ACGA	A-CCCC	G---	CCCGA-GCATGC	---A
C. wilfordii	GTATCCTC	----GCCGCGGGGATT	CAATCTT	----------ATACG	-------	ACGA	T-CCCC	G---	CCCAA-GCATGC	---A
Tylophora japonica	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--------	TATACA	-----	GTACGA	-----C	G---	CCCGA-GCGTGC	---A
T. floribunda	--ATCCTCGCATGCCGCGGGGATT	-------	GTTG	--------	TACACG	-----	GTACGA	-----C	G---	CCCGA-GCATGC	---A
T. aristolochioides	--AT	--------	GCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	-----C	G---	CCCGA-GCATGC	---A
T. ovata var. *brownii*	----------	GCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	ACCCCC	GG--	CCCGACGCATGC	---A
T. ovata var. *ovata*	----------	GCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	ACCCCC	GG--	CCCGACGCATGCAGCA	
T. tanakae	----------	GCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	ACCCCC	GG--	CCCGACGCATGC	---A
T. matsumurae	----------	GCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	ACCCCC	GGG-	CCCGACGCATGC	---A
T. brownii	----------	GCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	ACCCCC	GG--	CCCGACGCATGC	---A
Vincetoxicum magnificum	--ATCCTCGCATGCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	-----C	G---	CCCGA-GCATGC	---A
V. macrophyllum-1	--ATCCTCGCATGCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	-----C	G---	CCCGA-GCATGC	---A
V. macrophyllum-2	--ATCCTCGCATGCCGCGGGGATT	-------	GTTG	--------	TACACA	-----	GTACGA	-----C	G---	CCCGA-GCATGC	---A
V. inamoenum	--ATCCTCGCATGCCGCGGGGATT	-------	GTTGTG	------	TACACA	-----	GTACGA	-----C	G---	C	----------	A
V. katoi	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	-------	GTACGA	----CC	GGG-	CCCGA-GCATGC	---A
V. yamanakae	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	-------	GTACGA	----CC	GGG-	CCCGA-GCATGC	---A
V. yonakuniense	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	-------	GTACGA	-----C	GG--	CCCGA-GCATGC	---A
V. austrokiusianum	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	--ATACAGTACGA		-----G	GG--	CCCGA-GCATGC	---A
V. pycnostelma	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	-------	GTACGA	-----C	GG--	CCCGA-GCATGC	---A
V. ambiguum	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAAATACA	-------	GTACGA	-----C	GG--	CCCGA-GCATGC	---A
V. nipponicum-1	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	--ATACAGTACGA		-----C	GG--	CCCGA-GCATGC	---A
V. nipponicum-2	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAAATACA	-------	GTACGA	-----C	GG--	CCCGA-GCATGC	---A
V. sublanceolatum var. *sublanceolatum*	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	--ATACAGTACGA		-----C	GG--	CCCGA-GCATGC	---A
V. sublanceolatum var. *macranthum*	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAAATACA	-------	GTACGA	-----C	GGG-	CCCGA-GCATGC	---A
V. izuense	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAAATACA	--ATACAGTACGA		-----C	GG--	CCCGA-GCATGC	---A
V. hoyoense	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	--ATACAGTACGA		-----C	GG--	CCCGA-GCATGC	---A
V. japonica	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAAATACA	--ATACAGTACGA		-----C	GG--	CCCGA-GCATGC	---A
V. calcareum	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	--ATACAGTACGA		-----C	GG--	CCCGA-GCATGC	---A
V. acuminatum	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	-------	GTACGA	---CCC	GGGG	CCCGA-GCATGC	---A
V. amplexicaule	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	-------	GTACGA	----CC	GG--	CCCGA-GCATGC	---A
V. atratum	--ATCCTC	----GCCGCGGGGATT	-------	GTTG	--TATAA-TACA	-------	GTACGA	-----C	GG--	CCCGA-GCATGC	---A

Fig. 27. Ambiguously aligned regions in ITS1, ITS2 and ETS region in nrDNA. The numbers indicate the relative positions determined from the result of multiple alignments.

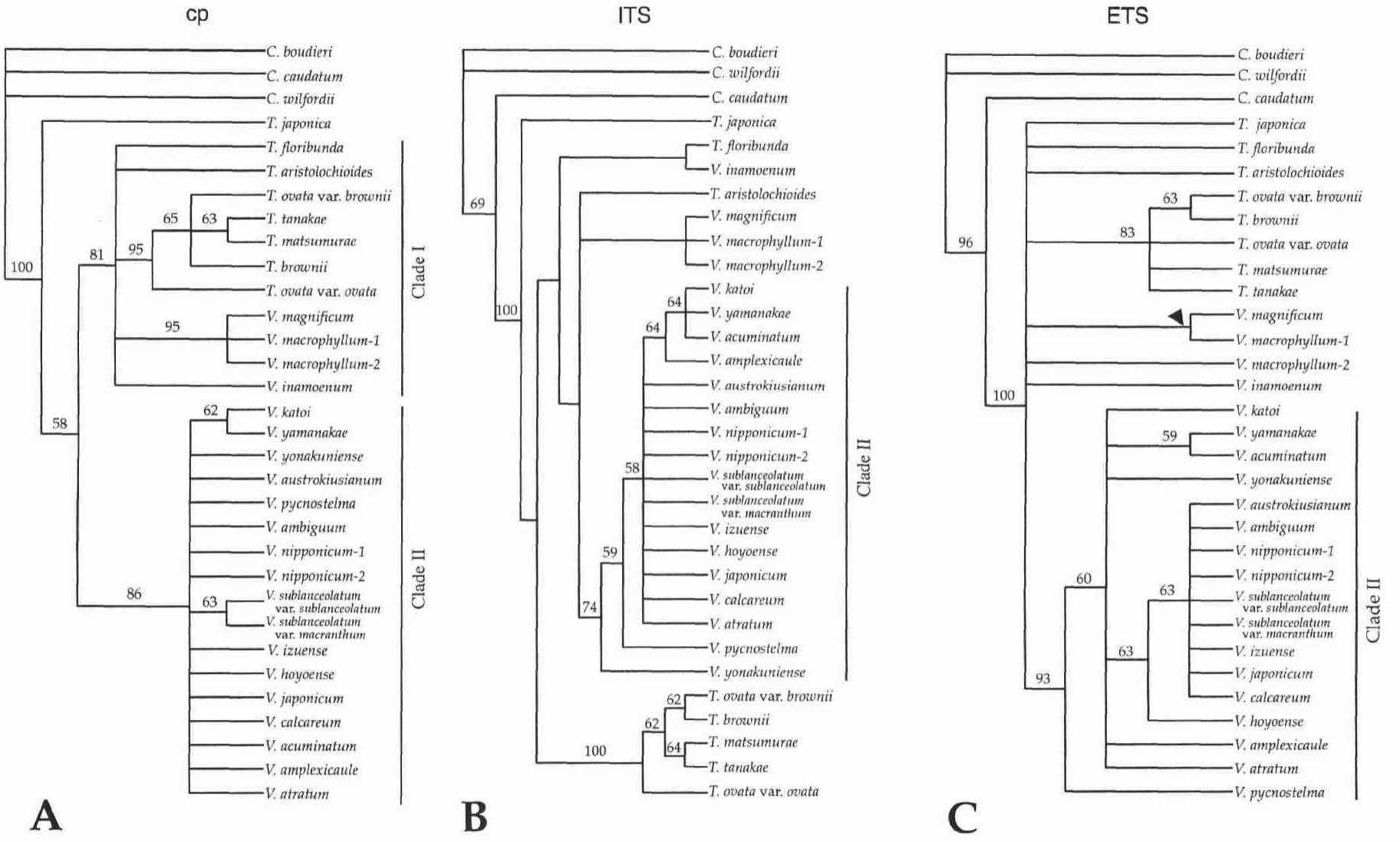

Fig. 28. Strict consensus trees of cpDNA, ITS and ETS regions. A. Strict consensus tree of the 10 equally parsimonious trees based on the combined five cpDNA regions. B. Most-parsimonious tree based on the ITS region. C. Strict consensus tree of 201 equally most-parsimonious trees based on the ETS region. Numbers above branches indicate bootstrap percentages above 50% for the analysis without indels. Dotted

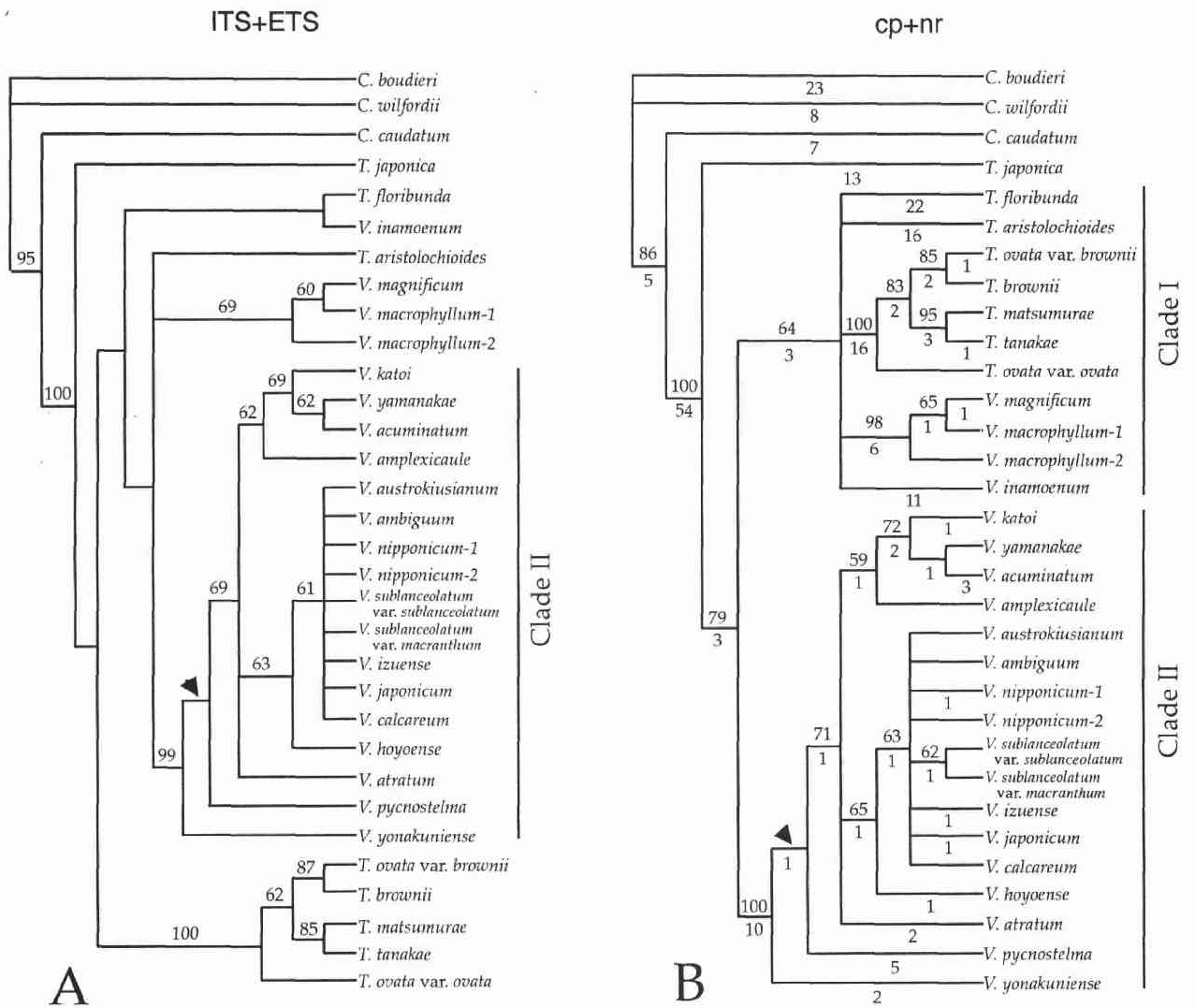

Fig. 29. Strict consensus trees of combined ITS and ETS region, and cpDNA and nrDNA. A. Strict consensus tree of four equally most-parsimonious trees based on the combined ITS and ETS regions. B. Strict consensus tree of 52 equally most-parsimonious trees based on the combined cpDNA and nrDNA. Numbers above branches indicate bootstrap values above 50% for the analysis and those below the branches indicate nucleotide substitutions. Dotted lines indicate clade not supported in the analysis including the indels. Arrowheads indicate clades supported by indels.

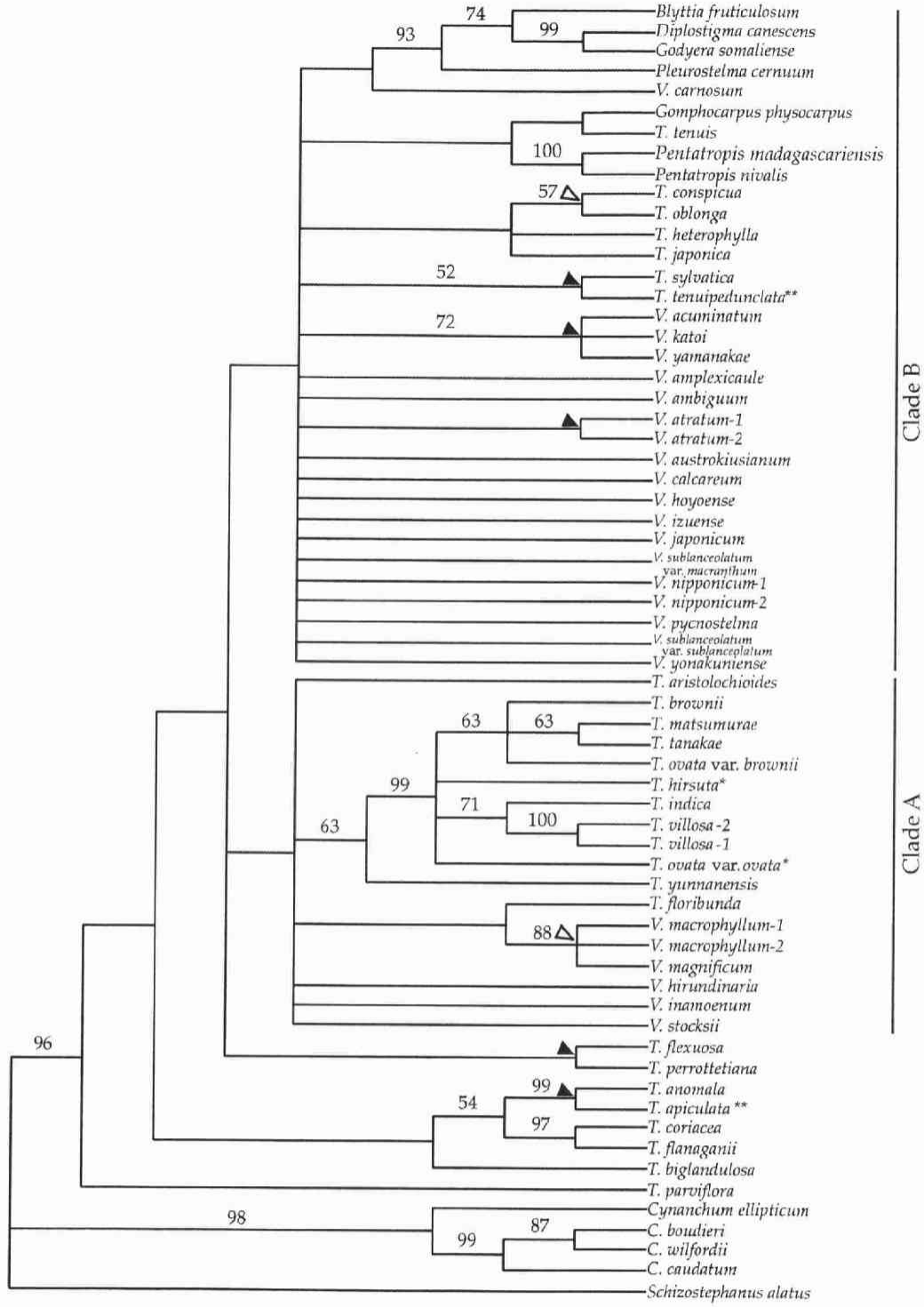

Fig. 30. Strict consensus trees of combined cpDNA and ITS region for *Blyttia fruticulosum*, four *Cynanchum* species, *Diplostigma canescens*, *Godyera somaliense*, *Gomphocarpus physocarpus*, two *Pentatropis* species, *Pleurostelma cernuum*, *Schizostephanus alatus*, 21 *Vincetoxicum* species, and 25 Tylophora species. Numbers above branches indicate bootstrap values above 50% for the analysis. Black and white arrowheads indicate clades not retrieved from cpDNA data analysis and ITS data analysis, respectively. Taxa followed by an asterisk (*) were monophyletically supported from ITS data alone and double asterisks were (**) from cpDNA data alone.

CHAPTER VII. EVOLUTIONARY TRENDS OF JAPANESE *VINCETOXICUM*

From the result of chapter VI, two lineages, i.e., Clade I and Clade II, were recognized in Japanese *Vincetoxicum*. In particular, 15 *Vincetoxicum* species in Clade II might have been under gone rapid radiation (Chapter VI). Polyploidy combined with hybridization has exerted a major influence on the evolution of higher plants (Stebbins, 1971) and considered to play one of the major roles for raising species diversities of certain genera in Japanese plants (e.g. *Trillium*: Kurabayashi, 1957; *Dendranthema*: Nakata et al., 1987). However, only three taxa of 15 *Vincetoxicum* species in Clade II had tetraploid of 2n=44 and the others had diploid of 2n=22 (chapter III), suggesting that polyploidy or hybridization is not major causes of rapid radiation in Clade II.

In this chapter, I further discuss evolutionary processes, especially the process of the rapid radiation of Clade II, by integrating the results of flower morphology, pollinator observation and phylogenetic analysis. The evolutionary patterns of morphology and the evolution of pollination systems were investigated by overlaying the appropriate morphological and ecological characters onto the molecular phylogenetic tree derived from the result of chapter VI with MacClade 4. 02 (Maddison and Maddison, 2000).

Pollination system

The phylogenetic distribution of pollinator types on one of the most parsimonious trees derived from molecular phylogenetic analysis based on the sequence of cpDNA and nr DNA are shown in Fig. 31. *Cynanchum caudatum* has generalized pollination and *C. wilfordii* has specialized wasp pollination (see Chapter IV). The basal condition in *Tylophora-Vincetoxicum* complex appears to be dipteran pollination. The most of all species in Clade I were pollinated exclusively by Diptera such as mosquitoes and flies, although six *Vincetoxicum* species in Clade II were also pollinated by the other insects orders. In Clade II, moth pollination is convergently arisen at least three times. Autogamy has evolved at once in Clade I.

Floral traits

The results of evolutionary analyses of four floral characters, i.e., corolla color, tube of corolla, corona types and length of guide rail, are shown in Fig. 32. Dark purplish corolla is dominated in *Tylophora-Vincetoxicum* complex and is considered as a primitive condition. In

Clade II, yellowish white or whitish flowers are arisen at least three times, and at least twice in Clade I. Four moth-pollinated *Vincetoxicum* species have whitish or yellowish white flowers, although not all whitish or yellowish white flowers species are moth-pollinated. The interstaminal parts of corona and tube structure of the corolla are shared two moth pollination species, although these traits are shared also with dipteran pollinated species. This result suggested that species pollinated by Diptera and moth has very similar basic morphologies. The most prominent character shared by four moth-pollinated species is relatively long guide rail. Therefore, this character seems to play an important role for switching to moth pollination.

Process of rapid radiation in 15 **Vincetoxicum** *species*

The rapid radiation of 15 *Vincetoxicum* species in Clade II might have been caused by a key innovation rather than by invasion of newly formed habitat with few competing species. The species in Clade II are different from their close relatives (Clade I) in their modes of pollination.

The Japanese flora has considered to be derived by invasions of plants from the Eurasian or Asian continents via Sakhalin, the Kuril islands, the Korean peninsula, and the Ryukyu islands throughout past geological epoch (Hotta, 1974; Maekawa, 1998; Fujii, 2003). Ten *Vincetoxicum* species in Clade II are endemic to Japan and might have originated or differentiated in Japan. Other five *Vincetoxicum* species in Clade II, *Vincetoxicum acuminatum, V. atratum, V. amplexicaule, V. nipponicum* and *V. pycnostelma*, distribute more wide geographic range. Most of these species might have migrated from Middle or South China via Korea or Ryukyu Islands to Southeast Japan and expanded its distribution area.

A possible hypothesis for the process of rapid radiation in *Vincetoxicum* is as follows. The widely spread and ancestral species partitioned their distribution areas and adapted to various environments, including forest floor (*V. acuminatum, V. calcareum, V. katoi, V. yamanakae*), edges of forest (*V. austrokiusianum, V. yonakuniense*), sunny meadows (*V. amplexicaule, V. atratum, V. pycnostelma*), marsh (*V. ambiguum, V. nipponicum, V. sublanceolatum*), and rocky beach (*V. hoyoense, V. izuense, V. japonicum*). Then, they adapted to local dominant pollinators, such as moths, and each population has isolated. In these *Vincetoxicum* species, a switch from dipteran to moth pollination may require only slight changes, for example guide rail length. These adaptations to different habitats and different pollination modes might have produced rapid morphological diversity in Clade II.

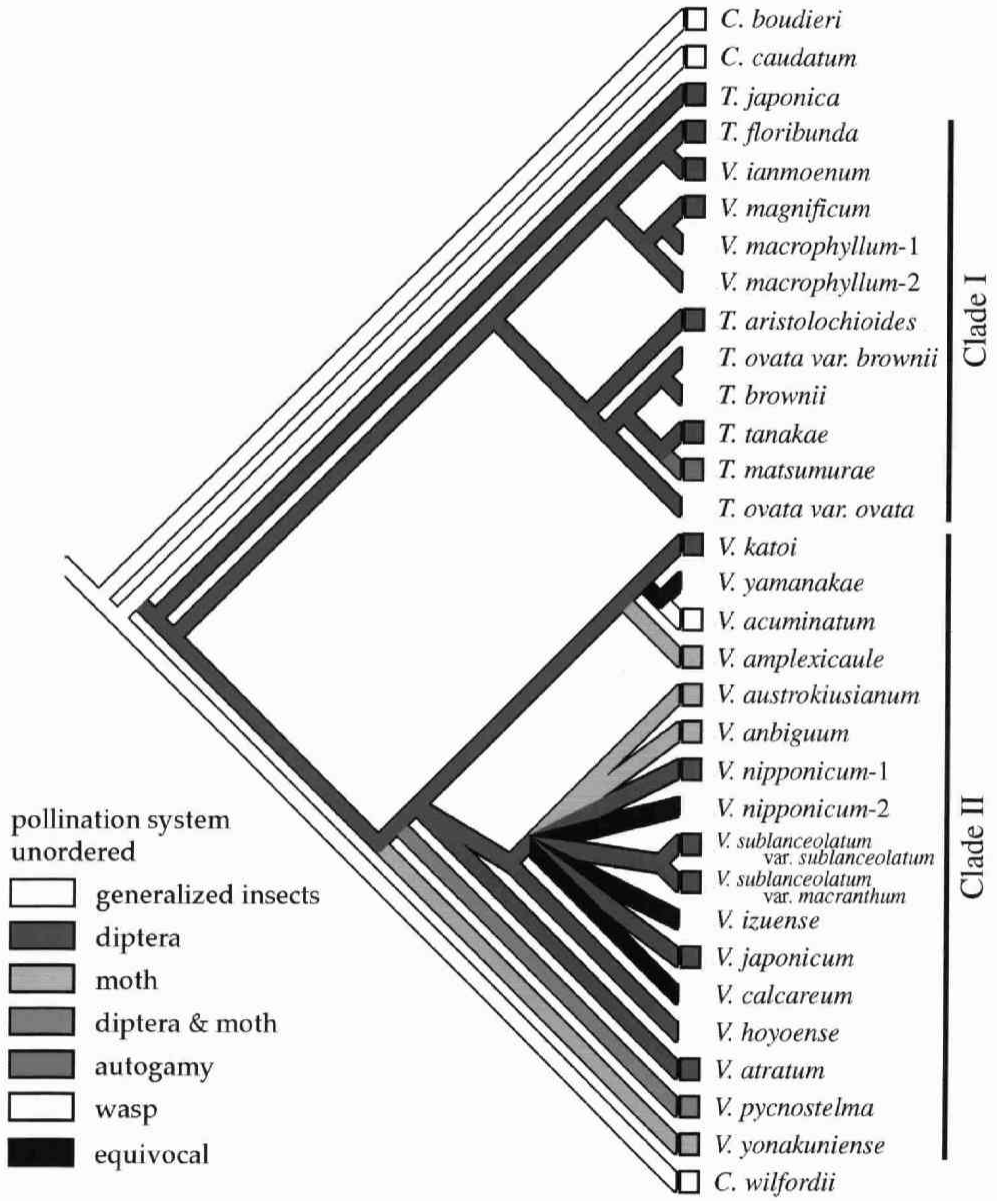

Fig. 31. Evolution of Pollination systems in three *Chynanchum* species, seven *Tylophora* species and 18 *Vincetoxicum* species.

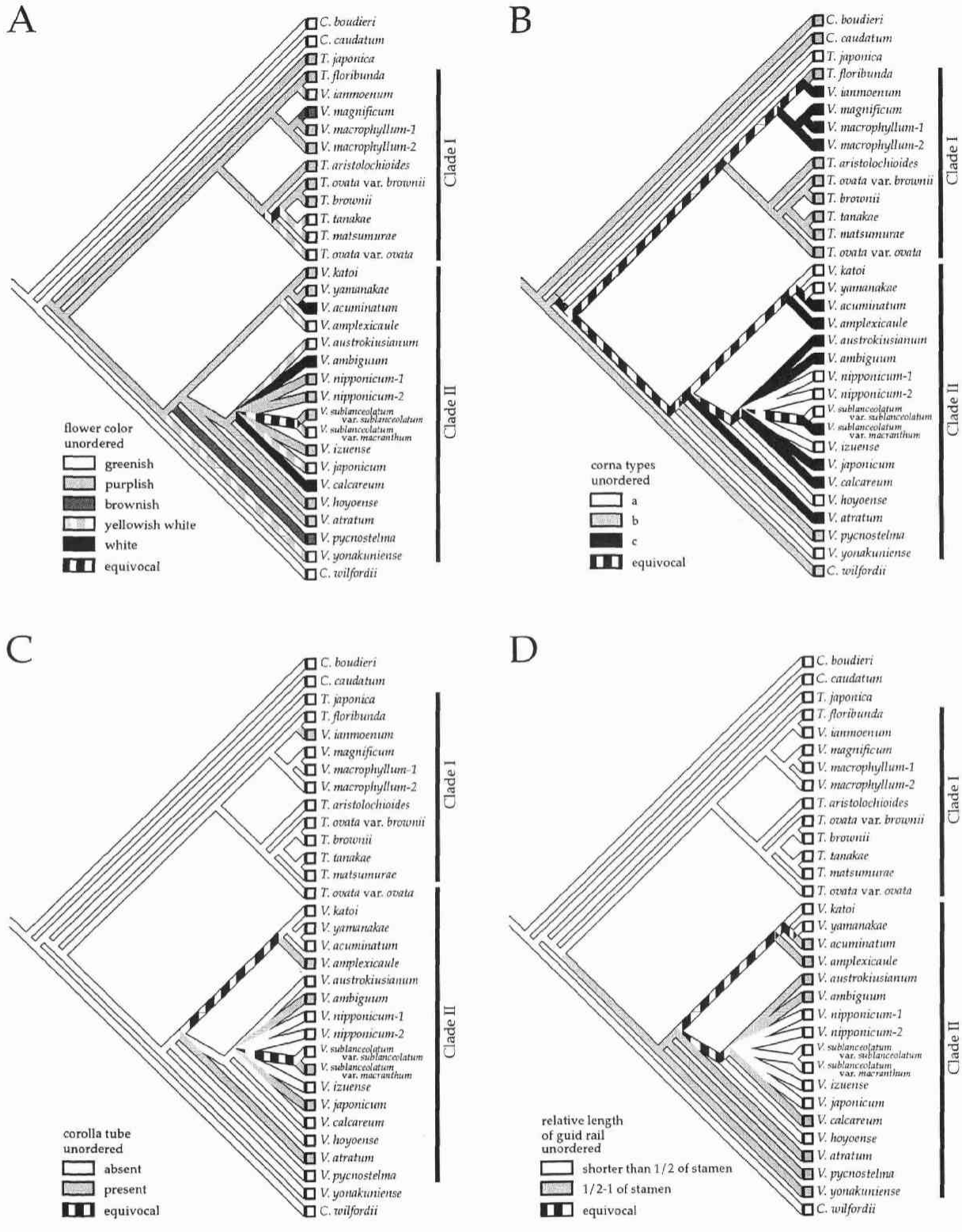

Fig. 32. Evolution of floral morphologies in three *Chynanchum* species, seven *Tylophora* species and 18 *Vincetoxicum* species. A. flower color. B. corona type. C. corolla tube. D. relative length of guide rail. State of each chracters are shown in Table 4 (Chapter IV).

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