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Rediscovery of *Zeuxine boninensis* (Orchidaceae) from the Ogasawara (Bonin) Islands and Taxonomic Reappraisal of the Species

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Abstract An initially unidentifiable orchid was discovered during a 2017 expedition to Minami-iwo-to Island, an oceanic island located ca. 1,300 km south of Tokyo, which belongs to the Ogasawara (Bonin) Islands. The plant was first identified as *Zeuxine sakagutii* Tuyama on the basis of morphological and macromolecular characters. No nucleotide sequence divergences in the nuclear ribosomal DNA ITS and the two plastid regions (*matK* and *trnL-F* intron) were found between the Ogasawara plant and *Z. sakagutii* samples from the Ryukyu Islands and Hainan Island, China. This result indicates a recent, long distance dispersal of this species to Ogasawara. *Z. boninensis* Tuyama, described from Ogasawara, was considered to be extinct. We did not find any differences between *Z. boninensis* and *Z. sakagutii* except for the labellum colour. Partial ITS sequences of *Z. boninensis* were retrieved from the specimens collected in 1935 and 1936 and they were 100% identical with those of *Z. sakagutii*. These results demonstrate conspecificity of these two entities, and *Z. boninensis* became the correct name. In addition, *Z. boninensis* var. *actinomorpha* T.Yukawa, a peloric flower type, is newly described from Amami-oshima Island, Ryukyu Islands, Japan.

Key words: Japan, new record, new variety, Ogasawara Islands, Orchidaceae, taxonomy, *Zeuxine*.

Introduction

Minami-iwo-to Island, a subtropical oceanic island, is located ca. 1,300 km south of Tokyo in the northwest Pacific Ocean (Fig. 1). This small island (3.5 km^2) belongs to the Volcano Islands group of the Ogasawara (Bonin) Islands and has the highest peak (916 m) of the Islands. Historically, Minami-iwo-to Island has never been inhabited and the island thus holds immense ecological value. Since the island has never been connected with a continent or an island and is demonstrated to have a recent origin of about 30 thousand years ago (Nakano *et al.*, 2009), the terrestrial biota of Minami-iwo-to Island is considered to have accrued through recent long-distance dispersal from other areas.

So far, only four scientific expeditions covering the summit area (1936, 1982, 2007, and 2017) were conducted on Minami-iwo-to Island. During the latest expedition of the island from 13 to 26 June 2017, a sterile, unidentifiable plant belonging

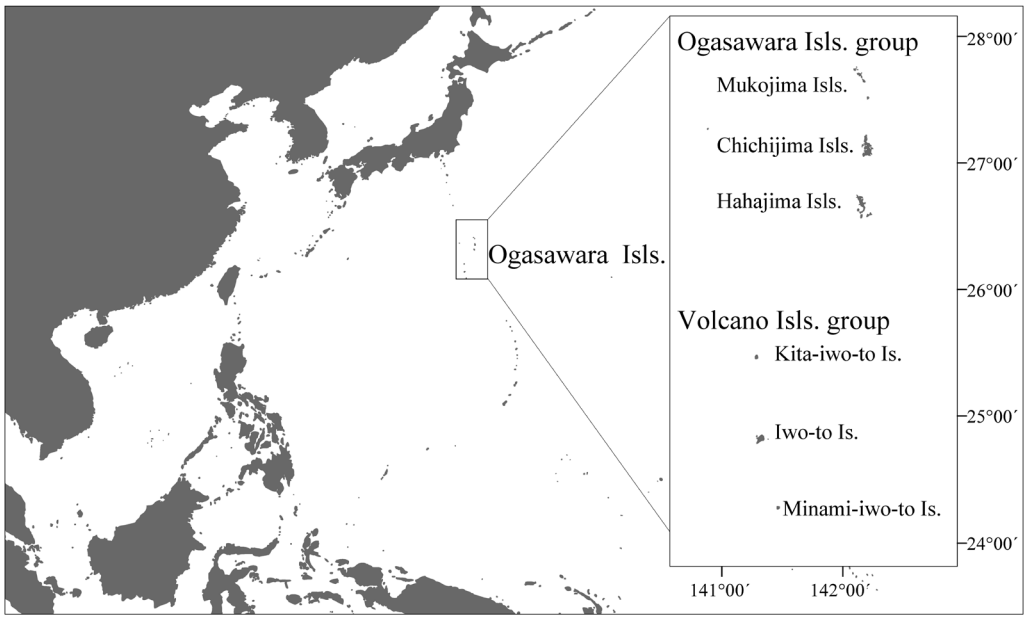

Fig. 1. Location of the Ogasawara Islands. Minami-iwo-to Island belongs to the Volcano Islands group.

to Orchidaceae was found (Fig. 2). Fujita *et al.* (2008) recorded six orchid species from the island, viz., *Calanthe triplicata* (Willemet) Ames, *Goodyera foliosa* (Lindl.) Benth. ex C.B.Clarke (as *Goodyera augustini* Tuyama), *Goodyera procera* (Ker Gawl.) Hook., *Goodyera* sp., *Liparis hostifolia* (Koidz.) Koidz. ex Nakai, and *Liparis nervosa* (Thunb.) Lindl. Although this most recent collection may correspond to the earlier *Goodyera* sp., accurate identification was not possible without reproductive organs. An ex situ collection of this plant flowered in Tsukuba Botanical Garden, National Museum of Nature and Science, in February 2018 and was used for morphological investigation and molecular identification.

Materials and Methods

The unidentifiable plants of Minami-iwo-to Island were collected on 21 June 2017. The habitat is a semi-shady, rocky slope facing south with shallow soil at around 700 m height above sea level often covered by cloud (Fig. 2). The plants were found at the edge of scrub-forest consisting of *Eurya japonica* Thunb., *Boehmeria nivea* (L.) Gaudich. var. *nivea*, and *Melastoma candidum* D.Don together with several orchidaceous species such as *Goodyera procera*, *Liparis hostifolia* and *Calanthe triplicata*. The plants were also collected at the edge of summit forest comprising *Machilus kobu* Maxim. and *Cyathea tuyamae* H.Ohba around 900 m height above sea level. Occasionally, the plants were found growing epiphytically on trunks of *Cyathea tuyamae*.

In total, seven individuals were collected and cultivated in Tsukuba Botanical Garden, National Museum of Nature and Science. The ex situ living collection bloomed in February 2018 (Fig. 3) and was subjected to morphological observation and DNA extraction. DNA was also extracted from herbarium vouchers or living plants of morphologically similar taxa for molecular identification. Materials used in molecular identification are listed in Table 1. Except for two old specimens of *Zeuxine boninensis* Tuyama (*T. Tuyama s. n.* collected in 1935 and *Y. Satake s. n.* collected in 1936), total DNA of plants was extracted from fresh or silica-gel-dried leaves with the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) following the manufactur-

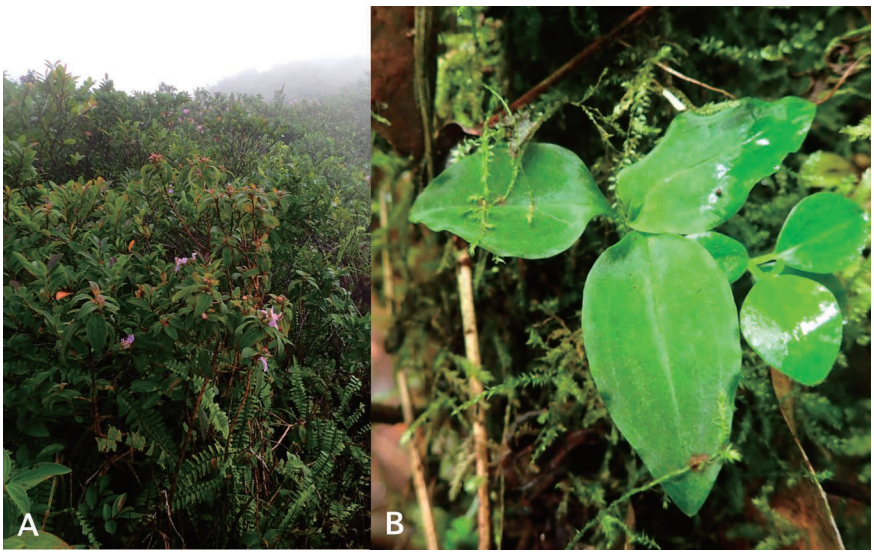

Fig. 2. Habitat of *Zeuxine boninensis* Tuyama on Minami-iwo-to Island, Ogasawara Islands, Japan. A. Scrub-forest at ca. 700 m height above sea level where this species grows. B. A sterile plant at the edge of forest. Photographs taken in June 2017.

Fig. 3. Flowering individual of *Zeuxine boninensis* Tuyama from Minami-iwo-to Island, Ogasawara Islands, Japan. A. Habit. B. Close-up of flowers. Photographs taken in February 2018 from an ex situ collection of *Takayama et al. 17062108* by Kazuhiro Suzuki.

er's instructions. Total DNA from the old herbarium specimens was extracted with a modified CTAB method of Doyle and Doyle (1987).

Nucleotide sequences were determined by amplifying the internal transcribed spacer (ITS) regions of the 18S–26S nuclear ribosomal DNA

Table 1. Materials used for DNA sequencing

Species	Locality	Voucher
Zeuxine boninensis	Japan, Ogasawara Isls., Hahajima Is.	Y. Satake *s. n.* (TI 6556)
	Japan, Ogasawara Isls., Hahajima Is.	T. Tuyama *s. n.* (TI 6559)
	China, Hainan Is.	H. Z. Tian d16 (HSNU)*
	China, Hainan Is.	H. Z. Tian Z3 (HSNU)*
	Japan, Ogasawara Isls., Minami-iwo-to Is.	K. Takayama *et al.* 17062108 (TNS)*
	Japan, Ryukyu Isls., Amami-oshima Is., Anengachi	H. Yamashita 18-2 (TNS)*
Zeuxine boninensis var. *actinomorpha*	Japan, Ryukyu Isls., Amami-oshima Is., Wase	H. Yamashita 18-1 (TNS)*
Zeuxine flava	China, Yunnan, Tongbiguan	C. Hu b28 (HSNU)

*Initially identified as *Z. sakagutii*.

and two regions of the plastid genome (*matK*, *trnL-F* spacer) via the polymerase chain reaction (PCR) from a total DNA extract. Experimental methods follow those described in Topik *et al.* (2005), Yukawa *et al.* (2005), and Tsutsumi *et al.* (2007) in which the primer information of the sequenced regions was also shown. Since PCR amplification of the above-mentioned old specimens of *Zeuxine boninensis* was unsuccessful, partial sequences in ITS 2 region were amplified with newly developed primers designed for the product sizes of ca. 150 bps (Forward: GCTTCATCCGTCACCAATTT, Reverse: CAAATGGCAGCATACACCAC). PCR was performed using EX Taq DNA polymerase with 5% dimethyl sulfoxide (DMSO) for initial denaturing for 3 min at 94℃, followed by 40 denaturation, annealing, and elongation cycles (30 sec at 94℃, 30 sec at 62℃ and 20 sec at 72℃), and a final elongation step (5 min at 72℃). The sequence data sets were aligned using MUSCLE implemented in MEGA 7.0 (Kumar *et al.*, 2016) and modified manually to minimize the number of gaps.

Results and Discussion

Morphological characters

General morphological characters of Minami-iwo-to collection matched with features of the genus *Zeuxine* (Fig. 3). Yukawa (2015) recognized eight species of *Zeuxine* in Japan, viz., *Z. affinis* (Lindl.) Benth. ex Hook.f., *Z. agyokuana* Fukuy., *Z. boninensis* Tuyama, *Z. nervosa* (Wall. ex Lindl.) Benth. ex Trimen, *Z. odorata* Fukuy., *Z. sakagutii* Tuyama, *Z. strateumatica* (L.) Schltr., and *Z. tenuifolia* Tuyama. These species are distributed in warm-temperate to subtropical regions of the country. Except for *Z. boninensis*, an endemic species of Japan, all the other species are also distributed in Taiwan where three more species such as *Z. arisanensis* Hayata, *Z. integrilabella* C.S. Leou, and *Z. philippinensis* (Ames) Ames were recorded (Lin *et al.*, 2016). Most of the above-mentioned species are also distributed in mainland China. However, *Z. flava* (Wall. ex Lindl.) Trimen, *Z. gengmanensis* (K.Y.Lang) Ormerod, *Z. goodyeroides* Lindl., *Z. grandis* Seidenf., *Z. membranacea* Lindl., and *Z. ovalifolia* L.Li & S.J.Li are additional taxa native to mainland China that are not found in either Japan or Taiwan (Zhou *et al.*, 2016). Micronesia is situated ca. 1,000 km southward from the Bonin Islands. In this region, three endemic *Zeuxine* species, viz., *Z. fritzii* Schltr., *Z. ovata* (Gaudich.) Garay & W.Kittr., and *Z. palawensis* Tuyama, were recorded (Costion and Lorence, 2012).

We compared morphological characters of the Minami-iwo-to collection with all of these *Zeuxine* species recorded from the surrounding regions and found our material to be identical to *Z. sakagutii*. The Minami-iwo-to plants share diagnostic characters of *Z. sakagutii* such as a petiolated, ovate-lanceolate leaf, velvet-like texture of adaxial leaf surface, a villose peduncle, a glabrous to sparsely hairy ovary, clasping lateral sepals, a yellow labellum epichile that is T-shaped, rectangular epichile lobules, a reddish-orange and narrowly lanceolate operculum, and a

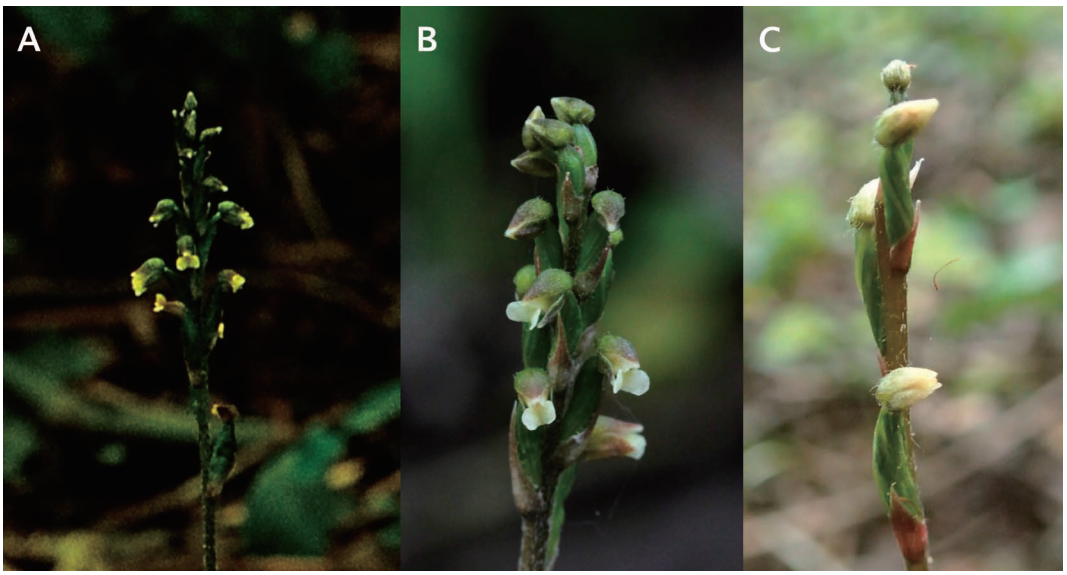

Fig. 4. *Zeuxine boninensis* Tuyama from Amami-oshima Island, Ryukyu Islands, Japan. A. Plant with typical flowers. B. Plant with yellowish cream labellum (*H. Yamashita 18-2*). C. Plant with peloric flowers, *Z. boninensis* Tuyama var. *actinomorpha* T. Yukawa (*H. Yamashita 18-1*). Photographed from in situ plants by Hiroshi Yamashita.

narrowly oblong viscidium (Fig. 4).

In the Ogasawara Islands, another congeneric entity, *Zeuxine boninensis* Tuyama is recorded from Hahajima Island, 250 km north from Minami-iwo-to Island. Since this species was considered to be extinct (Ministry of the Environment, 2018), observations have to be based on the protologue and specimens. We did not find any differences between these two species except for the labellum colour. In *Z. sakagutii*, the epichile is yellow and the hypochile is white, while Tuyama (1935) briefly described in the prologue that labellum colour is white in *Z. boninensis*. However, epichile colour of *Z. sakagutii* is variable and occasionally exhibits yellowish cream (Fig. 4B). In this respect, the reasoning to distinguish these two entities is not robust on the basis of morphological characters alone.

Zeuxine flava (Wall. ex Lindl.) Trimen, a close relative of *Z. sakagutii*, is distributed from mainland China to the Himalayas. Taxonomic confusion exists between *Z. flava* and *Z. sakagutii* (e.g., King and Pantling, 1898; Barretto *et al.*, 2011). As pointed out by Liu *et al.* (2015), they are clearly separated by posture of lateral sepals (patent in *Z. flava*, clasping in *Z. sakagutii*), shape of the epichile of the labellum (Y-shaped in *Z. flava*, T-shaped in *Z. sakagutii*), and shape of the viscidium (elliptic in *Z. flava*, narrowly oblong in *Z. sakagutii*).

During the course of this study, we found peloric flower individuals of *Zeuxine sakagutii* from Amami-oshima Island (Fig. 4C) and Yakushima Island, both located in the Ryukyu Islands, Japan. Except for a petaloid labellum devoid of the spur and adaxial processes and narrower lateral sepals in peloric plants, no differences were detected between the two types.

Macromolecular characters

In ITS, *matK*, and *trnL-F* intron regions, sequences were identical among the Minami-iwo-to material and four samples of *Zeuxine sakagutii* collected in the Ryukyu islands, Japan and Hainan Island, China (Table 1). A total of 154 bps in ITS2 region were successfully obtained from two herbarium specimens of *Z. boninensis* collected more than 80 years ago. These

sequences also 100% matched the corresponding region of the above-mentioned samples of *Z. sakagutii*. Since ITS2 region has been tested to have nucleotide substitutions among closely related species or even among conspecific individuals in some cases (e.g., Yukawa *et al.*, 2013; Takamiya *et al.*, 2014; Gale *et al.*, 2015; Tang *et al.*, 2015; Freudenstein *et al.*, 2017; Yukawa and Yamashita, 2017), this result demonstrates the high affinity between *Z. boninensis* and *Z. sakagutii*. In contrast, *Z. flava*, a potential sister species of *Z. sakagutii* (T. Yukawa and H. Z. Tian, unpublished), was discriminated from *Z. sakagutii* by a single substitution in ITS, four substitutions in *matK*, and eight substitutions in *trnL-F* intron. Besides, no molecular divergence was detected between typical and peloric individuals of *Z. sakagutii*.

Taking account of both the morphological and macromolecular evidence, we hereby conclude that the Minami-iwo-to plants belong to *Zeuxine boninensis* and *Z. sakagutii* is appropriate to be synonymous with *Z. boninensis*. Furthermore, no nucleotide sequence divergences between the Ogasawara plant and samples from other regions indicate a recent, long distance dispersal to Ogasawara.

Taxonomic Treatment

Zeuxine boninensis Tuyama, Bot. Mag., Tokyo 49: 369 (1935). TYPE: JAPAN, Ogasawara Isls., Hahajima Is., Mt. Sekimon, 9 April 1934, *T. Tuyama s. n.* (TI).

Zeuxine sakagutii Tuyama, Bot. Mag., Tokyo 50: 26 (1936), **syn. nov.**

Zeuxine gracilis (Breda) Blume var. *sakagutii* (Tuyama) T.Hashim., Ann. Tsukuba Bot. Gard. 5: 28 (1986).

Zeuxine taiwaniana S.S.Ying, Quart. J. Chin. Forest. 20: 57 (1987), **syn. nov.**

Zeuxine uraiensis S.S.Ying, Coloured Ill. Indig. Orchids Taiwan 3: 620 (1988), **syn. nov.**

Zeuxine flava auct. non (Wall. ex Lindl.) Trimen, King & Pantling, Ann. Roy. Bot. Gard. (Calcutta) 8: 289 (1898) p.p.; Nackejima, Biol. Mag. Okinawa 13: 36 (1975); Hatusima, Fl. Ryukyus, ad. & cor.: 916 (1975); Takahashi, Wild Orch. Jap. 4: 13 (1987); Averyanov, Turczaninowia 11: 157 (2008); Chen *et al.*, Fl. China 25: 74 (2008) p.p.; Barretto *et al.*, Wild Orch. Hong Kong: 155 (2011); Wu *et al.*, J. Guangxi Norm. Univ., Nat. Hist. Ed. 29: 58 (2011); Huang *et al.*, Guihaia 32: 760 (2012). Nakajima, Illust. Jap. Orch.: 128 (2012); Iwatsuki *et al.*, Fl. Jap. IVb: 240 (2016); Lin *et al.*, Taiwania 61: 122 (2016).

Zeuxine tenuifolia auct. non Tuyama, Maekawa, Wild Orch. Jap. Col.: 251 (1971).

Zeuxine leucochila auct. non Schltr., Garay & Sweet, Orch. South. Ryukyu Isls.: 83 (1974) p.p.; Satomi in Satake *et al.*, Wild Fl. Jap., Herb. Pl. 1: 215 (1982); Shimabuku, Check List Vascular Fl. Ryukyu Isls.: 704 (1990) p.p; Shimabuku, Check List Vascular Fl. Ryukyu Isls. Rev. ed.: 800 (1997) p.p.

Zeuxine parvifolia auct. non (Ridl.) Seidenf., Seidenfaden, Dansk Bot. Ark. 32: 82 (1978) p.p.; Chen *et al.*, Fl. China 25: 74 (2008) p.p.

Zeuxine affinis auct. non (Lindl.) Benth. ex Hook.f., Lin, Native Orch. Taiwan 3: 280 (1987).

Zeuxine clandestina auct. non Blume, Krishna *et al.*, Richardiana 11: 164 (2011).

var. ***boninensis***

Japanese name: Munin-kinuran.

Flowering period: February to May.

Distribution: JAPAN: Ogasawara Isls. (Hahajima Is., Minami-iwo-to Is.), Ryukyu Isls. (Yakushima Is., Tanaegashima Is., Suwanosejima Is., Amami-oshima Is., Okinawajima Is., Ishigakijima Is., Iriomotejima Is.); CHINA: Yunnan, Guangxi, Guangdong, Hainan, Hong Kong, Taiwan; VIETNAM; INDIA: Sikkim, West Bengal.

Specimens examined: JAPAN, Ogasawara Isls.: Tokyo Met. Hahajima Is., Mt. Sekimon, 9 April 1934, *T. Tuyama s. n.* (TI06553, TI06554, TI06555); Hahajima Is., 9 April 1936, *Y. Satake s. n.* (TI06556, TI06557); Hahajima Is., Mt. Sekimon, 9 April 1936, *Y. Satake s. n.* (TI06558); Hahajima Is., Mt. Sekimon, 26 Nov. 1935, *T. Tuyama*

s. n. (TI06559); Hahajima Is., Mt. Sekimon, May 1926, *Z. Toyoshima s. n.* (TI06560); Hahajima Is., April 1935, *M. Okabe s. n.* (TI06561); Minami-iwo-to Is., alt. 674 m, *K. Takayama et al. 17062108* ex hort. Tsukuba Bot. Gard., 27 February 2018 (TNS); Minami-iwo-to Is., alt. 876 m, *K. Takayama et al. 17062120* ex hort. Tsukuba Bot. Gard., 27 February 2018 (TNS). Ryukyu Isls.: Kagoshima Pref. Amami-oshima Is., Anengachi, 19 April 2018 *H. Yamashita 18-2* (TNS); Amami-oshima Is., Tastsugo-cho, Nagakumo-touge Pass, 14 April 1988, *H. Yamashita s. n.* (TNS9504297); Yakushima Is., Hinokuchi, 2 May 1985, *s. coll.* (TNS8503935); Yakushima Is., Hinokuchi, 9 May 1986, *Y. Hanei s. n.* (TNS9504113). Okinawa Pref. Iriomotejima Is., near Ohtomi, 6 April 1986, *Y. Hanei s. n.* (TNS8503929); Iriomotejima Is., near Ohtomi, 21 April 1986, *Y. Hanei s. n.* (TNS9504114); Iriomotejima Is., 26 March 1993, *K. Kanda & Y. Hanei s. n.* (TNS 8502358); Iriomotejima Is., along the crossing road, 26 March 1993, *K. Kanda & Y. Hanei s. n.* (TNS 8504564); Iriomotejima Is., 6 April 1994, *K. Kanda & Y. Hanei s. n.* (TNS8504741); Okinawajima Is., Nago-shi, Mt. Nago-dake, 12 April 1978, *s. coll.* (TNS8503930); Okinawajima Is., Kunigami-son, Yona Experimental Forest, University of the Ryukyus, 31 March 1995, *K. Inoue s. n.* (TNS8504740); Okinawajima Is., Mt. Yonaha-dake, 18 April 1995, *K. Inoue s. n.* (TNS8504742).

var. ***actinomorpha*** T.Yukawa, **var. nov.**

TYPE: JAPAN, Ryukyu Isls.: Kagoshima Pref. Amami-oshima Is., Amami-shi, Sumiyo-cho, Wase, alt. ca. 200 m, 18 April 2018, *H. Yamashita 18-1* (TNS-holotype).

This variety differs from the typical one by its petaloid labellum devoid of the spur and adaxial processes and narrower lateral sepals. Except for these characters, no differences were detected between them. Fig. 4C.

Japanese name: Amami-kinuran (nov.). This name refers to the type locality of this variety.

Flowering period: April to May.

Distribution: JAPAN: Ryukyu Isls. (Yakushima Is., Amami-oshima Is.).

Other specimens examined: JAPAN, Ryukyu Isls.: Kagoshima Pref. Amami-oshima Is., Sumiyo-son, Higashi-nakama, 11 April 1993, *H. Yamashita s. n.* (TNS8504485); Yakushima Is., Mt. Nogidake, ca. 1400 m, 17 April 1981, *H. Yoshioka et al. s. n.* (TNS8503925); Yakushima Is., Kurio, along Kuromi-gawa River, 9 May 1986, *Y. Hanei s. n.* (TNS8503925).

Note: Both typical and peloric flower individuals are found at several sites on Amami-oshima Island but these two types do not grow sympatrically (H. Yamashita, personal communication). The peloric type also has been found on Yakushima Island, another island of the Ryukyu Islands situated ca. 250 km north from Amami-oshima Island. Establishment of populations and expansion of distribution range in the northern Ryukyus demonstrate that this entity is an established biological unit rather than an aberrant individual caused by chance mutation. Thus it is appropriate to recognize this entity at the variety level.

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