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Hybrid of *Osmunda japonica* and *O. lancea* on Mt. Tenjo, Kozu Island, Izu Islands, Japan

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Abstract We found an *Osmunda lancea*-like plant on Mt. Tenjo, the Kozu Island, the Izu Islands, and identified it as *Osmunda* × *intermedia*, a hybrid between *O. japonica* and *O. lancea*, based on the morphology and molecular phylogeny. It is the first record of *O.* × *intermedia* from Izu Islands, and its habitat differs from the ordinary habitat of *O.* × *intermedia.* Morphology suggests that the individual is a later-generation hybrid.

Key words: Izu Islands, Kozu Island, *Osmunda* × *intermedia*.

Introduction

Osmunda lancea Thunb. is a rheophyte and is adapted to the ecologically specialized river-bank subject to periodical flooding with swift turbulence after heavy rains. It is characterized by the narrow-lanceolate pinnules resistant to fast current (Imaichi and Kato, 1992, 1993, 1997). This rheophilous species is endemic to Japan and distinguished from *O. japonica* Thunb., mainly by the shape of pinnule. *Osmunda japonica* has oblong pinnules with truncate bases and inhabits semi-open roadside slopes/excavations and secondary forests in eastern Asia. The pinnule of *O. lancea* is lanceolate and has a cuneate base. Phylogenetically, *O. japonica* and *O. lancea* formed a monophyletic clade and are quite close (Yatabe *et al.*, 1999; Metzgar *et al.*, 2008; Tsutsumi *et al.*, 2011). A previous study of the genetic population structure revealed that *O. japonica* and *O. lancea* are genetically differentiated in nuclear genes despite shared polymorphism in chloroplast genes, suggesting recent introgression between *O. japonica* and *O. lancea* (Yatabe *et al.*, 2009).

A natural semifertile hybrid between the two species is known as *O.* × *intermedia* (Honda) Sugim. with morphological intermediacy in its pinnule shape. The distribution almost overlaps that of *O. lancea* (Kurata and Nakaike, 1990). It occurs locally abundantly on and above stream banks but does not invade forest edges where *O. japonica* grows (Shimura, 1964). The analyses of the genetic population structure of those species inferred that later-generation hybrids are formed, based on the findings of putative F2 hybrids in *O.* × *intermedia* (Yatabe *et al.*, 2009). *Osmunda* × *intermedia* has a considerable variation, which may be caused by phenotype segregation of F2 or later progeny, multiple origins, and/or backcrosses (Kato, 2009).

The Izu Islands consist of about 100 Islands and are ca. 650 km long in the Pacific Ocean off-

shore the Izu peninsula in Japan. *Osmunda japonica* is common on the inhabited islands, although there is no record of rheophilous *O. lancea* from the Islands (Kurata and Nakaike, 1990). Recently we found an *O. lancea*-like plant with narrow-lanceolate pinnules around the top of Mt. Tenjo on the Kozu Island, an inhabited island of the Izu Islands locating about 55 km apart from the nearest Izu Peninsula. To reveal the taxonomic identity of the plant, we performed morphological and molecular phylogenetic analyses using several nuclear markers.

Materials and Methods

Living plant materials of an *Osmunda lancea*-like plant (KL1) and two *O. japonica* individuals (KJ1 and KJ2) were collected from the Kozu Island (Fig. 1, Table 1).

In a morphological analysis, five pinnules of

Fig. 1. Localities of *Osmunda lancea*-like plant (green square) and *O. japonica* (red squares) at Mt. Tenjo in the Kozu island. Sample IDs are identical to those of Table 1. The basemap is the 1 : 25,000 topographic map (Kozu island) published by Geospatial Information Authority of Japan. Scale bar = 300 m.

Table 1. *Osmunda* individuals found on the Kozu Island. Asterisks (∗) show samples used for molecular analyses

Species	Sample ID	Locality
O. lancea like plant	KL1*	Japan, Tokyo, Kodzu Isl., Mt. Tenjo (N34°12′59″, E139°09′16″, alt. ca. 500 m)
O. japonica	KJ1*	Japan, Tokyo, Kodzu Isl., Mt. Chichibu (N34°11′59″, E139°08′39″, alt. ca. 170 m)
	KJ2*	Japan, Tokyo, Kodzu Isl., Mt. Chichibu (N34°11′59″, E139°08′39″, alt. ca. 170 m)
	KJ3	Japan, Tokyo, Kodzu Isl., Mt. Tenjo (N34°13′09″, E139°09′11″, alt. ca. 500 m)
	KJ4	Japan, Tokyo, Kodzu Isl., Mt. Tenjo (N34°13′10″, E139°09′11″, alt. ca. 500 m)
	KJ5	Japan, Tokyo, Kodzu Isl., Mt. Tenjo (N34°12′51″, E139°09′13″, alt. ca. 500 m)
	KJ6	Japan, Tokyo, Kodzu Isl., Mt. Tenjo (N34°12′50″, E139°09′09″, alt. ca. 500 m)
	KJ7	Japan, Tokyo, Kodzu Isl., Mt. Tenjo (N34°12′50″, E139°09′09″, alt. ca. 500 m)
	KJ8	Japan, Tokyo, Kodzu Isl., Mt. Tenjo (N34°12′51 ″, E139°09′08″, alt. ca. 500 m)

Table 2. Primers, GenBank accession numbers and total lengths of nuclear EST markers analyzed in this study

Maker	Accession No.	Total length (bp)	Primer name	Primer sequence	References
EST_L141	FS993744	434	EST_L141F	CATTCGTGAGGGTTCAAGGT	Yatabe-Kakugawa *et al.* 2013
			EST_L141R	ACTGTCCCCTGATTTGATGG	Yatabe-Kakugawa *et al.* 2013
EST_L258	FS993861	317	EST_L258F	TCATGGCGACTGTGAAGAAG	Yatabe *et al.* 2009
			EST_L258R	CGCCCTTTGGATTTACGATA	Yatabe *et al.* 2009
EST_L261	FS993864	320	EST_L261F	ACGTGGTGGCTTCTGGTTAC	Yatabe-Kakugawa *et al.* 2013
			EST_L261R	GCAGCACCAAAACTCCTAGC	Yatabe-Kakugawa *et al.* 2013

the *O. lancea*-like plant (KL1) were picked out randomly in order to measure morphological characters as indicators of stenophylly using the same method as Yatabe *et al.* (2009). The number of ultimate veinlets of the basal basiscopic vein was counted, and the angle of pinnule-base was measured.

In a molecular phylogenetic analysis, DNA was extracted from silica-gel dried material using a QIAGEN DNeasy mini kit (Qiagen, Valencia California), following the manufacturer's instruction. Three nuclear DNA markers (EST_L141, EST_L258, EST_L261), which were selected from an expressed sequence tag library developed by Yatabe *et al.* (2009), and the chloroplast *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) were analyzed. Primers for amplification and sequencing and detailed information on the three nuclear markers are shown in Table 2. We used PHASE 2.1.1 to reconstruct probable pairs of haplotypes for each of the sequences with heterozygous sites (Stephens and Donnelly, 2003). We constructed a haplotype network using statistical parsimony methods in TCS v1.2.1 (Clement *et al.*, 2000) using samples from the Kozu Island, along with eight samples of *O. japonica* and three of *O. lancea* analyzed in Tsutsumi *et al.* (2011) (Appendix 1).

Results

We found only one *Osmunda lancea*-like individual, which inhabits in a wind beaten area around the top of Mt. Tenjo on the Kozu Island (Figs. 1 and 2, Table 1). It has narrow-lanceolate pinnules, and the base of the pinnules is cuneate, similar to that of *O. lancea* (Fig. 2). No fertile leaf was found. Several individuals of *O. japonica* were found around there (Figs. 1 and 2, Table 1), which are moderately small and inhabit under forests or in crevices of rocks.

The averages of the veinlet numbers of the basiscopic basal vein and the pinnule-base angles were plotted for the *O. lancea*-like individual, together with samples analyzed in Yatabe *et al.* (2009) (Fig. 3). The two values of the *O. lancea*-like individual fell in the range of those in populations of *O. japonica* and *O. lancea*. It was, however, an outlier in the putative F1 hybrids of

Fig. 2. *Osmunda lancea*-like plant and *O. japonica* at the top of Mt. Tenjo, Kozu Island. A–C. *O. lancea*-like plant. A. Habitat. B. Leaf. C. Base of pinnule. D–F. *O. japonica.*

O. × *intermedia*, which were identified by NewHybrids analysis using ten nuclear DNA markers in Yatabe *et al.* (2009).

The genotypes of *O. lancea*-like plant for the three examined markers (EST_L141, EST_L258, EST_L261) revealed that the *O. lancea*-like plant

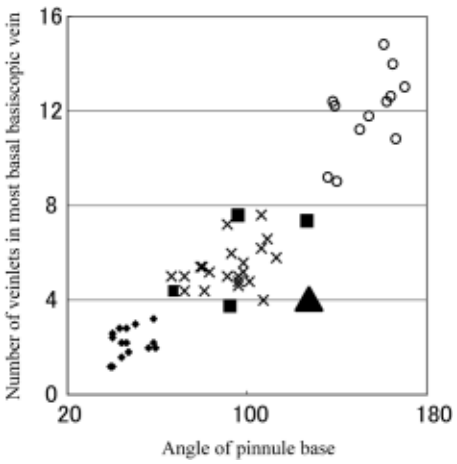

Fig. 3. Morphological characters of pinnules with data of Kozu island *O. lancea*-like plant (solid triangle) added on Fig. 3 of Yatabe *et al.* (2009). *Osmunda japonica* (open circles), *O.* × *intermedia* (crosses, identified as F1 hybrids by NewHybrids analysis based on ten nuclear DNA markers), *O. lancea* (solid diamonds), and possible later-generation hybrids (solid square, identified as F2 hybrids or backcross hybrids by NewHybrids analysis).

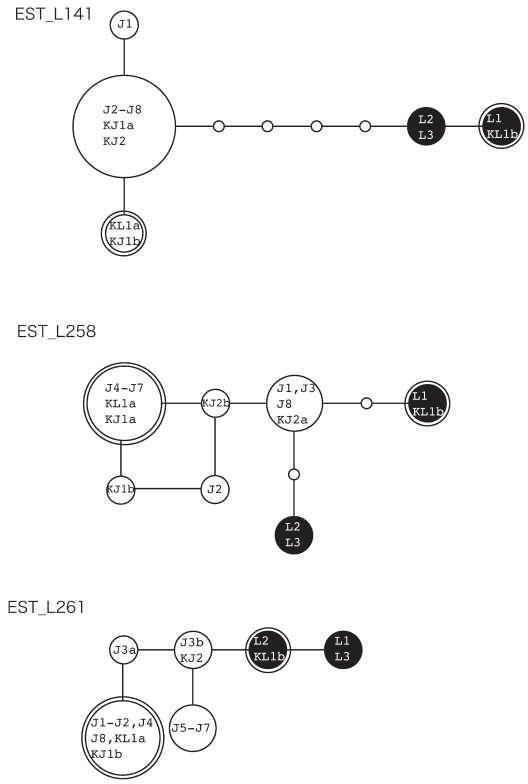

Fig. 4. Haplotype networks using three nuclear markers. Doubled circles indicate the haplotypes found in *O. lancea*-like plant. Size of each circle indicates the number of haplotypes (alleles) observed in all the examined individuals. White and black circle show haplotype derived from *O. japonica* and *O. lancea*, respectively. Sample information is shown in Appendix 1. J1–J8, *O. japonica*; L1–L3, *O. lancea*; KJ1–KJ2, *O. japonica* from the Kozu island; KL1, *O. lancea*-like plant from the Kozu island. Different alphabets following sample name indicate distinct alleles.

has two distinct alleles in all markers (Fig. 4); one is identical to a typical type of alleles in *O. lancea*, and the other is coincide with a typical type of alleles in *O. japonica*. There is no difference in the sequences of the chloroplast *rbcL* gene of the *O. lancea*-like plant from those of *O. japonica* and *O. lancea* (data not shown).

Discussion

Results of the morphological and molecular phylogenetic analyses suggested that the *Osmunda lancea*-like plant is *O.* × *intermedia*, a hybrid between *O. japonica* and *O. lancea.* It is the first record of *O.* × *intermedia* from the Izu Islands. It has untypical morphology among *O.* × *intermedia* plants; the angle of pinnule base is intermediate between those of *O. japonica* and *O.* × *intermedia*, although the number of veinlets in most basal basiscopic vein is similar to those of *O.* × *intermedia* (Fig. 3). It suggested that the Kozu Island plant is likely an F2 or later-generation hybrid. The genetically putative F2 hybrids or backcross individuals have extreme morphology in *O.* × *intermedia*, which is likely caused by segregation of genes for morphological characters (Yatabe *et al.*, 2009).

Osmunda japonica occurs in eastern Asia extending west to the western Himalaya and south to northern Vietnam (Kato, 2007). In Japan, it is widely distributed from northern Hokkaido to the Ryukyu Islands (Kurata and

Nakaike, 1990). Rheophilous *O. lancea* is endemic to Japan and has moderately narrow distribution area, from southern Hokkaido to Miyazaki Pref., southern Kyushu, and not distributed in the Ryukyu Islands (Kurata and Nakaike, 1990). The distribution of *O.* × *intermedia* almost overlaps that of *O. lancea* (Kurata and Nakaike, 1990). In the Izu Islands, however, *O. japonica* (one parent of *O.* × *intermedia*) is common on inhabited islands while *O. lancea*, another parent, is not recorded to occur (Kurata and Nakaike, 1990). It suggests that the Kozu Island *O.* × *intermedia* is considered not to be derived from outcrosses between the two extant neighboring parent plants. The Izu Archipelago is a chain of volcanic islands without any previous connection to the mainland. The oceanic islands were probably formed within the past million years (Kaneoka *et al.*, 1970). The Kozu Island is composed of at least 16 rhyolitic monogenetic volcanos and it has been inferred that the eruption of rhyolites began at least several tens of thousands years ago and was repeated intermittently (Isshiki, 1982). The latest and large eruption was at Mt. Tenjo in 838 AD according to Shoku Nihon Koki, a Japanese old historical literature, published in 869 AD (Isshiki *et al.*, 1965). One possible cause of the existence is that the establishment of *O.* × *intermedia* on the Kozu Island may be involved with a long-dispersal of spores of *O.* × *intermedia*, which is known as to be semifertile and has moderate spore germination rates (4–11%: Shimura, 1964). Another, less like possibility is that *O.* × *intermedia* was formed in situ by crossing of *O. japonica* and *O. lancea*, which became extinct or whose spores were dispersed recently.

Osmunda × *intermedia* usually grows in the upper rheophytic zone. However, the Kozu Island plant is found on the top of mountain. Its extreme morphology of narrow-lanceolate pinnules may happen to be appropriate to such a wind beaten area around the top of the mountain.

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Appendix 1. Localities, voucher information and sequence data deposited at DDBJ/GenBank for material of Osmundaceae used in molecular analyses. *; analyzed in Tsutsumi *et al.* (2012).

Osmunda × *intermedia*. **Japan**: KL1; Tokyo, Kodzu Isl., Mt. Tenjo; *K. Yamamoto et al. s.n.* (TNS); EST_L141, LC057166; EST_L258, LC057169; EST_ L261, LC057183.

Osmunda japonica. **Japan**: KJ1; Tokyo, Kodzu Isl., Mt. Chichibu; *N. Murakami et al. s.n.* (TNS); EST_L141, LC057164; EST_L258, LC057167; EST_L261, LC057181. KJ2; Tokyo, Kodzu Isl., Mt. Chichibu; *N. Murakami et al. s.n.* (TNS); EST_L141, LC057165; EST_L258, LC057168; EST_L261, LC057182. J1: Cult. in Tsukuba Botanical Garden (originated from Japan); *C. Tsutsumi s.n.* (TNS); EST_L141, LC057153; EST_L258, AB672830*; EST_L261, LC057170. J2; Miyazaki Pref., Kobayashi; *G. Kokubogata GK9416* (TNS); EST_L141, LC057154; EST_L258, AB672831*; EST_L261, LC057171. J3; Kagoshima Pref., Amami Island, Mt. Yuwan; *M. Kato et al. s.n.* (TNS: 764134); EST_L141, LC057155; EST_L258, AB672832*; EST_L261, LC057172. **China**: J4; Fujian, Mt. Wuyi; *A. Ebihara s.n.* (TNS: 762111); EST_L141, LC057156; EST_L258, AB672833*; EST_L261, LC057173. J5; Yunnan, Gongshan, Chichi; *L.-Y. Kuo 9468* (TAIF); EST_L141, LC057157; EST_L258, AB672834*; EST_L261, LC057174. **Bhutan**: J6; Sha-ngawang; *S. Matsumoto W.G.M.124* (TNS); EST_L141, LC057158; EST_L258, AB672835*; EST_L261, LC057175. **India**: J7; Meghalaya State, Shillong Peak ridge, by site of ruined "Peak Lodge"; *C. R. Fraser-Jenkins s.n.* (TNS: 740377); EST_L141, LC057159; EST_L258, AB672836*; EST_L261, LC057176. J8; Central Himalaya, from Fern Garden, Department of Botany, Government Postgraduate College, Pithoragarh, 262502. (Originated from Champawat); *C. R. Fraser-Jenkins s.n.* (TNS: 777785); EST_L141, LC057160; EST_L258, AB672837*; EST_L261, LC057177.

Osmunda lancea. **Japan**: L1; Cult. in Tsukuba Botanical Garden (transplanted from Shizuoka Pref.); *C. Tsutsumi s.n.* (TNS); EST_L141, LC057161; EST_L258, AB672838*; EST_L261, LC057178. L2; Kochi Pref.; *A. Ebihara et al., KC2007-1408* (TNS: 766627); EST_L141, LC057162; EST_L258, AB672839*; EST_L261, LC057179. L3; Miyazaki Pref.; *T. Minamitani s.n.* (TNS); EST_L141, LC057163; EST_L258, AB672840*; EST_L261, LC057180.