Morphological and Molecular Analyses of Rheophytic *Rhododendron ripense* and Its Allied Dryland Species *R. macrosepalum* (Ericaceae)

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(Received January 19, 2012; Accepted July 2, 2012)

The areas along rivers and streams are composed of particular environmental characteristics, and plants growing within this environment, called rheophytes, are subjected to flash floods after heavy rainfall as a strong selective pressure. To identify potential hybridization of the rheophytic *Rhododendron ripense* Makino and its allied dryland species *R. macrosepalum* Maxim., we conducted morphological and molecular analyses of these plant species. These analyses revealed a high rate of hybridization and introgression from *R. ripense* into *R. macrosepalum*, suggesting that hybrid plants arising from *R. ripense* and *R. macrosepalum* may develop broader leaves than *R. ripense* and may affect the flooding of river waters along the riverside.

Keywords : hybridization, phytochrome, rheophyte, *Rhododendron*

INTRODUCTION

Interspecific hybrids are very useful materials to introduce new genetic divergence from wild species into crops and horticultural species, and have been used for many horticultural and ornamental plants (Allard, 1960). The genus *Rhododendron* belongs to the family Ericaceae and contains many horticultural species, and most of which are hybrids that are propagated from cuttings or graftings. In fact, the hybrids of *Rhododendron* are immensely varied in color, size, shape and winter hardiness as a result of their complex genetic background. Plants can react to changes in their environment with a variety of short-term physiological and morphological responses, which occur within a framework of adaptations fashioned by natural selection and species diversity. Of the various environments, environments containing riparian vegetation are temporally and spatially heterogeneous as a result of fluvial disturbances and comprise numerous different habitat types, including channels, channel banks, floodplains, and wetland habitats. Riparian plant species exhibit a diversity of morphological adaptations, which enable them to persist in these variable and dynamic habitats. Among them, plant species in areas that sustain flash floods, known as rheophytes (van Steenis, 1981), have narrow leaves to adapt to the flow of river water (Kato and Imaichi, 1992; Imaichi and Kato, 1992). *Rhododendron ripense* Makino is a rheophytic species and semi-evergreen shrub endemic to Japan that is distributed in western Honshu, Shikoku, and northeastern Kyushu (Yamazaki, 1993). This species has brancelets and petioles with spreading glandular

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pilose hairs, densely mixed with ascending eglandular strigose hairs (Yamazaki, 1993). Recently, a comparative morphological and anatomical study of this species and its closely related dryland species (*R. macrosepalum* Maxim.) indicated that *R. ripense* (approx. 35.0 cm width) had narrower leaves than *R. macrosepalum* (approx. 55.3 cm width), and this stenophyllization in *R. ripense* appeared to be caused by a decrease in the number of cells found in the leaf-width direction (Ueda et al., 2012). These species could be distinguished with their leaf width, and there is a strong correlation between gross morphology and anatomy in leaves (Ueda et al., 2012).

In general, geographic and habitat isolation are among the most important factors responsible for establishing the level of reproductive segregation that leads to measurable genetic differentiation (Coyne and Orr, 2004). Moreover, since dispersal of individuals and gametes is generally more likely among geographically close demes, landscape discontinuities and distributional gaps result in increased neutral divergence (Devaux and Lande, 2008). Normally, the distributions of *R. ripense* (in western Japan) and *R. macrosepalum* (in eastern Japan) do not overlap; however, these distributions do overlap in some localities in the eastern Shikoku and Chugoku districts. Even though the habitats in which these species grow are different (riverside for *R. ripense* and forest for *R. macrosepalum*), the distance between populations of *R. ripense* and *R. macrosepalum* is very short in some areas, suggesting that there may be a natural hybridization between the species. Two other species, *R. x hortense* Nakai and *R. x mucronatum* (Blume) G. Don, are thought to be putative hybrids or represent a putative hybrid species originating from *R. ripense* and *R. macrosepalum* (Inobe, 1971; Yamazaki and Yamazaki, 1972). Natural hybridization is perceived as an important phenomenon in plant speciation (Arnold, 1997; Rieseberg et al., 2000). In particular, the family Ericaceae, including the genus *Rhododendron*, comprises many horticulture species via hybridization (Tolstead and Glencoe, 1991; Kehr, 1996; Barlup, 2002; Sakai et al., 2006; Jones et al., 2007).

Various molecular markers have been used to assess the genetic variability of wild plants, and chloroplast and nuclear markers provide information that allows researchers to estimate the putative parents involved in hybridization and introgression (Wendel and Doyle, 1998; Nishizawa and Watano, 2000). Previous studies indicated that polymorphisms in the intergenic region between *trnW* and *trnP* in chloroplast DNA (cpDNA) and the *phytochrome* gene in nuclear DNA (nrDNA) have proven to be effective tools with which to clarify the relationship of closely related taxa (Tsukaya et al., 2003; Samuel et al., 2005; Muleo et al., 2009). Among *phytochrome* genes, *phytochrome C* (*Phy C*) is used to construct phylogenetic tree of various taxa (Mathews and Donoghue, 2000; Samuel et al., 2005; Jabaily and Sytsma, 2010). To examine the rheophyte *R. ripense*, its allied dryland species *R. macrosepalum*, and their interspecific hybridization, we analysed their morphology, the intergenic region between *trnW* and *trnP* of cpDNA, and the *phytochrome C* (*PhyC*) gene of nrDNA using individual plants from specific areas in Shikoku, Japan.

MATERIALS AND METHODS

Plant materials and morphological analysis

All *R. ripense* and *R. macrosepalum* samples examined in this study were collected from the field in 2011 (Fig. 1). A total of 82 individuals, representing 8 populations, were sampled from across the range of both species (Tables 1 and 2). In particular, the 2 species could be distinguished by the number of stamen per plant, a qualitative morphological characteristic; *R. ripense* generally has more than 10 stamens, while *R. macrosepalum* has 5 (Yamazaki, 1993). Therefore, the number of stamens was counted for each individual plant.

Molecular analysis

For the molecular analysis, total DNA was isolated from 200 to 300 mg dry weight of leaves using a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, USA), according to the

Fig. 1 Sampling localities used in this study. See Table 1 for other abbreviations. □: *Rhododendron ripense*; ■: *R. macrosepalum*.

Table 1 Sampling localities of *Rhododendron ripense* and *R. macrosepalum*.

Species	No.	Locality	Latitude	Longitude	Sample nos. ^z	Individual number
<i>R. ripense</i>	1	Ehime Prefecture Minamiuwa-Gun, Ainan-Cho, Souzu River	N33°0'31"	E132°36'24"	2–10	8
	2	Kochi Prefecture Sukumo City, Matsuda Town, Matsuda River	N33°3'11"	E132°41'39"	11–28	10
	3	Kochi Prefecture Nagaoka-Gun, Motoyama-Cho, Asemi River	N33°46'51"	E132°41'39"	30–44	10
<i>R. macrosepalum</i>	4	Kochi Prefecture Aki-Gun, Umaji Village, Umaji	N33°33'14"	E134°2'58"	134–160	16
	5	Kochi Prefecture Kounan City, Noichi-Cho, Onishi	N33°34'15"	E133°42'12"	101	1
	6	Kochi Prefecture Kami City, Kahoku-Cho, Irooi	N33°39'3"	E133°45'70"	105–133	23
	7	Kochi Prefecture Aki-Gun, Yasuda-Cho, Segiri	N33°31'19"	E134°2'00"	102–104	3
	8	Tokushima Prefecture Kaiyo-Gun, Kaiyo-Cho, Hirai	N33°41'21"	E134°16'15"	163–180	11

^z Sample nos. correspond to Table 2.

manufacturer's protocol. We amplified the intergenic region between *trnW* and *trnP* from cpDNA and the *PhyC* gene from nrDNA with primers designed by Nishizawa and Watano (2000) and Samuel et al. (2005), respectively. Isolated DNA was amplified by PCR in a 50 µL reaction volume containing approximately 50 ng total DNA, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1.25 units *Taq* DNA polymerase (Takara), and 0.5 µM of each primer. We used the following thermal cycle profile for amplification: 1.5 min at 94°C, 2 min at 48°C, and 3 min at 72°C for 45 cycles, followed by a 15-min final extension at 72°C. After amplification, PCR products representing the intergenic region between *trnW* and *trnP* and those corresponding to the *PhyC* gene were subjected to electrophoresis on 1.0% low-melting-temperature agarose gels to remove by-products and purify the amplified products. We sequenced the purified PCR products using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied BioSystems) and an ABI Prism 3100 Genetic Analyzer (Applied BioSystems) according to the manufacturer's instructions.

Table 2 Morphological and molecular data for individuals collected at in Kochi Prefecture, Shikoku, Japan.

Individual no.	Morphology	<i>cp trnW-P</i>	<i>PhyC</i>	Individual no.	Morphology	<i>cp trnW-P</i>	<i>PhyC</i>
2	R/M	R	R	116	R/M	M	R/M
3	R/M	R	R	117	M	M	R/M
4	R	R	R	118	M	M	R
5	R	R	R	119	M	M	R/M
6	R	R	R	121	M	M	R
8	R	R	R	123	M	M	R
9	R	R	R	125	M	M	R
10	R	R	R	126	R/M	M	R/M
11	R	R	R	127	R/M	M	R/M
12	R	R	R	128	R/M	M	R/M
13	R	R	R	130	M	M	R/M
14	R	R	R	131	M	M	R/M
15	R	R	R	133	M	M	R
16	R	R	R	134	M	M	R/M
17	R	R	R	138	M	M	R
19	R/M	R	R	139	M	M	R
20	R	R	R	141	M	M	R
28	R	R	M	143	R/M	M	M
30	R	R	R	144	R/M	M	R
31	R	R	R	145	M	M	R
33	R	R	R	146	R/M	M	R/M
34	R	R	R	148	M	M	R/M
35	R/M	R	R	149	M	M	R
36	R/M	R	R	150	R/M	M	R/M
37	R	R	R	155	R/M	M	R
39	R	R	R	157	R/M	M	R/M
41	R	R	R	158	M	M	R/M
44	R	R	R	159	R/M	M	R/M
101	M	M	R	160	M	M	R
102	M	M	M	163	M	M	R
103	M	M	M	164	M	M	R/M
104	R/M	M	R/M	166	M	M	R/M
105	R/M	M	R	168	M	M	R/M
107	M	M	R/M	172	M	M	M
108	R/M	M	M	174	M	M	M
109	M	M	R	175	R/M	M	R
110	M	M	R	176	R/M	M	R/M
111	M	M	M	177	M	M	R
112	M	M	R	179	M	M	R/M
113	M	M	R	180	M	M	R/M
114	M	M	M				
115	M	M	R				

R, M, and R/M indicate *Rhododendron ripense*, *R. macrosepalum*, and heterozygous for *R. ripense* and *R. macrosepalum*, respectively, in each column.

RESULTS AND DISCUSSION

In this study, we recorded more than 10 stamens in *R. ripense* plants and 5 stamens in *R. macrosepalum* plants. We observed 21 intermediate types. With regards to the intergenic region between *trnW* and *trnP* from cpDNA, the length was 254 bp in both *R. ripense* and *R. Macrosepalum* (accession nos. AB692784, AB692785). The cpDNA sequences differed from each other by 2 nucleotide substitutions (Fig. 2) and were in accordance with the morphological identification conducted using stamen numbers. We found apparent difference in the cpDNA sequences

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R. ripense      1 CTGACTAAAGCGCTTTCTTATCAGAATAGATAAGACTGGAAAAAAGATTCTTTTGG 60
R. macrosepalum 1 CTGACTAAAGCGCTTTCTTATCAGAATAGATAAGACTGGAAAAAAGATTCTTTTGG 60
*****
R. ripense      61 AACGTC AATT CATCTTGTATACATATATATATATATATTATTATAGTAGTATCATAAAAATG 120
R. macrosepalum 61 AACGTC AATT CATCTTGTATACATATATATATATATATTATTATAGTAGTATCATAAAAATG 120
*****
R. ripense      121 ATATACTATGTATGTCCAATATGAATCTGATCCTTCGCTGCTTTTCAAAGGCGAAGTAAT 180
R. macrosepalum 121 ATATACTATGTATGTCCAATATGAATCTGATCCTTCGCTGCTTTTCAAAGGCGAAGTAAT 180
*****
R. ripense      181 AGGTAGGGATGACAGGATTTGAACCCGCGACATTTTGTACCCAAAACAAACGCGCTACCA 240
R. macrosepalum 181 AGGTAGGGATGACAGGATTTGAACCCGCGACATTTTGTACCCAAAACAAACGCGCTACCA 240
*****
R. ripense      241 AGCTGCGCCACATC 254
R. macrosepalum 241 AGCTGCGCCACATC 254
*****

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Fig. 2 Comparison of the intergenic sequences found between *trnW* and *trnP* (cpDNA) in *Rhododendron ripense* and *R. macrosepalum*. Mutation and nonmutation sites are shown in the box and asterisks, respectively.

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R. ripense      1 TAGAATGATATGTGATTGTTTGGCCCTCCAGTTAGGGTTATTCAAGACAAGAGATTGA 60
R. macrosepalum 1 TAGAATGATATGTGATTGTTTGGCCCTCCAGTTAGGGTTATTCAAGACAAGAGATTGA 60
*****
R. ripense      61 TGAACCATTTAGTCTTGGTGGATCCACATTAAAGGTCCTCATGGGTGTCATGCTCAGTA 120
R. macrosepalum 61 TGAACCATTTAGTCTTGGTGGATCCACATTAAAGGTCCTCATGGGTGTCATGCTCAGTA 120
*****
R. ripense      121 CATGGCGAATATGGGGTCCATTGCATCTCTTGTGTTATCCGTCACAATCAACAAGGAAGA 180
R. macrosepalum 121 CATGGCGAATATGGGGTCCATTGCATCTCTTGTGTTATCCGTCACAATCAACAAGGAAGA 180
*****
R. ripense      181 CCAGGAGTCAGAGAGTGATCCACAAAGGGGAAGAAAACGTGGGGTTTGGTTGTTGTCA 240
R. macrosepalum 181 CCAGGAGTCAGAGAGTGATCCACAAAGGGGAAGAAAACGTGGGGTTTGGTTGTTGTCA 240
*****
R. ripense      241 TCACACGAGCCCTAGGTTTGTCCGTTTCCCTTGAGGTACGCGTGTGAGTTCCTTAGTTCA 300
R. macrosepalum 241 TCACACGAGCCCTAGGTTTGTCCGTTTCCCTTGAGGTACGCGTGTGAGTTCCTTAGTTCA 300
*****
R. ripense      301 AGTTTTTGGTGTTTCAAGGAAGTAGAGATGGCGGTTCAACTGAGGGAGAAGCA 360
R. macrosepalum 301 AGTTTTTGGTGTTTCAAGGAAGTAGAGATGGCGGTTCAACTGAGGGAGAAGCA 360
*****
R. ripense      361 TATTTTGGCGACTCAAGCAGTGCTTTGTGACATGCTCCTAAGAGATGCTCCCGTTGGAAT 420
R. macrosepalum 361 TATTTTGGCGACTCAAGCAGTGCTTTGTGACATGCTCCTAAGAGATGCTCCCGTTGGAAT 420
*****
R. ripense      421 TATCAATCAATCACCAACGATGGATCTTGTCAATTGTGATGGAGCTGCAATTTATTA 480
R. macrosepalum 421 TATCAATCAATCACCAACGATGGATCTTGTCAATTGTGATGGAGCTGCAATTTATTA 480
*****
R. ripense      481 CAGGAACAAAGTTTGGTCTCTTGGGATCACCCTTCAGAGGCACAAATCAGAGATATAGC 540
R. macrosepalum 481 CAGGAACAAAGTTTGGTCTCTTGGGATCACCCTTCAGAGGCACAAATCAGAGATATAGC 540
*****
R. ripense      541 CGAATGGCTTATTGAGTACCACGGGGGAGTACAGGGTTAAGTACTGATAGCCTTATGGAA 601
R. macrosepalum 541 CGAATGGCTTATTGAGTACCACGGGGGAGTACAGGGTTAAGTACTGATAGCCTTATGGAA 601
*****

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Fig. 3 Comparison of the *PhyC* gene sequences (nrDNA) of *Rhododendron ripense* and *R. macrosepalum*. Mutation and nonmutation sites are shown in the box and asterisks, respectively.

between the *R. ripense*-type (R-type) and *R. macrosepalum*-type (M-type) plants (Table 2).

The length of the *PhyC* gene from nrDNA was 601 bp in both *R. ripense* and *R. macrosepalum* (Fig. 3: accession nos. AB692782, AB692783). We found 2 alleles in the *PhyC* sequence. All 37 *R. ripense* individuals, with the exception of sample number 28, had a *PhyC* allele, which we designated the R-type (Table 2). We also found another *PhyC* sequence, which differed from the R-type sequence by a single nucleotide substitution (Fig. 3). This sequence was mainly found in *R. macrosepalum* individuals in this study, and therefore, we named this sequence after the M-type *PhyC* sequence. One individual *R. ripense* plant and 8 *R. macrosepalum* plants were homozygous for the M-type *PhyC* sequence (Table 2). In addition, 23 individual *R. macrosepalum* plants had both R- and M-type *PhyC* sequences. The heterozygous type appeared to be a hybrid

between R-type and M-type sequences in the *PhyC* gene from nrDNA. These sequences have been deposited in the DDBJ/EMBL/GenBank International DNA databases (Table 3).

Hybridization can arise from unidirectional gene flow from one species to another or through bidirectional gene flow with both species serving as maternal and paternal parents. The direction of gene movement has important implications in our understanding of the evolutionary potential and sustainability of hybrid progenies. Contrasts between maternally inherited cpDNA and nrDNA inherited through both parents can provide confirmation of hybridization and evidence for the direction of gene flow (Rieseberg et al., 1990; Rieseberg and Ellstrand, 1993; Martinsen et al., 2001; Wu and Campbell, 2005; Wallace, 2006). For example, both parents contributed to the cytoplasmic diversity of the hybrid sunflower *Helianthus deserticola*, leading to high levels of differentiation among the population (Gross et al., 2003). The polyploid *Tragopogon* complex, *T. miscellus*, exhibits different floral morphologies depending on whether *T. dubius* or *T. pratensis* is the maternal parent (Tate et al., 2009). In our study, a combination of morphological traits and cpDNA/nrDNA analysis suggested that natural hybridization between *R. ripense* and *R. macrosepalum* occurred in some areas. Interestingly, all hybrids between the 2 species had M-type cpDNA sequences, and putative introgression was found in *R. ripense*, where sample No. 28 was found to have both R-type cpDNA and M-type nrDNA (Table 2). This result indicated that asymmetric hybridization could occur from *R. ripense* into *R. macrosepalum*. Some studies of natural hybrids have demonstrated asymmetric hybridization (Wolfe and Elisens, 1995; Buggs and Pannell, 2006; Hamzeh et al., 2007), suggesting that this phenomenon is common in natural systems (Currat et al., 2008). Asymmetric hybridization is much stronger in organelle genes than nuclear genes because the former experience lower rates of gene flow due to uniparental inheritance, and selection is a common explanation for asymmetric patterns of introgression (Lehman et al., 1991; Borge et al., 2005). For example, selection for pollen characteristics, such as large quantities of pollen production, gametic compatibility, and dispersal methods, could result in preferential gene flow to a species with lower pollen production or a less-effective means of pollen dispersal. In fact, Currat et al. (2008) found strong evidence of asymmetrical introgression in a wide variety of taxa. Our results describing asymmetrical introgression indicated that there were very few hybrids that were formed by the movement of *R. macrosepalum* genes into *R. Ripense*.

In summary, we demonstrated interspecific hybridization between rheophytic *R. ripense* and its allied dryland species *R. macrosepalum* by analyzing the combined morphological, cpDNA, and nrDNA data. The results, based on molecular information, provide an unbiased interpretation of hybridization. It will be interesting to see whether a more comprehensive sampling and additional genetic evidence will support the working hypothesis developed in this study. A molecular basis for further studies of *R. ripense* and *R. macrosepalum* plants has been established.

We wish to thank Drs. J. Yokoyama, O. Miura, R. Ueda, Y. Tsuchiya, Y. Yoshimi, S. Isomoto, H. Miyata, Y. Kumekawa, N. Kakimoto, S. Takei, and T. Sunami for providing much help. This study was partly supported by the River Fund in charge of the Foundation of River and Watershed Environment Management (FOREM), Japan (to T. Fukuda).

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