

在来種ネズミモチと移入種トウネズミモチ(モクセイ科) の保全遺伝学的研究

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Abstract

Genetic introgression from introduced, non-native species into native populations is a growing challenge for biological conservation, and one that raises unique practical and ethical issues. *Ligustrum lucidum* is native to China, and cultivated or used in gardens in various areas in Japan. Recently, some studies reported that this species escaped from cultivated areas and was sympatric with *L. japonicum*. This indicates that *L. japonicum* faces the ecological and genetic risk of hybridization and introgression with non-native *L. lucidum*. Therefore, we examined whether hybridization between *L. japonicum* and the non-native *L. lucidum* has occurred in a coexisting population using phenological and molecular analyses. The phenological results indicate that there is very little overlap in the flowering times of the two species. Moreover, molecular analyses using PCR-RFLP of chloroplast and nuclear DNA sequences could not detect any hybridization or introgression of *L. lucidum* and *L. japonicum* in the population.

Key words : hybrid, *Ligustrum*, molecular, native, PCR-RFLP

Introduction

The number of non-native species introduced worldwide has more than doubled as compared with estimates approximately three decades ago (Williamson and Fitter 1996a; 1996b). This is predominantly due to the growth in global trade and human mobility (Sala et al. 2000; Gozlan 2008). Biological invasions are now considered a major environmental issue of public concern. The introduction of a non-native species into an ecosystem is likely to present an ecological risk if the species is able to integrate successfully into the ecosystem (Gozlan and Newton 2009). This can result in interactions that are detrimental for native species or even cause malfunction of whole ecosystems. The introduced species such as *Festuca arundinacea* Schreb., *Pyracantha angustifolia* (Franch.) C.K.Schneid. and *Trifolium repens* L. could affect biodiversity

via competition for pollinator, hybridization, reduction of native species, habitat modification, and transmission of novel diseases (Nakatsubo 1997; Ishida et al. 1998; Okubo and Oka 2005). There are many such examples with some causing serious consequences for the conservation of biodiversity (Yoshioka 2005).

Ligustrum japonicum Thunb. is an evergreen shrub or small tree that grows 2-5 m tall and is native to central and southern Japan and Korea (Noshiro 2003). Its leaves are 5-10 cm long and 2-5 cm wide. The upper side of the leaf is glossy dark green, whereas the lower side is paler and glaucous to yellowish green. These leaves are thick and leathery with an entire margin. This species is believed to be closely related to the Chinese *L. lucidum* Aiton (Yoshinaga and Kameyama 2001; Matsui 2002), which grows to a height of up to 25 m.

Its leaves are a glossy dark green, 6–17 cm long, and 3–8 cm wide (Noshiro 2003). *Ligustrum lucidum* is non-native to Japan. It has been used as a garden shrub, and can therefore be found in various areas in Japan, such as in parks and grounds, along streets and rivers, and in other places. Thus, *L. lucidum* is an introduced species in Japan and growing in a new, artificial habitat (Yoshinaga and Kameyama 2001). Recently, there has been a marked increase in the problems arising from the accidental or deliberate escape of non-native plant species into the wild. In fact, some studies have reported the escape of *L. lucidum* from its cultivated areas, and the two *Ligustrum* species are now sympatric in Japan (e.g., Hashimoto et al. 2003). The escapes of *L. lucidum* can pose a considerable ecological threat, not only through direct competition, but also through hybridization with native species (Yoshinaga and Kameyama 2001). Although any natural hybrids between *L. japonicum* and *L. lucidum* have not been reported up to now, artificial crossing experiments showed that the two species could produce hybrids (Van Huylenbroeck et al. 2004).

Genetic methods provide powerful tools to identify cryptic species or species with few readily identifiable phenotypic characteristics (Marhold et al. 2002). Furthermore, the use of both chloroplast and nuclear genetic markers allows the identification of hybrid species, because it can reveal the contributions of both the father and mother plants to the hybrid genome. Hybridization and introgression are most commonly identified by the heterogeneity of nuclear DNA (nrDNA) and incongruence between chloroplast DNA (cpDNA) and nuclear DNA (nrDNA) that may indicate different parental contributions to the hybrid genome. This suggests that it is useful to use both cpDNA and nrDNA to detect interspecific gene flow and subsequent chloroplast capture. Therefore, in order to test whether *L. japonicum* and *L. lucidum* produce hybrids in a region of natural sympatry, we conducted molecular analyses based on cpDNA and nrDNA sequences of individuals derived from areas where the two species coexist. Additionally, we observed flowering phenology of the

two species to examine the potential for hybridization.

Materials and methods

Phenological analysis of *Ligustrum japonicum* and *L. lucidum*

During the flowering season of 2009, we conducted a field survey to examine flowering times of *L. japonicum* and *L. lucidum*. Our research area is Mt. Godaisan of Kochi city, Japan (N 33°32'; E 133°35'), where both species coexist. The numbers of *L. japonicum* and *L. lucidum* individuals used for the analysis were 18 and 27, respectively. During the flowering season from June to July, flowering observations were conducted two to four times per week for each species. In the flowering time, we counted the number of panicles and calculated the flowering ratio (flowering panicles per total panicles) of each species.

Sequencing analysis

For the molecular analyses, total DNA was isolated from 200–300 mg of dried or fresh leaves using a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, USA), according to the manufacturer's protocol. After this, we amplified the *trnT-trnL* from cpDNA and the internal transcribed spacer (ITS) region from nrDNA using primers designed by Taberlet et al. (1991) and White et al. (1990), respectively. For partial samples, we used our original primers (a1: 5'-CTT TCT ATC CAT AGG AAT TC-3' and b1: 5'-ATT CAT TAG ATA CTG ATT AC-3' for the *trnT-trnL*, F1: 5'-CGA AAC CTG CAA AGC AGA CC-3' and R1: 5'-CTG TCG AAG TGC AAA GCA CC-3' for the ITS). 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) with 50 mM KCl and 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 extension at 72°C. We preliminary sequenced the ITS and the *trnT-trnL* regions from the parental species, and used the result in order to ensure that PCR-RFLP can effectively demonstrate

hybridization events. After amplification, PCR products of the *trnT-trnL* as well as of the ITS region, were subjected to electrophoresis in 1% low-melting-temperature agarose gels with the aim to remove by-products and purify the amplified products. We sequenced the purified PCR products using BigDye Terminator ver. 3.1 (Applied BioSystems) and ABI Prism 3100 Genetic Analyzer (Applied BioSystems) according to the manufacturer's instructions. For sequencing, we used the same primers as those used for amplification.

PCR-RFLP analysis

A total of 421 individuals, including seed-

lings, from the coexisting population of *L. japonicum* and *L. lucidum* in the Mt. Godaisan were analysed. We carried out PCR-RFLP analysis after checking the sequencing results and alignments. The alignments indicated that *Mse* I restriction sites might be useful to distinguish the two species in the *trnT-trnL* of cpDNA and in the ITS of nrDNA. Amplified products were digested with *Mse* I at 37°C for more than 1 hour. Digested DNA fragments were separated on 1.5% agarose gels, and the size of each band was determined.

Phylogenetic analysis

To reveal the genetic relationships between

Table 1. *Ligustrum* taxa used for isolation of DNA sequences, which, together with the listed database accession numbers of sequences, were used in the phylogenetic analysis

Species name	OTU name	Accession No.	Reference
<i>Ligustrum acutissimum</i> Koehne	<i>L. acutissimum</i>	AF361295	Li et al. (2002)
<i>L. compactum</i> (Wall. ex G.Don) Hook.f. & Thomson ex Decne.	<i>L. compactum</i>	AF361292	Li et al. (2002)
<i>L. confusum</i> Decne.	<i>L. confusum</i>	JF830353	Gu et al. (2011)
<i>L. delavayanum</i> Har.	<i>L. delavayanum</i>	JF830384	Gu et al. (2011)
<i>L. expansum</i> Rehder	<i>L. expansum</i>	JF830359	Gu et al. (2011)
<i>L. gracile</i> Rehder	<i>L. gracile</i>	JF830349	Gu et al. (2011)
<i>L. henryi</i> Hemsl.	<i>L. henryi</i>	JF830374	Gu et al. (2011)
<i>L. ibota</i> Siebold	<i>L. ibota</i>	AF361297	Li et al. (2002)
<i>L. japonicum</i> Thunb.	<i>L. japonicum</i> -1	AB610486	This study
<i>L. japonicum</i> Thunb.	<i>L. japonicum</i> -2	AB610487	This study
<i>L. japonicum</i> Thunb.	<i>L. japonicum</i> -3	AB610488	This study
<i>L. japonicum</i> Thunb.	<i>L. japonicum</i> -4	AF361299	Li et al. (2002)
<i>L. liukiense</i> Koidz.	<i>L. liukiense</i>	AB610489	This study
<i>L. lucidum</i> Aiton	<i>L. lucidum</i> -1	AB610490	This study
<i>L. lucidum</i> Aiton	<i>L. lucidum</i> -2	AB610491	This study
<i>L. lucidum</i> Aiton	<i>L. lucidum</i> -3	AB610492	This study
<i>L. massalongianum</i> Vis.	<i>L. massalongianum</i>	AF361293	Li et al. (2002)
<i>L. micranthum</i> Zucc.	<i>L. micranthum</i>	AB610493	This study
<i>L. obtusifolium</i> Siebold. & Zucc.	<i>L. obtusifolium</i> -1	AF361294	Li et al. (2002)
<i>L. obtusifolium</i> Siebold. & Zucc.	<i>L. obtusifolium</i> -2	HM751203	Kim and Kim (2010)
<i>L. ovalifolium</i> Hassk.	<i>L. ovalifolium</i> -1	AB610494	This study
<i>L. ovalifolium</i> Hassk.	<i>L. ovalifolium</i> -2	AF361296	Li et al. (2002)
<i>L. quihoui</i> Carrière.	<i>L. quihoui</i>	FJ980379	Chen and Han (unpubl.)
<i>L. robustum</i> Blume	<i>L. robustum</i>	JF830380	Gu et al. (2011)
<i>L. sempervirens</i> Lingelsh.	<i>L. sempervirens</i>	AF361300	Li et al. (2002)
<i>L. sinense</i> Lour. var. <i>sinense</i>	<i>L. sinense</i> var. <i>sinense</i>	JF830362	Gu et al. (2011)
<i>L. strongylophyllum</i> Hemsl.	<i>L. strongylophyllum</i>	JF830401	Gu et al. (2011)
<i>L. vulgare</i> L.	<i>L. vulgare</i> -1	AF361298	Li et al. (2002)
<i>L. vulgare</i> L.	<i>L. vulgare</i> -2	EU314901	Wallander (2008)
Outgroup			
<i>Fraxinus floribunda</i> Wall.	<i>Fraxinus floribunda</i>	AF135190	Wallander (2008)
<i>Osmanthus fragrans</i> Lour.	<i>Osmanthus fragrans</i>	EF362771	Tsai and Huang (unpubl.)
<i>O. heterophyllum</i> (G.Don) P.S.Green	<i>O. heterophyllum</i>	EF362771	Lu et al. (unpubl.)
<i>Syringa vulgaris</i> L.	<i>Syringa vulgaris</i>	AF361291	Li et al. (2002)
<i>S. oblata</i> Lindl. ex Carrière	<i>S. oblata</i>	AF361288	Li et al. (2002)

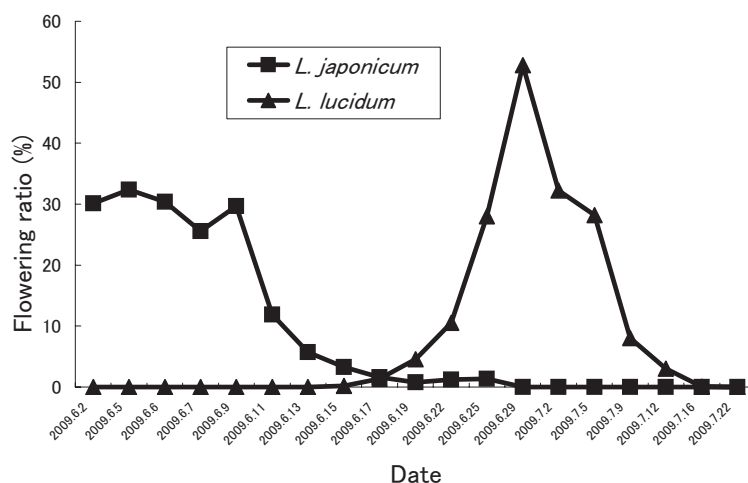


Fig. 1. Phenologies of *Ligustrum japonicum* and *L. lucidum*. Squares indicate *L. japonicum* and triangles indicate *L. lucidum*.

the species, we examined 29 sequences of *Ligustrum* species in our phylogenetic analysis using the ITS of nrDNA (Table 1). *Fraxinus floribunda* Wall., *Osmanthus fragrans* Lour., *O. heterophyllus* (G.Don) P.S.Green, *Syringa vulgaris* L., and *S. oblata* Lindl. were selected as outgroups on the basis of the result of previous phylogenetic analyses including the genus *Ligustrum* (Li et al. 2002). Vouchers of plants used in this study have been deposited in the Herbarium, Makino Botanical Garden, Kochi (MBK).

To construct phylogenetic trees, the sequences were aligned using Clustal W (Thompson et al. 1994) with default settings, followed by manual adjustment. The positions of insertions and deletions (InDels) were excluded for phylogenetic analysis. To assess the relationships between

sequences, we constructed phylogenetic trees from the DNA sequences using neighbour-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) methods. The best-fit model of sequence evolution was calculated using the Akaike information criterion (AIC) with PAUP 4.0b10 (Swofford 2002) and Modeltest v3.7 (Posada and Crandall 1998). For the BI analysis, on the other hand, MrModeltest v2.3 (Nylander et al. 2004) was used. NJ and ML analyses were performed under the best-fit model using PAUP 4.0b10 and TreeFinder (Jobb et al. 2004), respectively. For the NJ and ML analyses, confidence in tree topology was tested using non-parametric bootstrap analysis (Felsenstein 1985) with 1000 replicates. For BI analyses, posterior probabilities were obtained from a Metropolis-coupled Markov chain

Table 2. Origins of *Ligustrum* species and voucher numbers in MBK

Species name	OTU name	Source locality	Voucher
<i>Ligustrum japonicum</i>	<i>L. japonicum</i> -1	Mt. Godaisan, Kochi City, Kochi Prefecture, JAPAN	----- ¹⁾
<i>L. japonicum</i>	<i>L. japonicum</i> -2	Inukai Shrine, Sogabe Town, Kameoka City, Kyoto Prefecture, JAPAN	MBK 0178658
<i>L. japonicum</i>	<i>L. japonicum</i> -3	Heitateyama Park, Okayama City, Okayama Prefecture, JAPAN	-----
<i>L. liukiense</i>	<i>L. liukiense</i>	Isen Town, Kagoshima Prefecture, JAPAN	MBK 0055167
<i>L. lucidum</i>	<i>L. lucidum</i> -1	Mt. Godaisan, Kochi City, Kochi Prefecture, JAPAN	-----
<i>L. lucidum</i>	<i>L. lucidum</i> -2	Heitateyama Park, Okayama City, Okayama Prefecture, JAPAN	-----
<i>L. lucidum</i>	<i>L. lucidum</i> -3	Golden Temple, Municipality, Kunning, Yunnan, CHINA	MBK 0024365
<i>L. micranthum</i>	<i>L. micranthum</i>	Between Takinoura and Mt. Mikaeriyama, Ani island, Ogasawara Archipelago, JAPAN	MBK 0055164
<i>L. ovalifolium</i>	<i>L. ovalifolium</i> -1	Shinajima island, Minamichita Town, Aichi Prefecture, JAPAN	MBK 0057269

1) No voucher. MBK: Herbarium of the Makino Botanical Garden, Kochi.



Fig. 2. Phylogeny of *Ligustrum* species based on ITS sequences of nuclear DNA using Bayesian method. Numbers below branches indicate posterior probabilities (>90%) for Bayesian tree and bootstrap values (>50%) for NJ and ML trees.

Monte Carlo simulation (two independent runs; four chains with 1.0×10^6 generations each; chain temperature: 0.2; trees sampled every 100 generations; deviation of split frequencies < 0.01), with parameters estimated from the data set. A consensus topology was calculated for 7500 trees after discarding the first 2500 trees (burn-in = 25%) using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). Corrected evolutionary distances were computed using PAUP 4.0b10 and the best-fit model including all codon positions.

Results

Phenological analyses of *Ligustrum japonicum* and *L. lucidum*

The flowering time of *L. japonicum* and *L. lucidum* differed markedly in the examined area (Fig. 1). The flowering time of *L. japonicum* covered the period from mid-May until mid-June, but that of *L. lucidum* started mid-June and finished mid-July in 2009. The flowering of *L. lucidum* occurred approximately one month after *L. japonicum* flowered. Flowering times of *L. japonicum* and *L. lucidum* over-

lapped in the middle of June. However, the overlap lasted only nine days, and the flowering ratios of the both species were very low in the overlapping period.

PCR-RFLP analyses of *Ligustrum japonicum* and *L. lucidum*

We determined the sequences of the *trnT-trnL* of cpDNA and the ITS region of nrDNA for 13 *L. lucidum* and 12 *L. japonicum* samples collected from different localities (Table 2). The length of the *trnT-trnL* alignment is 574 bp. Based on the sequence data, we found that the *trnT-trnL* sequences of *L. lucidum* and *L. japonicum* have two and three *Mse* I sites (TTAA), respectively. Moreover, the ITS sequences of *L. lucidum* and *L. japonicum* have three and two *Mse* I sites, respectively. These sequences have been deposited in the DDBJ/EMBL/GenBank International DNA databases (*trnT-trnL*: AB634833-4 and ITS: AB610486-92). Based on these findings, we anticipated that we could use these restriction sites for species identification based on DNA samples of the different individuals. If hybrids are present, the nrDNA sequence results at the polymorphic sites cannot be determined using direct sequences method of PCR products because of heterozygosity. Therefore, we conducted PCR-RFLP to examine whether hybrids are present in the population.

We identified 322 *L. japonicum* individuals and 99 *L. lucidum* individuals based on the PCR-RFLP analyses of cpDNA and nuclear ITS sequences. If hybrid and introgression plants exist, they would show a combined band pattern of nrDNA (hybridization), or incongruent results for cpDNA and nrDNA (introgression). However, we could not detect these band patterns in any of the individuals from the coexisting population.

Phylogenetic relationships of *Ligustrum* species

We reconstructed phylogenetic trees of nuclear ITS sequences based on the NJ, ML, and BI methods (Fig. 2). These different methods generated mostly congruent topologies. Thus the BI tree with the support of Bayesian posterior probabilities, NJ bootstrap, and ML bootstrap are shown in Fig. 2. The Accession numbers

used in this phylogenetic analysis are shown in Table 1. In all trees, species of *Ligustrum* formed a monophyletic group. Moreover, all individuals of *L. japonicum* were included in a monophyletic group with *L. liukiuense* Koidz. and *L. micranthum* Zucc., which had relative high bootstrap values (BI: 94%; NJ: 85%; ML: 76%). While, all individuals of *L. lucidum* formed a monophyletic group with *L. gracile* Rehd. From each one of the trees it was deduced that *L. japonicum* was not the most closely related species to *L. lucidum*.

Discussion

In our PCR-RFLP analyses, we failed to detect any hybrids between the native (in Japan) *L. japonicum* and the non-native *L. lucidum*. Moreover, Kiyota et al. (2011) also could not detect any hybrids between *L. japonicum* and *L. lucidum* by amplified fragment length polymorphism (AFLP) analysis using multiple populations in Kanto district. Therefore, these results suggest that the two species would not generate hybrids under the natural condition in various areas of Japan. The lack of hybrids even in coexisting sites may be explained by the considerable differences of the flowering phenology of them (Fig. 1). Therefore, the risks to introduce *L. lucidum*, which is included in a list of “Other Alien Species” provided on the Invasive Alien Species Act in Japan would be mainly its competitive habits such as motives for pollinators and seed dispersers, high fitness for environments in city, and expansion of its distributions (Yoshinaga and Kameyama 2001). In addition, our phylogenetic results indicated that *L. lucidum*, which can make the artificial hybrid with *L. japonicum*, have close relationships with *L. gracile* (Fig. 2), implying that hybridization between *L. japonicum* and Chinese *L. gracile*, which flowers for May to August (Chang et al. 1996) could occur. Therefore care should be taken at import and utilization of *L. gracile* in Japan, because the flowering times of *L. gracile* and *L. japonicum* overlap. In the future, it needs further analyses to protect Japanese *Ligustrum* from genetic disturbance of non-native species.

This is the first report contributing to our

knowledge of the phylogenetic positions of *L. liukiense* and *L. micranthum*. Moreover, our phylogenetic result indicated that *L. liukiense* and *L. micranthum*, which are endemic species of Japan, were closely related to *L. japonicum*, suggesting that these three species originated in Japan. The flowering phenologies of *L. liukiense* and *L. micranthum* are earlier than those of *L. japonicum* (Noshiro 2003), therefore, we consider that the risk of hybridization between *L. lucidum* and *L. liukiense* or *L. micranthum* is very low. However, it may be easy to affect the ecological pressure by escaping and/or artificial colonization of *L. lucidum* to *L. liukiense* and *L. micranthum* because their distribution areas are very small.

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- 齋藤倫広¹・早川宗志^{1,2}・南谷幸雄¹・村松優子³・平田亜也³・植田諒介¹・松山佳那子³・大賀教平³・横山菜々子³・室井美和子³・伊藤 桂¹・荒川 良¹・福田達哉^{1,*}: 在来種ネズミモチと移入種トウネズミモチ (モクセイ科) の保全遺伝学的研究
- 移入種から在来種への遺伝子移入は生物学的保全のための懸念課題であり、実用的かつ倫理的な問題を提起している。中国原産のトウネズミモチ (*Ligustrum lucidum*) は日本の広い地域で園芸として栽植されているが逸出により分布を拡大しており、在来種のネズミモチ (*L. japonicum*) と同所的に生育していることが近年報告されている。このため、ネズミモチはトウネズミモチとの雑種形成や浸透交雑の生態学的リスクにさらされていると考えられる。そこで本研究では、在来種のネズミモチと移入種のトウネズミモチの間での雑種形成による遺伝的攪乱の有無を明らかにすることを目的として、開花期調査および分子遺伝学的調査を行った。開花期の調査結果より、両種の開花期がずれていることが明らかとなった。PCR-RFLP解析の結果、ネズミモチとトウネズミモチの間に交雑個体および浸透交雑個体を検出することはできなかったため、両種間での交雑は起こっていないと考えられる。
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