

INTERSECTIONAL GENE FLOW BETWEEN INSULAR ENDEMICS OF *ILEX* (AQUIFOLIACEAE) ON THE BONIN ISLANDS AND THE RYUKYU ISLANDS¹

HIROAKI SETOGUCHI^{2,4} AND ITTA WATANABE³

²Department of Natural Environmental Sciences, Faculty of Integrated Human Studies, Kyoto University, Kyoto 606-8501, Japan; and

³Makino Herbarium, Faculty of Science, Tokyo Metropolitan University, Minami-Osawa, Hachioji, Tokyo 192-03 Japan

Hybridization and introgression play important roles in plant evolution, and their occurrence on the oceanic islands provides good examples of plant speciation and diversification. Restriction fragment length polymorphisms (RFLPs) and *trnL* (UAA) 3'exon-*trnF* (GAA) intergenic spacer (IGS) sequences of chloroplast DNA (cpDNA), and the sequences of internal transcribed spacer (ITS) of nuclear ribosomal DNA were examined to investigate the occurrence of gene transfer in *Ilex* species on the Bonin Islands and the Ryukyu Islands in Japan. A gene phylogeny for the plastid genome is in agreement with the morphologically based taxonomy, whereas the nuclear genome phylogeny clusters putatively unrelated endemics both on the Bonin and the Ryukyu Islands. Intersectional hybridization and nuclear gene flow were independently observed in insular endemics of *Ilex* on both sets of islands without evidence of plastid introgression. Gene flow observed in these island systems can be explained by ecological features of insular endemics, i.e., limits of distribution range or sympatric distribution in a small land area.

Key words: Aquifoliaceae; cpDNA; hybridization; *Ilex*; introgression; island; ITS.

Hybridization and introgression play important roles in plant evolution, as has been discussed at length (reviewed in Rieseberg and Brunsfeld, 1991; Rieseberg and Wendel, 1993; Rieseberg, 1995). The detection of cytoplasmic DNA and nuclear DNA variation has generated many empirical demonstrations of hybridization and introgression (e.g., Whittemore and Schaal, 1991; Wendel, Stewart, and Rettig, 1991; Dorado, Rieseberg, and Arias, 1992; Arnold, 1993; Brubaker, Koontz, and Wendel, 1993; Kron, Gawen, and Chase, 1993; Rieseberg and Wendel, 1993; Watano, Imazu, and Shimizu, 1995, 1996). In many cases, the hybridization and introgression investigated were interspecific and detected as asymmetric gene flow between cytoplasmic and nuclear loci. Cytoplasmic gene flow is frequently observed with or without evidence of nuclear introgression (Rieseberg, Choi, and Ham, 1991; reviewed in Rieseberg and Soltis, 1991; Rieseberg and Wendel, 1993). On the other hand, only two examples of nuclear introgression without cytoplasmic gene flow have been reported (Wagner et al., 1987; Arnold, Buckner, and Robinson, 1991).

The island system provides good examples of plant

speciation and diversification. Many cases of hybridization and/or introgression in insular plants have been reported previously (Gillet and Lim, 1970; Borgen, 1976; Brochmann, 1984, 1987; Helenurm and Ganders, 1985; Lowrey and Crawford, 1985; Carr and Kyhos, 1986; Lowrey, 1986; Witter and Carr, 1988; Liston, Rieseberg, and Mistretta, 1990; Pacheco, Stuessy, and Crawford, 1991; Francisco-Ortega et al., 1996; Francisco-Ortega, Jansen, and Santos-Guerra, 1996), and these processes generally have been regarded as important factors in the evolution and speciation of insular plants. The chief advantages of an island system are that it usually presents a small land area and that endemics are sympatrically or adjacently distributed. Hybridization and introgression between various species frequently occur in the regions of sympatry, or at the edges of zones where species distributions come into contact with each other (Rieseberg and Brunsfeld, 1991) and insular endemics are growing due to conditions that readily induce gene flow. The insular endemics are small in terms of population size and show low genetic diversity (e.g., Helenurm and Ganders, 1985; Lowrey and Crawford, 1985; Crawford, Stuessy, and Silva, 1987; Witter and Carr, 1988; Wendel and Percival, 1990; Ito and Ono, 1990; Ito et al., 1990; Crawford et al., 1992; Soejima et al., 1994; Ito, Soejima, and Ono, 1997). Thus, island systems are good arenas in which to investigate the gene flow among endemic species. The Bonin Islands and Ryukyu Islands in Japan (Fig. 1) are typical island systems in that they consist of a small land-mass rich with endemic species. *Ilex* (Aquifoliaceae) is one of the three genera (*Ilex*, *Ficus*, and *Symplocos*) that occur in insular speciation in both the Bonin Islands system and the Ryukyu Islands system.

The Bonin (Ogasawara) Islands are located in the western part of the North Pacific Ocean, ~1000 km south of Tokyo (Fig. 1), and consist of 20 islands scattered be-

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⁴ Author for correspondence.

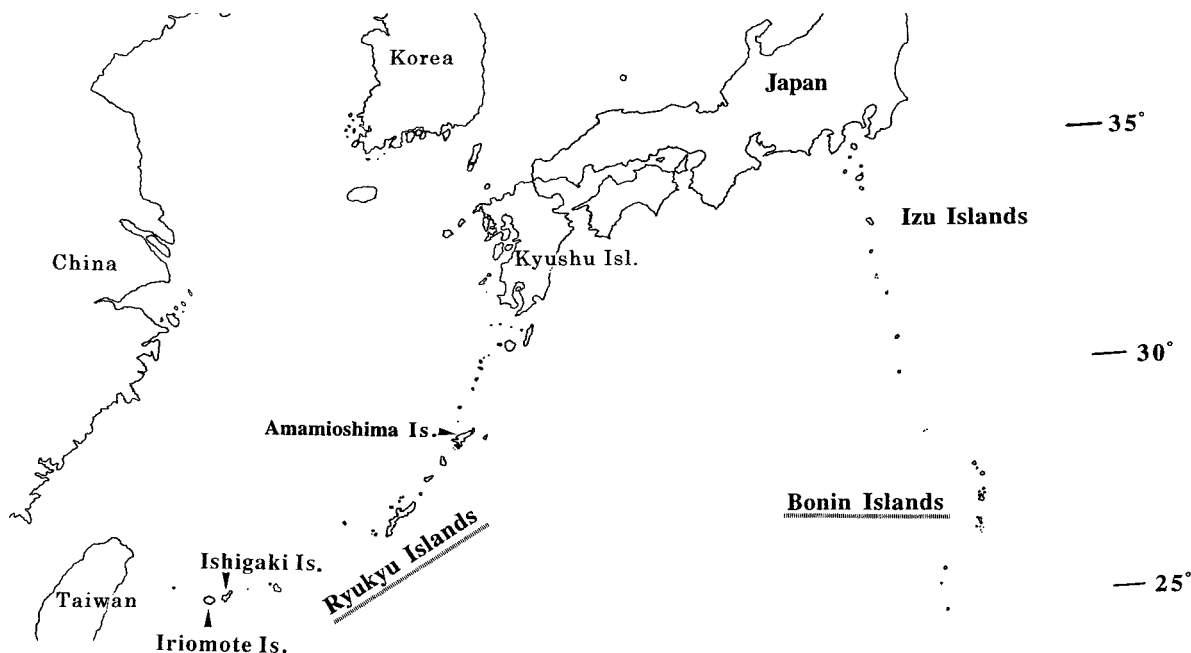


Fig. 1. The study area encompassing the Bonin and the Ryukyu Islands is shown.

tween 26°30' and 27°40' N and between 142°00' and 142°15' E. The archipelagos are of Tertiary origin and are ~3–5 million yr old (Asami, 1970). The Bonin Islands have been studied extensively with regard to plant speciation and diversification. The plant groups of oceanic islands that are isolated from, and have never been connected to, continental landmasses show a tendency to undergo rapid adaptive radiation from one or several ancestral species that migrated by means of long-distance dispersal. All four genera investigated (*Crepidiastrum*, *Machilus*, *Pittosporum*, and *Symplocos*) are monophyletic groups that originated from an ancestral species on the Bonin Islands (Ito and Ono, 1990; Ito et al., 1990; Soejima et al., 1994; Ito and Pak, 1996). Only four *Ilex* species are extant there, all of them endemic to the islands. *Ilex matanoana* Makino, a small tree, is the only species from section *Polyphyllae* that is present on the islands, whereas *I. mertensii* Maxim., *I. percoriacea* Tsuyama, and *I. beecheyi* (Loes.) Makino, all medium-sized trees, are endemics that fall under the section *Ilex*. The sections *Polyphyllae* and *Ilex* show the following distinctive differences in their morphological characteristics: the color of their mature fruits (black in section *Polyphyllae*, red in section *Ilex*); the position of flowers in the axils (peduncles in the axils of the current year's branches in section *Polyphyllae*, peduncles in the axils of the preceding year's branches in section *Ilex*); and the presence or absence of dots on the abaxial side of leaves (present in section *Polyphyllae*, absent in section *Ilex*). The morphological differences between *Ilex matanoana* (section *Polyphyllae*) and the other three species (section *Ilex*) are very distinct. No plants of intermediate morphology between the two sections have been noted, and morphological evidence does not suggest intersectional hybridization and introgression of the *Ilex* species on the Bonin Islands.

The Ryukyu Islands, continental islands located in an

arc between Kyushu of Japan and Taiwan, consist of 120 islands scattered between 24°00' and 30°50' N, and between 123°00' and 131°10' E (Fig. 1). The islands are the product of repeated formation and division of the landbridge across the Chinese continent and on through Taiwan, Okinawa, Amamioshima, and Kyushu by means of transgression and regression during the Pliocene and Quaternary periods (Kimura, 1996). The hypothesis that the landbridge was present at several times is also supported by the vicariance of *Trimeresurus* (a land snake; Hanzawa, 1935), *Cuora flavomarginata* (a land turtle), and several mammals (Hasegawa, 1980). Eight species of *Ilex* (*Ilex maximowicziana* Loes., *I. rotunda* Thunb., *I. integra* Thunb., *I. dimorphophylla* Koidz., *I. warburgii* Loes., *I. liukiensis* Loes., *I. goshiensis* Hayata, and *I. macrocarpa* Oliver) from four sections are distributed in the islands (see Table 1), and four of these eight species are insular endemics of the Ryukyu Islands. *Ilex maximowicziana* (section *Polyphyllae*), a small tree, is endemic to Ishigaki Island and Iriomote Island of the Ryukyu Islands. *Ilex dimorphophylla* Koidz. (section *Ilex*) is an endemic species to Amamioshima Island of the Ryukyu Islands, and *I. warburgii* Loes. and *I. liukiensis* Loes. are endemic to the southern part of the Ryukyu Islands. The morphological differences among the sections are distinctive. The sectional differences between *Polyphyllae* and *Ilex* are particularly distinct, as mentioned above, and no plants of intermediate morphology between these two sections have been noted in the Ryukyu Islands.

In the present study, we attempted to clarify the presence of the intersectional gene flow of *Ilex* both on the Bonin Islands and the Ryukyu Islands by examining restriction fragment length polymorphisms (RFLPs) of chloroplast DNA (cpDNA), sequences from the intergenic spacer (IGS) between the *trnL* (UAA) 3'exon and *trnF* (GAA) gene of cpDNA, and sequences from the internal transcribed spacer (ITS) of nuclear ribosomal

TABLE 1. Total extracted DNA and vouchers of the specimens examined in the present study.

Section	Species	Source	Voucher (MAK)
<i>Polyphyllae</i>	<i>Ilex crenata</i> Thunb.	Hachioji, Tokyo	Setoguchi IL 9332
	<i>I. maximowicziana</i> Loes.	Ishigaki Isl.	Setoguchi and Yokoyama IL9345, Setoguchi IL9820, IL9821, IL9822, IL9824, IL9826
		Iriomote Isl.	Setoguchi IL9811, IL9812, IL9815, IL9816, IL9817, IL9818, IL9819
	<i>I. mutchagara</i> Makino	Okinawa Isl.	Niizato s.n.
	<i>I. matanoana</i> Makino	Bonin Isl.	Setoguchi et al. IL9317, IL9412, IL9414, IL9701, IL9702, IL9703, IL9704, IL9705, Hirota s.n. (no voucher)
<i>Paltoria</i>	<i>I. pedunculosa</i> Miq.	Cultivated ^a	Setoguchi IL9305
	<i>I. rotunda</i> Thunb.	Iriomote Isl.	Setoguchi and Yokoyama IL9325
<i>Ilex</i>	<i>I. integra</i> Thunb.	Hachioji, Tokyo	Setoguchi IL9302
	<i>I. dimorphophylla</i> Koidz.	Cultivated ^a	Setoguchi IL9301, IL9437
		Amami Isl.	Setoguchi IL9804, IL9805, IL9806
	<i>I. latifolia</i> Thunb.	Cultivated ^a	Setoguchi IL9303
	<i>I. buergeri</i> Miq.	Cultivated ^b	Yamada s.n.
	<i>I. kiusiana</i> Hatusima	Cultivated ^b	Yamada s.n.
	<i>I. warburgii</i> Loes.	Ishigaki Isl.	Setoguchi and Yokoyama IL9327
	<i>I. beecheyi</i> (Loes.) Makino	Bonin Isl.	Setoguchi et al. IL9416, IL9420, IL9422, IL9706, IL9707, IL9708
	<i>I. mertensii</i> Maxim.	Bonin Isl.	Setoguchi et al. IL9415, IL9430, IL9431, IL9718, IL9719, IL9720
	<i>I. percoriacea</i> Tuyama	Bonin Isl.	Setoguchi et al. IL9401, IL9425, IL9426, IL9712, IL9713
	<i>I. liukiuensis</i> Loes.	Ishigaki Isl.	Setoguchi and Yokoyama IL9312
Rugosae	<i>I. rugosa</i> Fr. Schm.	Mt. Kitadake	Watanabe s.n.
Prinos	<i>I. serrata</i> Thunb.	Hachioji, Tokyo	Setoguchi IL9331
Micrococca	<i>I. micrococca</i> Maxim.	Cultivated ^c	Setoguchi and Watanabe IL9402
Prinoides	<i>I. macropoda</i> Miq.	Cultivated ^a	Setoguchi IL9304
(Outgroup)	<i>Nemopanthus mucronatus</i> (L.) Trec.	Cultivated ^d	Boufford s.n.

^a Cultivated in the Jindai Botanical Park, Tokyo, Japan.^b Cultivated in Fukuoka-ken Forest Experimental Station, Fukuoka, Japan.^c Cultivated in Tama Forest Science Garden, Tokyo, Japan.^d Cultivated in the Harvard Forest, Massachusetts, USA.

DNA (rDNA). Because of the rapid radiation that can occur among island endemics (see Crawford, Witkus, and Stuessy, 1987), morphological diversification may evolve with little diversification at the molecular level. Therefore RFLPs of the plastid genome often are not informative for inferring phylogenetic relationships among the species of insular plant genera, especially if rapid radiation has occurred in the islands (e.g., Crawford et al., 1992, 1993). On the other hand, the IGS sequence between the *trnL* (UAA) 3' exon and *trnF* (GAA) gene usually shows intraspecific variations (e.g., Gielly and Taberlet, 1994; Fujii et al., 1995). The internal transcribed spacer (ITS) of nuclear rDNA also shows substantial nucleotide variation among closely related species and has been used successfully for phylogenetic analysis, even on the oceanic islands (e.g., Sang et al., 1994, 1995; Kim et al., 1996).

MATERIALS AND METHODS

Plant samples—*Ilex* L. is the largest genus in the Aquifoliaceae, comprising ~400 species in the tropical to temperate regions of the world (Loesner, 1942). Twenty-five species from seven sections of *Ilex* have been reported in Japan (Yamazaki, 1989). To elucidate the horizontal gene transfer between insular endemics, *Ilex matanoana*, *I. mertensii*, *I. percoriacea*, and *I. beecheyi* from the Bonin Islands and *I. maximowicziana* and *I. dimorphophylla* from the Ryukyu Islands were sampled. We collected leaves from five to 13 individuals for each species

(Table 1). We also sampled species of *Ilex* in Japan to compare the genotype of cpDNA and nuclear DNA of the sections *Polyphyllae* and *Ilex*, and finally 20 of the 25 species of *Ilex* in Japan and one outgroup species were sampled for molecular analysis (Table 1). All voucher specimens were deposited at MAK. We used *Nemopanthus* for the outgroup to root the phylogenetic trees in the present study. *Nemopanthus* is considered to be the most closely related sister group of *Ilex* (Savolainen et al., 1994) and distinguished morphologically from *Ilex* by its rudimentary calyx lobes, free petals, and lack of stipules.

Total DNA extraction—Fresh leaves were frozen using liquid nitrogen and pulverized to fine powder. Prior to the DNA extraction, leaf powder was suspended in HEPES buffer (pH 8.0) and centrifuged at 10000 rpm, 20°C for 5 min to remove the sticky polysaccharide (Setoguchi and Ohba, 1995). Total DNA was isolated from collected pellets using CTAB (hexadecyltrimethylammonium bromide) method of Haselbe and Iwatsuki (1990), and purified in CsCl/ethidium bromide gradients (Palmer, 1986).

RFLPs analysis—DNAs were digested with 21 restriction endonucleases having 6-bp recognition sequences: *Acc* I, *Ava* I, *Bam* HI, *Ban* II, *Bgl* II, *Cla* I, *Dra* I, *Eco* T14I, *Eco* T22I, *Eco* RI, *Eco* RV, *Fba* I, *Hae* II, *Hind* III, *Kpn* I, *Pst* I, *Sal* I, *Sca* I, *Sna* BI, *Vsp* I, and *Xba* I. DNA fragments were separated by electrophoresis using 0.7–1.0% agarose gels and blotted to Hybond-N+ membranes (Amersham, Buckinghamshire, UK). The probes used in Southern hybridization were from the clone bank of tobacco cpDNA (Sugiura et al., 1986). They were grouped into six regions as follows: region 1—B7, B20; region 2—B19, B22, B29; region 3—Ba1; region 4—B8, B10, B15; region 5—

Ba2; and region 6—B25, B28. Labeling of probes, hybridization, and detection were performed using ECL systems (Amersham, Buckinghamshire, UK).

Amplification and sequencing of ITS of nuclear rDNA and trnL (UAA) 3'exon-trnF (GAA) IGS region of cpDNA—Double-stranded DNA of the complete ITS regions (ITS 1, 5.8S rDNA, and ITS 2 in nuclear DNA [~ 600 bp]) of nuclear rDNA and the trnL (UAA) 3'exon-trnF (GAA) IGS region of cpDNA (~ 380 bp) were amplified over 30 cycles of symmetric polymerase chain reaction (PCR) using the primers ITS-4 and ITS-5 of White et al. (1990) for ITS, and primers e and f of Taberlet et al. (1991) for the IGS region. PCR (polymerase chain reaction) cycle conditions in the first cycle consisted of 2 min at 94°C for denaturation, 1 min at 45°C for primer annealing, and 1 min at 72°C for primer extension. Denaturation time at 94°C was reduced to 1 min during the next 28 cycles. An extension time at 72°C was increased to 5 min in the last cycle. PCR products were purified by electrophoresis in 1.0% agarose gel using $1 \times$ TAE buffer. The gel was stained with ethidium bromide and the DNA was eluted using GeneClean II (Bio 101, Vista, California, USA). Purified DNAs were sequenced by standard methods of Taq dye deoxy terminator cycle sequencing kit (Perkin Elmer, Foster City, California, USA) using ITS-2 and ITS-5 primers for ITS 1, and ITS-3 and ITS-4 for ITS 2 sequence determination following White et al. (1990) on an Applied Biosystems Model 373A automated sequencer (Applied Biosystems, Foster City, California, USA). Sequence data were aligned manually with the GENETYX program (The Software Development, Tokyo, Japan). Insertions and/or deletions (indels) were generally placed to increase the number of matching nucleotides in a sequence position. After machine aligning the sequences, we further manually adjusted the alignment. The DNA sequences have been deposited with GenBank.

Phylogenetic analysis—Restriction site data of cpDNA were analyzed together with IGS sequences between the trnL (UAA) 3'exon-trnF (GAA) cpDNA, because only a few informative site mutations were found in the IGS sequences. Informative site mutations of IGS sequence were coded as presence/or absence characters in the matrix of RFLPs (Appendix 1). Indels were excluded from this study.

Variable nucleotide sites of ITS region of nuclear rDNA were analyzed separately. Both data sets were phylogenetically analyzed by unweighted Wagner parsimony (Farris, 1970) using PAUP version 3.1.1 (Swofford, 1993). The heuristic algorithm with the stepwise addition option was used to find the shortest trees. Bootstrap analysis (Felsenstein, 1985) of 100 replicates and decay analysis (Bremer, 1988) were conducted to assess the internal support for clades found in each analysis.

RESULTS

RFLPs of cpDNA and phylogenetic analysis—We detected 32 restriction site mutations, ten of which were phylogenetically informative (Table 2). Length mutations were excluded from this study. Restriction sites were scored according to presence (scored as 1) or absence (scored as 0) of restriction sites, as shown in Appendix 1 (from numbers 1 to 32). The RFLPs of the four endemics to the Bonin Islands and *I. maximowicziana* and *I. dimorphophylla* (endemics to the Ryukyu Islands) were examined using 5–13 individuals to find intraspecific divergence in cpDNA (see Table 1); however, no difference in the restriction pattern was discovered among any individuals within the species.

IGS sequences between the trnL (UAA) 3'exon-trnF (GAA) IGS region of cpDNA—The complete sequence of the IGS between trnL (UAA) 3'exon-trnF (GAA) re-

TABLE 2. Restriction site changes among the 20 species of *Ilex* and the allied genus *Nemopanthes*.

No.	Enzymes	Probe regions ^a	Fragment size changes (kb)	Taxa
1	<i>Bam</i> HI	3	1.2 = 0.9 + 0.3	1~20
2	<i>Ban</i> II	2	4.0 = 3.1 + 0.9	19
3	<i>Ban</i> II	3	9.4 = 6.7 + 2.7	16
4	<i>Ban</i> II	3	2.9 = 1.8 + 1.1	16
5	<i>Bgl</i> II	2	9.2 = 1.9 + 7.3	7~18
6	<i>Bgl</i> II	2	2.7 = 2.0 + 0.7	6~18
7	<i>Bgl</i> II	3	8.0 = 5.0 + 3.0	6
8	<i>Bgl</i> II	4	4.3 = 2.3 + 2.0	16
9	<i>Bgl</i> II	5	2.2 + 1.0 = 3.2	1~20
10	<i>Cla</i> I	3	2.0 + 1.3 = 3.3	20
11	<i>Cla</i> I	6	2.9 + 1.2 = 4.1	16
12	<i>Dra</i> I	3	3.5 = 3.0 + 0.5	6
13	<i>Dra</i> I	5	2.5 = 1.9 + 0.6	1~4
14	<i>Dra</i> I	6	8.5 = 5.5 + 3.0	6
15	<i>Eco</i> RI	3	6.6 = 4.9 + 1.7	10
16	<i>Eco</i> RI	3	6.6 = 2.8 + 3.8	16
17	<i>Eco</i> RI	5	2.0 + 1.6 = 3.6	1~5, 19
18	<i>Eco</i> RI	6	2.0 + 1.5 = 3.5	1~4, 6~19
19	<i>Eco</i> T14I	1	0.7 + 0.6 = 1.3	1~20
20	<i>Eco</i> T14I	2	4.4 + 2.0 = 6.4	7~18
21	<i>Eco</i> T22I	2	7.2 = 3.9 + 3.3	8
22	<i>Eco</i> T22I	6	13.1 = 9.2 + 2.0 + 1.9	8
23	<i>Hind</i> III	3	8.8 = 6.4 + 2.4	5
24	<i>Hind</i> III	3	6.9 = 4.3 + 2.6	16
25	<i>Sal</i> I	4	5.2 = 3.2 + 2.0	1~20
26	<i>Sca</i> I	1	7.2 = 4.5 + 2.7	5
27	<i>Sna</i> BI	1	2.7 + 1.7 = 4.4	7~17
28	<i>Sna</i> BI	2	2.0 = 1.2 + 0.8	1, 3
29	<i>Sna</i> BI	2	3.7 + 0.3 = 4.0	1~17
30	<i>Sna</i> BI	3	3.6 + 1.7 = 5.3	6, 17
31	<i>Sna</i> BI	3	1.8 + 1.3 = 3.1	20
32	<i>Sna</i> BI	6	3.3 + 1.5 = 4.8	6

^a The original probe regions (Sugiura et al., 1986) were grouped into the six regions (see Materials and Methods of the present paper).

gion of cpDNA (~ 380 bp) was determined for the 20 ingroup taxa and one outgroup. Appendix 2 shows the IGS sequences of the 21 taxa examined. The DNA sequences have been deposited with GenBank under accession numbers GBAN-U92620–GBAN-U92640 (the prefix GBAN- has been added to link the online version of *American Journal of Botany* with GenBank but is not part of the actual GenBank accession number) in the order presented in Table 1. The IGS sequences were very uniform with one indel of 1 bp among *Ilex* species. Two indels were required to align *Ilex* and outgroup sequences. As a result, only two site changes were found to be phylogenetically informative (represented as “/” in Appendix 2). We could not find any intraspecific divergence in the ITS sequence in *I. maximowicziana* and *I. dimorphophylla* (endemics to the Ryukyu Islands) and the four endemics to the Bonin Islands.

Phylogenetic analysis of combined data of RFLPs and sequences of IGS between the trnL (UAA) 3'exon-trnF (GAA) gene of cpDNA—Informative site change data of IGS sequence were encoded as presence/absence characters and were combined with the RFLPs data matrix. The three most parsimonious trees resulting from phylogenetic analysis using Wagner parsimony had a length of 39, consistency index of 0.706 (excluding autapomorphic characters), and retention index of 0.909.

RFLPs + *trnL* (UAA) 3'exon - *trnF* (GAA)
IGS sequence tree (cpDNA)

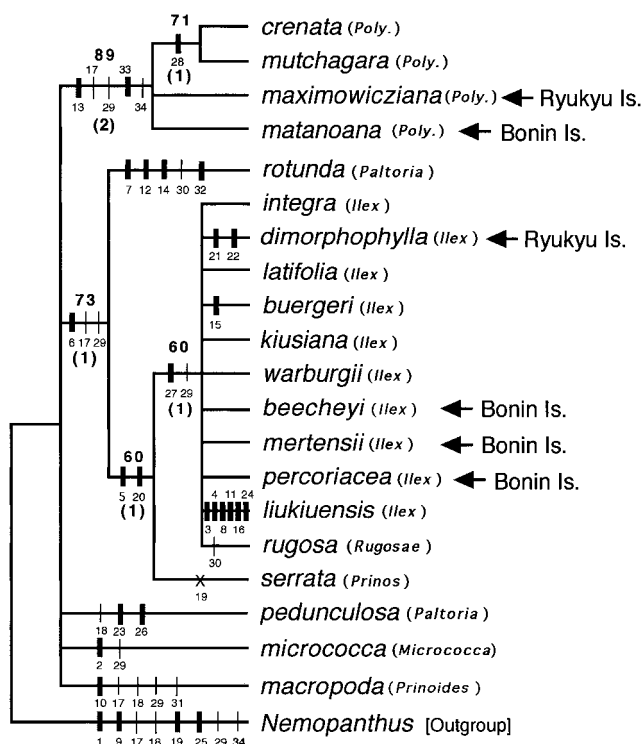


Fig. 2. Phylogeny of *Ilex* inferred from RFLPs and the *trnL* (UAA)3'exon-*trnF*(GAA) IGS sequence of cpDNA. This is the strict consensus tree of the three most parsimonious ones of length 39, CI = 0.706 (excluding uninformative data), and RI = 0.909. The numbers in parentheses and above branches represent decay and the percentage of the 100 bootstrap values (>50%), respectively. Heavy bars indicate a site change with a consistency index = 1.00, thin bars a site change of consistency index < 1.00, and crosses indicate reversals. Character numbers correspond to those in Appendix 1.

Figure 2 shows the strict consensus of the three most parsimonious trees. The strict consensus tree indicates that species in the Bonin Islands can be divided into two groups, with *I. matanoana* belonging to section *Polyphyllae*, and *I. beecheyi*, *I. mertensii*, and *I. percoriacea* placed within section *Ilex* in an unresolved polytomy. Furthermore, *I. Maximowicziana* from the Ryukyu Islands (endemic to the Ishigaki and Iriomote Islands) is placed within section *Polyphyllae*, and *I. dimorphophylla* from the Ryukyu Islands (endemic to the Amamiyoshima Island) is placed within section *Ilex*. The monophyly of the section *Polyphyllae* is supported by bootstrap value of 89%, and section *Ilex* clusters with sections *Rugosae*, *Prinos*, and *Paltoria*, supported by bootstrap value of 73%.

Phylogenetic analysis of ITS sequences of nuclear rDNA—The complete sequences of ITS 1 (~380 bp, including 32 bp of the 18S gene of rDNA) and ITS 2 (~380 bp, including 99 bp of the 5.8S gene of rDNA and 28 bp of the 26S gene of rDNA) were determined for the 20 species of *Ilex* and *Nemopanthus mucronatus*. The sequence boundaries for the two ITS regions and three coding regions (18S, 5.8S, and 26S) of rDNA were

ITS tree (nuclear ribosomal DNA)

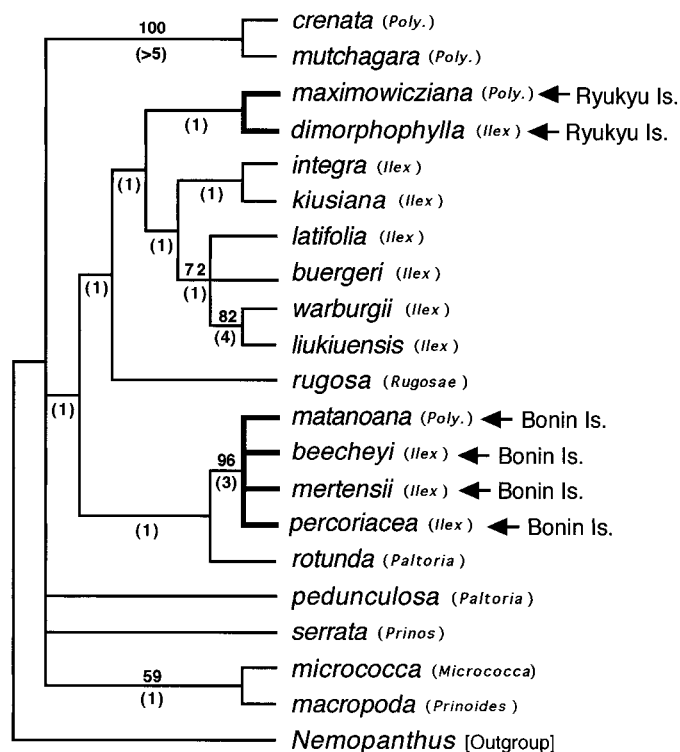


Fig. 3. ITS phylogeny of *Ilex*. This is the strict consensus tree of the 72 most parsimonious ones of length 97, CI = 0.619 (excluding uninformative site changes), and RI = 0.701. The numbers in parentheses and above branches represent decay and percentage of the 100 bootstrap values (>50%), respectively.

determined by the published sequences for *Daucus carota* and *Vicia faba* (Yokota et al., 1989). The DNA sequences have been deposited with GenBank under accession numbers U92578-U92598 (for ITS 1) and U92599-U92619 (for ITS 2) in the order presented in Table 1. No difference in sequence was discovered among any individuals within the species of *I. maximowicziana*, *I. dimorphophylla* (endemic to the Ryukyu Islands), and the four endemics of the Bonin Islands. The ITS 1 and 2 sequences of the 21 taxa examined are shown in Appendices 3 and 4, respectively. Alignments of the ITS 1 and 2 sequences of the 21 taxa required the introduction of 22 indels (11 indels for each of ITS 1 and 2). Regions of ITS 1 (positions 130–140 bp in Appendix 3) and ITS 2 (positions 120–150 bp in Appendix 4) were difficult to align due to large indels and nucleotide diversification. Therefore, these regions were excluded from the phylogenetic analysis. We used a total of 48 site mutations for the phylogenetic analysis, 22 from the ITS 1 and 26 from the ITS 2 region (represented as “^” in Appendices 3 and 4).

Unweighted Wagner parsimony resulted in 72 trees of equally high parsimony of 97 steps, consistency index of 0.619, and retention index of 0.701. The strict consensus is shown in Fig. 3. This tree supports the monophyly of all four species endemic to the Bonin Islands and their membership in the section *Ilex* alliance with a bootstrap value of 96% and decay index of 3. In addition, the mon-

ophylly of two endemics to the Ryukyu Islands, *I. maximowicziana* (section *Polyphyllae* and distributed on the Ishigaki and Iriomote Island) and *I. dimorphophylla* (section *Ilex* and distributed on the Amamioshima Island), is also indicated by the tree, although the bootstrap value is below 50% and decay index is 1.

DISCUSSION

Intersectional gene flow between insular endemics—

The phylogenetic analyses based on the two sets of molecular data disagreed with regard to the phylogenetic relationships of all the endemics of the Bonin Islands and two of the insular endemics of the Ryukyu Islands. The RFLPs and IGS sequences of cpDNA agree with the morphologically based taxonomy, whereas the ITS phylogeny groups together putatively unrelated endemics from the two island systems.

The cpDNA tree indicates that *Ilex matanoana* (Bonin Islands) and *I. maximowicziana* (Ryukyu Islands) are part of the section *Polyphyllae* alliance, whereas the other three species of the Bonin Islands (*I. mertensii*, *I. percoriacea*, and *I. beecheyi*) and *I. dimorphophylla* (Ryukyu Islands) are included in the section *Ilex* alliance. On the other hand, the ITS of nuclear rDNA phylogeny clusters all four species on the Bonin Islands together and two endemics of the Ryukyu Islands, *I. maximowicziana* and *I. dimorphophylla*, together. The Bonin Islands cluster is strongly supported, with a bootstrap value of 96% and a decay index of 3, but for the cluster that included the two Ryukyu Islands species the reliability is low. Each of these two monophyletic clusters is unrelated to its sectional attributes (unrelated to other species in their sections), in which there exist many morphological differences between sections *Polyphyllae* and *Ilex* as described above in the introduction.

The discrepancy between the cpDNA and nuclear rDNA trees can be explained in two ways, i.e., a diploid hybrid origin of *Ilex matanoana* (on the Bonin Islands) and *I. maximowicziana* (on the Ryukyu Islands), or an intersectional introgression on both of the islands.

The first hypothesis considers the *Ilex matanoana* and *I. maximowicziana* as diploid hybrids between species of sections *Polyphyllae* and *Ilex*. In the Bonin Islands, *I. matanoana* is considered to be the progeny of the section *Polyphyllae* \times alliance of the three endemics of section *Ilex* on the Bonin Islands. On the other hand, *I. maximowicziana* is considered to be the progeny of section *Polyphyllae* \times *I. dimorphophylla*. Morphological character expression in plant hybrids is not always intermediate (Rieseberg and Ellstrand, 1993), and *I. matanoana* and *I. maximowicziana*, which show typical morphological characteristics of section *Polyphyllae* (no trace of section *Ilex*), could be hybrids. In this case, past representatives of the *Polyphyllae* could have served as the hybrid progenitors on the Bonin and Ryukyu Islands.

The second hypothesis for the discrepancy between the cpDNA and nuclear rDNA trees is the intersectional introgression of nuclear DNA between sections *Polyphyllae* and *Ilex* both on the islands without evidence of cpDNA introgression. On the Bonin Islands, introgression of nuclear rDNA from the three endemics of section *Ilex* (*I. mertensii*, *I. percoriacea*, and *I. beecheyi*) into *I. matanoana*

(section *Polyphyllae*) might have occurred. Likewise, introgression from *I. dimorphophylla* into *I. maximowicziana* may have occurred in the Ryukyu Islands.

In any case, the following three points can be noted from the present results: (1) nuclear gene flow is observed without evidence of cpDNA gene flow; (2) the introgression or hybridization is intersectional; and (3) the introgression or hybridization is independently repeated on the two island systems, the Bonin Islands and the Ryukyu Islands.

In the present study, the gene phylogeny for the cpDNA is consistent with the morphologically based taxonomy, whereas the nuclear rDNA phylogeny groups together several putatively unrelated endemics on the Bonin and the Ryukyu Islands. Nuclear gene flow is observed in sympatric endemics of *Ilex* on both sets of islands without evidence of cpDNA gene flow. In many cases, the event of hybridization and introgression is detected as an asymmetric gene flow between cytoplasmic and nuclear genomes. Cytoplasmic gene flow is frequently observed with or without evidence of nuclear introgression (reviewed in Rieseberg and Soltis, 1991; Rieseberg and Wendel, 1993). On the other hand, only two examples in which nuclear introgression occurred without cytoplasmic introgression have been reported (Wagner et al., 1987; Arnold, Buckner, and Robinson, 1991). Therefore, the gene flow observed in the present study can be considered unusual among plant populational studies. Nuclear genes might be capable of crossing some species borders that cpDNA is unable to cross, as Wagner et al. (1987) have suggested. However, no explanation has been proposed about the mechanisms that make cytoplasmic gene flow more probable than nuclear gene flow. Further genetic study focused on the mechanism is needed.

Most molecular evidence of plant gene flow has been reported as occurring between closely related species, although intersectional gene flow has been reported in rare instances (e.g., *Gossypium*; Wendel, Stewart, and Rettig, 1991). Although we have not yet surveyed the genetic distances among these species, low genetic divergence was characteristic, not only of the island endemics, but also of the whole of the genus *Ilex* that was investigated in the present study. We could detect only 32 restriction site mutations, ten of which were phylogenetically informative in the RFLPs analysis of cpDNA taken from seven sections of *Ilex*. Although the IGS sequences between the *trnL* and *trnF* genes of cpDNA sequence often show intraspecific variations (e.g., Gielly and Taberlet, 1994; Fujii et al., 1995, 1997), we could detect only two site mutations in these sequences within the genus. The phylogenetic analysis based on ITS sequences were also not successful, and most of the branch lengths of each node equaled 1. Thus, the low genetic divergence between the sections of *Ilex* may have been one of the factors facilitating the intersectional gene movement.

It is also noteworthy that a trace of intersectional hybridization and introgression was independently surveyed in the two island systems, the Bonin Islands and the Ryukyu Islands, in the present study. Generally, hybridization and introgression between various species frequently occur in the regions of sympatry or at the edges of zones, where species distributions come in contact with each other (Rieseberg and Brunsfeld, 1991). In the present

study, the insular endemics of *Ilex* were seen to be sympatrically distributed in a very narrow environment in the Bonin islands. These sympatric endemics that share a restricted geographic or habitat range could have been made to hybridize, although they are, nonetheless, putatively unrelated species that are categorized in different sections. Once hybridization (and sequential backcrossing) has occurred in a localized pocket, the introgressed gene could easily accumulate within the small population of these endemics. Thus, the gene flow between insular endemics that occurs through hybridization and introgression should come about without difficulty because of certain ecological features of insular endemics, i.e., limits of their distribution range or sympatric distribution in a small land area, and small population size.

Unfortunately, the breeding system or pollination of the endemic species of *Ilex* has not been studied on the two island systems. Further ecological studies are needed to discuss the plausibility of interspecific gene flow.

Biogeographic perspective on the Ryukyu Islands—

From the biogeographic point of view, the intersectional gene flow observed in the Ryukyu Islands may shed light on the geographic history of the islands. *Ilex dimorphophylla* (section *Ilex*) is endemic to Amamiyoshima Island, and *I. maximowicziana* (section *Polyphyllae*) is endemic to Ishigaki Island and Iriomote Island (see Fig. 1). Although the distance between Amamiyoshima Island and the Ishigaki–Iriomote Islands is over 500 km, our gene flow findings suggest that both species hybridized in the past.

The Ryukyu Islands are the product of the repeated formation and division of the landbridge through the mainland of China, Taiwan, and Kyushu during the Pliocene and Quaternary periods. The hybridization between *Ilex dimorphophylla* and *I. maximowicziana* can be more easily explained by the presence of a landbridge that connected Amamiyoshima Island, Ishigaki Island, and Iriomote Island until 20 000 yr ago (Kimura, 1996). However, the present study is the first report on horizontal gene transfer among endemics of the Ryukyu Islands, and further case studies are needed to confirm the effect of the landbridge on plant speciation. In addition, although another six *Ilex* species are distributed on these islands, we could not detect any trace of gene flow among those species in the present study. Further precise studies are needed to examine the presence or absence of gene movement among the remaining *Ilex* species in the Ryukyu Islands.

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APPENDIX 1. RFLPs (numbers 1–32) and *trnL* (UAA) 3'exon-*trnF* (GAA) IGS sequence (numbers 33, 34) data matrix for 21 species of *Ilex* and the outgroup *Nemopanthus*. Site changes in IGS sequences (represented as “^” in Appendix 2) were encoded as presence/absence characters (0/1).

Taxon	Characters			
	1	0	2	3
<i>I. crenata</i>	10000000011010000001000010110111 10			
<i>I. maximowicziana</i>	10000000011010000001000010100111 10			
<i>I. muchagara</i>	10000000011010000001000010110111 10			
<i>I. matanoana</i>	10000000011010000001000010100111 10			
<i>I. pedunculosa</i>	10000000011000000101001011100111 00			
<i>I. rotunda</i>	10000110011101001001000010100010 01			
<i>I. integra</i>	10001100011000001000000010000111 01			
<i>I. dimorphophylla</i>	10001100011000001000110010000111 01			
<i>I. latifolia</i>	10001100011000001000000010000111 01			
<i>I. buergeri</i>	10001100011000101000000010000111 01			
<i>I. kiusiana</i>	10001100011000001000000010000111 01			
<i>I. warburgii</i>	10001100011000001000000010000111 01			
<i>I. beecheyi</i>	10001100011000001000000010000111 01			
<i>I. mertensii</i>	10001100011000001000000010000111 01			
<i>I. percoriacea</i>	10001100011000001000000010000111 01			
<i>I. liukiuensis</i>	10111101010000011000000110000111 01			
<i>I. rugosa</i>	10001100011000001000000010000011 01			
<i>I. serrata</i>	10001100011000001000000010101111 01			
<i>I. micrococca</i>	11000000011000000001000010101111 01			
<i>I. macropoda</i>	10000000001000001101000010101101 01			
<i>Nemopanthus</i>	00000000111000001111000000101111 00			

APPENDIX 2. Aligned DNA sequences of intergenic spacer (IGS) between the *trnL* (UAA) 3'exon and *trnF* (GAA) region of cpDNA for 20 species of *Ilex* and one outgroup taxa examined in the present study. “^” indicates sites that are phylogenetically informative within *Ilex*.

	1	2	3	4	5	6
	0	0	0	0	0	0
<i>I. crenata</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. maximowicziana</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. mutchagara</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. matanoana</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. penduculosa</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. rotunda</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. integra</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. dimorphophylla</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. latifolia</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. buergeri</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. kiusiana</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. warburgii</i>	CCCATTGACCCCTAA-TATTTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. beecheyi</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. mertensii</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. percoriacea</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. liukiensis</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. rugosa</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. serrata</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. micrococca</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>I. macropoda</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
<i>Nemopanthus</i>	CCCATTGACCCCTAACTATTATCCTATCCCTTTTCGTTAGCGGTTCAAAATTCGTTATCT					
	7	8	9	1	1	1
	0	0	0	0	0	0
<i>I. crenata</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. maximowicziana</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. mutchagara</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. matanoana</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. penduculosa</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. rotunda</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. integra</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. dimorphophylla</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. latifolia</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. buergeri</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. kiusiana</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. warburgii</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. beecheyi</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. mertensii</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. percoriacea</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. liukiensis</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. rugosa</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. serrata</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. micrococca</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>I. macropoda</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					
<i>Nemopanthus</i>	TTCTCATTCACTCTATTCTTTTACAAAAGGATATGAGCGGAAACGGTTTTCTCTTTTCAC					

^

APPENDIX 2. Continued.

	1	1	1	1	1	1
	3	4	5	6	7	8
	0	0	0	0	0	0
<i>I. crenata</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. maximowicziana</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. mutchagara</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. matanoana</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. penduculosa</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. rotunda</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. integra</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. dimorphophylla</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. latifolia</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. buergeri</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. kiusiana</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. warburgii</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. beecheyi</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. mertensii</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. percoriacea</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. liukuensis</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. rugosa</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. serrata</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. micrococca</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>I. macropoda</i>	AAACAGGTTTT-ATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
<i>Nemopanthis</i>	AAACAGGTTTTGATATATGTGATACACGTCCAAATGAACATCTTTGAACAAGGAATCCC					
	1	2	2	2	2	2
	9	0	1	2	3	4
	0	0	0	0	0	0
<i>I. crenata</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. maximowicziana</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. mutchagara</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. matanoana</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. penduculosa</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. rotunda</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. integra</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. dimorphophylla</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. latifolia</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. buergeri</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. kiusiana</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. warburgii</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. beecheyi</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. mertensii</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. percoriacea</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. liukuensis</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. rugosa</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. serrata</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. micrococca</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>I. macropoda</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					
<i>Nemopanthis</i>	CATTTGAATGCTTCATGGTTGATATGATTATTCATAATCAAACCTTACAAAGCCTTCCTTT					

^

APPENDIX 2. Continued.

	2	2	2	2	2	3
	5	6	7	8	9	0
	0	0	0	0	0	0
<i>I. crenata</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. maximowicziana</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. mutchagara</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. matanoana</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. penduculosa</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. rotunda</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. integra</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. dimorphophylla</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. latifolia</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. buergeri</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. kiusiana</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. warburgii</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. beecheyi</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. mertensii</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. percoriacea</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. liukuensis</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. rugosa</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. serrata</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. micrococca</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>I. macropoda</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTT----CATATT					
<i>Nemopanthus</i>	TGAAGATCCAAGAAATTCAGTACATGGATAAGACTTTGTAATATACTTTCATACATATT					

	3	3	3	3	3	3
	1	2	3	4	5	6
	0	0	0	0	0	0
<i>I. crenata</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. maximowicziana</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. mutchagara</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. matanoana</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. penduculosa</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. rotunda</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. integra</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. dimorphophylla</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. latifolia</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. buergeri</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. kiusiana</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. warburgii</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. beecheyi</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. mertensii</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. percoriacea</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. liukuensis</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. rugosa</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. serrata</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. micrococca</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>I. macropoda</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					
<i>Nemopanthus</i>	TTTTTAATTGACATAGACCCAGTCATCTAGTAAAAATGAGGATGCTGCATAGGAAACGGT					

APPENDIX 2. Continued.

	3	3
	7	8
	0	0
	.	.
<i>I. crenata</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. maximowicziana</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. mutchagara</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. matanoana</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. penduculosa</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. rotunda</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. integra</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. dimorphophylla</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. latifolia</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. buergeri</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. kiusiana</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. warburgii</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. beecheyi</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. mertensii</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. percoriacea</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. liukiuensis</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. rugosa</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. serrata</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. micrococca</i>	CGGGATAGCTCAGCTGGTAGA	
<i>I. macropoda</i>	CGGGATAGCTCAGCTGGTAGA	
<i>Nemopanthus</i>	CGGGATAGCTCAGCTGGTAGA	

APPENDIX 3. Aligned DNA sequences of ITS1 and neighboring rRNA coding regions for the 20 *Ilex* and one outgroup taxa examined in the present study. “^” indicates sites that are phylogenetically informative within *Ilex*. Polymorphic sites are represented by following symbols: K = G or T, M = A or C, Y = C or T.

	18S rDNA	ITS1					
	1	2	3	4	5	6	
	0	0	0	0	0	0	
<i>I. crenata</i>	TTCCGTAGGTGAA-CTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAMACC-GGCGAA						
<i>I. maximowicziana</i>	TTCCGTAGGTGAACCTGCGGAAGGATTATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. mutchagara</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACC-GGCGAA						
<i>I. matanoana</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. penduculosa</i>	TTCCGTAGGTGAA-CTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. rotunda</i>	TTCCGTAGGTGAACCTGCGGAAGGATTATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. integra</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. dimorphophylla</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. latifolia</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. buergeri</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. kiusiana</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCYGGCGAA						
<i>I. warburgii</i>	TTCCGTAGGTGAACCTGCGGAAGGATTATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. beecheyi</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. mertensii</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. percoriacea</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. liukuensis</i>	TTCCGTAGGTGAACCTGCGGAAGGATTATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. rugosa</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. serrata</i>	TTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATGCCTGC-AAAGTAGACCCYGGCGAA						
<i>I. micrococca</i>	TTCCGTAGGTGAACCTGCGGAAGGATTATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>I. macropoda</i>	TTCCGTAGGTGAACCTGCGGAAGGATTATTGTCGATGCCTGC-AAAGTAGACCCGGCGAA						
<i>Nemopanthus</i>	TTCCGTAGGTGAACCTGCGGAAGGATTATTGTCGATGCCTGC-AAAGTAGACCCAGCGAA						
			^				
	7	8	9	1	1	1	
	0	0	0	0	0	0	2
<i>I. crenata</i>	CTTGTTAAA--ACATGCCGGGGGTTTGAGAAGGGGTGCGCGAGCCCCCAATAAGGTT						
<i>I. maximowicziana</i>	CTCGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. mutchagara</i>	CTTGTTTAA--ACATGCGGGGGGKTTGAAAAAGGGTTGCGGAACCCCCCAATCAGCT						
<i>I. matanoana</i>	CTCGTTAAAATATATGGCTGGGGGTCTGGGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. penduculosa</i>	CTTGTTAAA--ATATGCCGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--AGACACACT						
<i>I. rotunda</i>	CTCGTTAAA--ATATGCGTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--GACACATT						
<i>I. integra</i>	CTTGTTAAAATATATGCTGGGGGTTTGAGAAGGGGTGTCGAGCCCCC--AACAAAAT						
<i>I. dimorphophylla</i>	CTTGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--AACGCACT						
<i>I. latifolia</i>	CTTGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. buergeri</i>	CTTGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. kiusiana</i>	CTYGTAAATATGT--CCTGGGGGTGAGACAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. warburgii</i>	CTTGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. beecheyi</i>	CTCGTTAAAATATATGGCTGGGGGTCTGGGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. mertensii</i>	CTCGTTAAAATATATGGCTGGGGGTCTGGGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. percoriacea</i>	CTCGTTAAAATATATGGCTGGGGGTCTGGGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. liukuensis</i>	CTTGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. rugosa</i>	CTTGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--AACACACT						
<i>I. serrata</i>	CTTGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGTCCCCC--GACACACT						
<i>I. micrococca</i>	CTTGTTAAA--ATATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--GACACACT						
<i>I. macropoda</i>	CTTGTTAAA--ATATGTTTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--GACACACT						
<i>Nemopanthus</i>	CTTGTTTAA--ACATGCTGGGGGTTTGAGAAGGGGTGCGCGAGCCCCC--GACACACT						
	^	^	^	^	^	^	^

APPENDIX 3. Continued.

	1	1	1	1	1	1
	3	4	5	6	7	8
	0	0	0	0	0	0
<i>I. crenata</i>	CCCC-ACCTCC-----GGATTTGGCTTGTTTCCCCC-TGGCGGGGAACTCGGCCAG-					
<i>I. maximowicziana</i>	CCCC-AGCCCCCTC-GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. mutchagara</i>	CCCCCACCTC-----GGGATCTGGCTCGTTTCCCCCTGGCGGGGAACTCGGCCAAG-					
<i>I. matanoana</i>	CCCC-AGCCCCGCTC-GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. penduculosa</i>	CCCC-ACCC-----GGGATCTGGCTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. rotunda</i>	CCCC-ACCCCC--GGGACTTGGCCCGGGTTCCCC--TTGCGGGGA-CTCGGTCAAG-					
<i>I. integra</i>	TCCCC-AGCCCCCTTGGGATTTGGCTTGCGTTCCCC--TAGGGGGGA-TTGGCCAAG-					
<i>I. dimorphophylla</i>	CCCC-AGCCCCCTCGGG-TTTGGCTTGCGTTCCCC--TAGCGGGGA-CGCGCCAAG-					
<i>I. latifolia</i>	CCCC-AGCCCCCTC-GGGATTTGGCTTGCGTTCCCC--TAGGGGGGA-CTCGGTCAAG-					
<i>I. buergeri</i>	CCCC-AGCCCCCTC-GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. kiusiana</i>	CCCC-AGGCCCTC-GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. warburgii</i>	CCCC-AGCGCCCTC-GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. beecheyi</i>	CCCC-AGCCCGCTC-GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTTGGCCAAG-					
<i>I. mertensii</i>	CCCC-AGCCCGCTC-GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. percoriacea</i>	CCCC-AGCCCGCTC-GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. liukiuensis</i>	CCCC-AGCGCCCTC-GGGTTTTGGCTTGCGTTCCCC--TAGTGGGGA-CTCGGTCAAG-					
<i>I. rugosa</i>	CCCC-ACCTC-----GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTTGGTCAAG-					
<i>I. serrata</i>	CCCC-ACCCCC--GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. micrococca</i>	CCCCCACCTC-----GGGATTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>I. macropoda</i>	CCCC-ACCTCG-----GGG-TTTGGCTTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
<i>Nemopanthus</i>	CCCC-ACCTC-----GGGATTTGGATTGCGTTCCCC--TAGCGGGGA-CTCGGTCAAG-					
		^	^ ^	^ ^	^ ^	^ ^
	1	2	2	2		
	9	0	1	2		
	0	0	0	0		
<i>I. crenata</i>	CTCCCGACAAACGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. maximowicziana</i>	CTCCCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. mutchagara</i>	CTCCCGACAAACGAACCCC-GGCGCTGTTTGCGCCAAGGAACA					
<i>I. matanoana</i>	CTCCCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. penduculosa</i>	CTCCCGGCAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAATC					
<i>I. rotunda</i>	CTCCCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. integra</i>	TTCCGGAAAA-CGAACCCG-GGCGCTTTTGTGCGCCAAGGAACC					
<i>I. dimorphophylla</i>	CTCCCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. latifolia</i>	CTCCCGACAA-CGAACCCC-GGCGCTATCTGCGCCAAGGAACC					
<i>I. buergeri</i>	CTCCCGACAA-CGAACCCC-GGCGCTATTTGCGCCAAGGAACC					
<i>I. kiusiana</i>	CTCCCGACAA-CGAACCCC-GGCGCTATTTGCGCCAAGGAACC					
<i>I. warburgii</i>	CTCCCGACAA-CGAACCCC-GGCGCTATCTGCGCCAAGGAACC					
<i>I. beecheyi</i>	CTCCCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. mertensii</i>	CTCCCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. percoriacea</i>	CTCCCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. liukiuensis</i>	CTCCCGACAA-CGAACCCC-GGCGCTATCTGCG-----GGAACC					
<i>I. rugosa</i>	CTACCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
<i>I. serrata</i>	CTCCCGACAA-CGAACCCC-GGCGCTATCTGCGCCAAGGAACC					
<i>I. micrococca</i>	CTCCCGACAA-CGAACCCC-CGGCGCTATTTTGCGCCAAGGAACC					
<i>I. macropoda</i>	CTCCCGACAA-CGAACCCC-GGCGCTATTTTGCGCCAAGGAACC					
<i>Nemopanthus</i>	CTCCCGACAA-CGAACCCC-GGCGCTGTTTGCGCCAAGGAACC					
		^ ^				

APPENDIX 4. Aligned DNA sequences of ITS2 and neighboring rRNA coding regions for the 20 *Ilex* and one outgroup taxa examined in the present study. “^” indicates sites that are phylogenetically informative within *Ilex*. Polymorphic sites are represented by following symbols: M = A or C, S = G or C, Y = C or T.

	5.8S rDNA					
	1	2	3	4	5	6
	0	0	0	0	0	0
<i>I. crenata</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. maximowicziana</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. mutchagara</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. matanoana</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. penduculosa</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. rotunda</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. integra</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. dimorphophylla</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. latifolia</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. buergeri</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. kiusiana</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. warburgii</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. beecheyi</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. mertensii</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. percoriacea</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. liukuensis</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGATCGCAAGTTGCGCCCAA					
<i>I. rugosa</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. serrata</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. micrococca</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>I. macropoda</i>	TTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
<i>Nemopanthus</i>	CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTGAACGCAAGTTGCGCCCAA					
	^					
	ITS2					
	7	8	9	1	1	1
	0	0	0	0	1	2
<i>I. crenata</i>	AGCCATTAGGCCAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCG-CCCCAACCCCC					
<i>I. maximowicziana</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCCCGTCGCCACCAACCCC-					
<i>I. mutchagara</i>	AGCCATTAGGCCAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCG-CCCCAACCCCC					
<i>I. matanoana</i>	ACCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCCCAACCCCC-					
<i>I. penduculosa</i>	AGCCATTAGGCTAAGGGCAAGTCTGCCTGGGCGTCACGCATCACGTCG-CCCCAACCCC-					
<i>I. rotunda</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCCCAACCCCCG					
<i>I. integra</i>	AGCCATTAGGCCAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCCCAACCCCC-					
<i>I. dimorphophylla</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCCCGTCGCCACCAACCCC-					
<i>I. latifolia</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCACCAACCCC-					
<i>I. buergeri</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCACCAACCCC-					
<i>I. kiusiana</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCCCCCAACCCCC-					
<i>I. warburgii</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCACCAACCCC-					
<i>I. beecheyi</i>	ACCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCCCAACCCCC-					
<i>I. mertensii</i>	ACCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCCCAACCCCC-					
<i>I. percoriacea</i>	ACCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCCCAACCCCC-					
<i>I. liukuensis</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCACCAACCCC-					
<i>I. rugosa</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCACCAACCCC-					
<i>I. serrata</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACACATCACGTCG-CCCCAACCCC-					
<i>I. micrococca</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCG-CCCCAACCCC-					
<i>I. macropoda</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCG-CCCCAACCCC-					
<i>Nemopanthus</i>	AGCCATTAGGCTAAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCG-CCCCAACCCCC					
	^ ^ ^ ^					

APPENDIX 4. Continued.

	1	1	1	1	1	1
	3	4	5	6	7	8
	0	0	0	0	0	0
<i>I. crenata</i>	C-AACGGCCTAGCCG-----GATATTGCGGGAGTTGGGGCGG--AGATTGGCCTCCCGT					
<i>I. maximowicziana</i>	--GATGCCCATCTG-----GATATTGCGGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. mutchagara</i>	--ACCGGCCAGCCG-----GATACTGCGGGAGTTGGGGCGG--AGATTGGCCTCCCGT					
<i>I. matanoana</i>	--AATTGC-----GGGAGTCGGGGCGG-AAATTGGCCTCCCGT					
<i>I. penduculosa</i>	--AATGCCAAGCTTGCTG--GGTATTGCGGGAGCTGGGGCGG--AAATTGGCCTCCCGT					
<i>I. rotunda</i>	ACAATGCCCGCTGGCAGCCGATATTGCGGGAGTTGCGGGCGG-AGATTGGCCTCCCGT					
<i>I. integra</i>	--AATT-----GCGGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. dimorphophylla</i>	--AATGCCAGCCG-----GATATTGCGGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. latifolia</i>	--AATGCCAGCTG-----GATATTGAGGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. buergeri</i>	--AATGCCAGCTG-----GATATTGCGGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. kiusiana</i>	--AATTGC-----GGGATTAGGGGCGGAAATTGGCCTCCCGT					
<i>I. warburgii</i>	--AATGCCCGCTG-----GATATTACGGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. beecheyi</i>	--AATTGC-----GGGAGT-GGGGGCGG-AAATTGGCCTCCCGT					
<i>I. mertensii</i>	--AATTGC-----GGGAGTCGGGGCGG-AAATTGGCCTCCCGT					
<i>I. percoriacea</i>	--AATTGC-----GGGAGTCGGGGCGG-AAATTGGCCTCCCGT					
<i>I. liukuensis</i>	--AATGCGCGCTG-----GATATTACGGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. rugosa</i>	--AATGCCTAGCTG-----GATATTGCGGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. serrata</i>	--AATGCCTAGTTGGTTA--GGCATTGTTGGAGTTGGGGCGG-AAATTGGCCTCCCGT					
<i>I. micrococca</i>	--AATGCCTAGCTGGCTG--GATATTGCGGGAGTTGGGGGCGGAAATTGGCCTCCCGT					
<i>I. macropoda</i>	--AATGCCTACCTGGCTG--GATATTGCGAGGAGTTGGGGCGG--AAATTGGCCTCCCGT					
<i>Nemopanthus</i>	C-AATTCCTATGTACCTG--GATATTGTGGGAGATGGGGCGG--AAATTGGCCTCCCGT					
	^			^	^	
	1	2	2	2	2	2
	9	0	1	2	3	4
	0	0	0	0	0	0
<i>I. crenata</i>	CCGCGCGACCGTGAGCGGTTGGCCCAAAAAA---CGAGTTCTTGACGATGGACGTACGGA					
<i>I. maximowicziana</i>	TC--ACGAACGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. mutchagara</i>	CCGCGCGACCGTGAGCGGTTGGCCCAAAAAA---CGAGTTCTTGACGATGGACGTACGGA					
<i>I. matanoana</i>	CC--ACGAACGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. penduculosa</i>	CC--ACGACCGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. rotunda</i>	CC--ACGAACGTGCGCGGTTGGCCCAAAAAAAG---CGAGTTCTTGACGACGGACGTACGGA					
<i>I. integra</i>	CC--ACGAACGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. dimorphophylla</i>	TC--ACGAACGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. latifolia</i>	CC--ATGAACGCGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATAGACGTACGG					
<i>I. buergeri</i>	CC--ATGAACGCGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGG					
<i>I. kiusiana</i>	ACATCTGAACGTGCGCGGTTGGCCCGAAAAA---TGRGTTCTTGAATAAGGACGTACGGA					
<i>I. warburgii</i>	CCATATGAACGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGG					
<i>I. beecheyi</i>	CC--ACGAACGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. mertensii</i>	CC--ACGAACGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. percoriacea</i>	CC--ACGAACGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. liukuensis</i>	T--ATGAACGCGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGG					
<i>I. rugosa</i>	CC--ACGAACGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. serrata</i>	CC--ACGATTGTGCGCGGTTGGCCCAAAAAA---TGAGTTCTTGACGATGGACGTACGGA					
<i>I. micrococca</i>	CC--ACGACCGTGCGCGGTTGGCCCAAAAAAATAAGAGTTCTTGACGATGGACGTACGGA					
<i>I. macropoda</i>	CC--ACGACTGTGTGTGGTTGGCCCAAAAAA---GAATTCCTGACGACGGACGTACGGA					
<i>Nemopanthus</i>	AC--ACGACCGTGAGCGGTTGGCCCAATAA---TGAGTTCTTGACGATGGACGTACGGA					
	^ ^ ^			^	^	^

	26S rDNA					
	3	3	3	3	3	3
	1	2	3	4	5	6
	0	0	0	0	0	0
<i>I. crenata</i>	TCCGACCCTG-TGCACC--TGATTCGCTCTAGGG-CGA-GGTGCTTC-GACCGCGACCCC					
<i>I. maximowicziana</i>	TCTGCGCGGACCTGCG--GCACCTTC-TTCAC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. mutchagara</i>	TCCGACCCTG-TGCACC--TGATTCGCTCTCGGG-CGACGGTGCTTC-GACCGCGACCCC					
<i>I. matanoana</i>	TCTGACCGTGACCTGT--GCACC-TCC-TTCGC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. penduculosa</i>	TCTGATCGTGACCTTC--GCACC-TTCTCTAATGGGATGGTGCTTCGACCGCGACCCC					
<i>I. rotunda</i>	TCTGACCGCGACCTGT--GCGCC-TTCCTTAGG-GGGCGGCGCTCC-GACCGCGACCCC					
<i>I. integra</i>	TCTGACCGCGACCTGT--GCACC-TTC-TTCAC-GGATGGTGCTTC-GACCGCGACCCC					
<i>I. dimorphophylla</i>	TCTGACCGCGACCTGT--GCACCTTC-TTGAC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. latifolia</i>	TCTGACCGCGACCTGT--GCACCTTCCTTCAC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. buergeri</i>	TCTGACCGGACCTGT--GCACCTTCCTTCAC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. kiusiana</i>	TCTGACCGCAACTCTGT--GCAGCATCCTTCAT-GGATCGTGCTTC-AACCGCGACCCC					
<i>I. warburgii</i>	TCTGACTGCGACCTGT--GCACCTTCCTTCAC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. beecheyi</i>	TCTGACCGTGACCTGT--GCACC-TCC-TTCGC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. mertensii</i>	TCTGACCGTGACCTGT--GCACC-TCC-TTCGC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. percoriacea</i>	TCTGACCGTGACCTGT--GCACC-TCC-TTCGC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. liukiuiensis</i>	TCTGACTGCGACCTGT--GCACCTTCCTTCAC-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. rugosa</i>	TCTGACCGTGACCTGT--GCACC-TTC-TTCAT-GGATGGTGCTTC-GACCGCGACCCC					
<i>I. serrata</i>	TCTAACCGTGACCTGT--GCACC-TTC-TTTAG-GGATGGTGCTCC-GACCGCGACCCC					
<i>I. micrococca</i>	TCTGATCGTGACCTGC--GCGCC-TCC-TTCGG-GGATGGCGCTTT-GACCGCGACCCC					
<i>I. macropoda</i>	TCTGACCGTGACCTGT--GCAC--TTC-TTTCG-GGATGGTGCTTC-GACCGCGACCCC					
<i>Nemopanthus</i>	TCTAACCGTGACCTATATGCACC-TTC-TTCAG-CGATGGTGCTTC-GACCGCGACCCC					