Genetic structure of interspecific hybridization between the long corolla tube species Isodon longitubus and its short corolla tube congener I. inflexus

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Abstract

Interspecific hybridization between the short corolla tube species *Isodon inflexus* and the long corolla tube species *I. longitubus* was analyzed using genetic and morphometric markers. Bayesian clustering analysis using microsatellites revealed that plants in the contact zone consisted of two *I. inflexus* individuals, 33 *I. longitubus* individuals, and 13 hybrid individuals (F_2-like and backcrosses to both *I. inflexus* and *I. longitubus*). Using the sequences of the *psbA-trnH* inter genic spacer in chloroplast DNA, three haplotypes were found among pure *I. inflexus*, while four haplotypes were found among pure *I. longitubus*. Most hybrid individuals had a haplotype found only in *I. inflexus*, suggesting that the initial F_1 might have been formed by hybridization with *I. inflexus* as the maternal parent, and that in later hybrid formation, hybrids or *I. inflexus* tended to serve as the maternal parent. Although strong prezygotic isolation mechanisms contribute to preventing hybridization between the species, human habitat disturbance might have created the contact zone. Although mature hybrids in the contact zone contained equal proportions of backcrosses to both *I. inflexus* and *I. longitubus*, seedlings comprised more individuals originating from backcrosses to *I. longitubus*. The dominance of backcrosses to *I. longitubus* was probably caused by the quantitative difference between the parental species in the contact zone. In the contact zone, signs of contemporary hybridization in the nuclear genome might have been diluted by repeated backcrossing. The present study could illustrate the process of unidirectional introgression leading to chloroplast capture, a phenomenon frequently observed in *Isodon* species in Japan.

Introduction

Plant-pollinator interactions are among the most critical factors contributing to floral diversification and reproductive isolation in flowering plant species (Fenster et al. 2004; Scheistl and Schlüter 2009). Differences in flower traits are generally interpreted as adaptations to attract some animal pollinators but exclude others (Grant and Grant 1965; Crepet 1984).

Floral traits such as color (Bradshaw and Schemske 2003; Hopkins and Rausher 2012; Sobel and Streisfeld 2015) and size (Whittall and Hodges 2007; Anderson and Johnson 2008) can confer strong premating isolation by influencing the behavior and mechanical fit of pollinator groups. Various groups of pollinators mediate pollen transfer among closely related plant species with markedly different floral morphologies. Notable examples include North American *Ipomopsis*, *Diplacus*, and *Aquilegia*, which are highly diversified in morphology and pollinated by hummingbird, bee, and hawkmoth species (Grant 1993). Although extreme floral differences are the evolutionary consequences of adaptation to different groups of pollinators, reproductive isolation between species is sometimes imperfect. Therefore, hybridization between plant species with extremely different floral morphologies is an attractive model for studying the coevolution of plants and animals (Cronk and Yang 2016).

In contrast, in northeast Asia where specialized vertebrate nectarivores are absent (Funamoto 2019), bee species dominate as pollinators even for plant species with diversified floral morphologies (Inoue and

Kato 1992). In particular, for plant species with narrow tubular flowers, the morphological correspondence between the length of corolla tubes and pollinators' mouth-part length has been considered to play a key role in reproductive isolation between congeneric plant species (Suzuki et al. 2007). An example of hybridization among species with extremely different floral morphologies (spur-less and long-spur species) has been demonstrated in *Epimedium* species, which are primarily pollinated by different groups of bees (Suzuki 1984).

Isodon (Benth.) Schrad. ex Spach (Lamiaceae) comprises approximately 100 species and is widely distributed from the Far East to Africa (POWO 2023). In Japan, seven species and six varieties, belonging to two sections (*Amethystoides* and *Isodon*) are currently recognized (Murata and Yamazaki 1993). All Japanese species in the section *Amethystioides* have short corolla tubes and are pollinated by syrphid flies, halictid bees, wasps, and flesh flies (Suzuki 1992). In contrast, the species in the section *Isodon* have corolla tubes of various lengths and are primarily pollinated by bumblebees. In the section *Isodon*, corolla length generally corresponds to the proboscis length of the visiting bumblebee species (Suzuki et al. 2007).

Molecular phylogenetic analyses have revealed that Japanese *Isodon* species have experienced frequent chloroplast capture across the taxa, even between different sections (Maki et al. 2010; Ogishima et al. 2019). These results suggest incomplete reproductive isolation between species with short and long corolla tubes. In Japanese *Isodon*, contemporary interspecific hybridization seems rather frequent, as eight hybrid species have been recorded including those among short- and long- corolla tube species (Murata and Yamazaki 1993). In contrast, morphological differences are maintained, and no hybrid-origin species was detected in previous phylogenetic analyses (Ogishima et al. 2019). This suggests that even if interspecific hybridization occurs, the hybrid zones are maintained only temporarily.

An asymmetrical introgression tends to result in many hybrids with a discrepancy between nuclear and cytoplasmic genes (e.g. Rieseberg 1995; Burgess et al. 2005). The chloroplast capture observed in Japanese *Isodon* species may be a consequence of asymmetrical introgression between two species. However, there has been no investigation to test how interspecific hybridization occurs at the population level in Japanese *Isodon* species. Recently, a hybrid population between the long corolla species *Isodon longitubus* (Miq.) Kudô and the short corolla species *I. inflexus* (Thunb.) Kudô was discovered in the Shikoku District, Japan. This population allows us to investigate the population structure of hybrid populations between these two *Isodon* species.

In the present study, we examined the contact zone population of *I. inflexus* and *I. longitubus* using morphological characters and a combination of chloroplast and nuclear DNA markers to address the following specific questions: (i) What is the genetic composition of plants in the contact zone? (ii) Do hybrids show evidence of asymmetrical introgression?

Materials and Methods

Sampling

Isodon inflexus is distributed in China, Korea, and Japan and occurs in grass lands or sunny forest edges from Hokkaido to Kyushu in Japan (Murata and Yamazaki 1993) (Fig. 1A). *Isodon longitubus* is a perennial herb endemic to Japan and occurs on forest floors or in forest edges in western Honshu, Shikoku, and Kyushu (Murata and Yamazaki 1993) (Fig. 1C). Putative hybrids between *I. longitubus* and *I. inflexus* were discovered at the roadside of an artificial *Cryptomeria japonica* forest (N 33.764, E 132.98). The habitat of the hybrids was in the contact zone of the two species in Toon, Ehime Prefecture (Fig. 2). In this habitat, *I. inflexus* is found in forest edges or sunny road slopes at elevations below 550 m above sea level (asl). Above this elevation *I. longitubus* occurs dominantly on rather shady roadsides. At the contact zone, individuals with intermediate corolla tube lengths were found along an approximately 200 m span at the roadside of the forest edge (Fig. 2). Individuals with intermediate corolla tube length co-occurred with the parental species.

Samples were collected from all individuals in the area where plants with intermediate characteristics were found. We collected 47 individuals from the contact zone. The flowers were fixed in FAA (formalin, acetic acid, 70% ethanol in a ratio of 5:5:90) for morphological observation. The leaves were used for measurements and for DNA extraction. After flowering, we collected mature seeds from 19 individuals in the contact zone. These seeds were placed on damp filter papers in Petri dishes and stored at 4˚C for 1 month to break dormancy. Then they were kept at room temperature (25–27˚C) until germination. One or two seedlings per maternal plant were used for DNA analysis. A total of 22 seedlings were used for subsequent DNA analyses. For comparison, samples were collected from a population of *I. inflexus* at 300m asl and *I. longitubus* at 800m asl along the roadside (Fig. 2). Additionally, we collected an *I. inflexus* population in Tsurugi (N 33.99, E 134.085), Tokushima Prefecture and an *I. longitubus* population in Omogo, Ehime Prefecture (N 33.73, E 133.11). The sampling size for each population is shown in Table 1. Voucher specimens representing the populations were deposited in the Herbarium of Tohoku University (TUS; T. Yamashiro 11843, 11873, 11885, 11886, and 11888).

Molecular analyses

Total genomic DNA was isolated from fresh leaves using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. We genotyped the following 13 microsatellite loci: Isodon6, Isodon7, Isodon9, Isodon36, Isodon47, Isodon64, and Isodon70 (Yamashiro et al. 2013); Iso9 and Iso37 (Ogishima et al. 2022); Iso16, Iso43, Iso46, and Iso47 (Ogishima et al. unpublished) (Online Resource 1). We amplified these loci in three multiplex PCR mixes (set1: Isodon6, Isodon7, Isodon9, and Isodon64; set2: Isodon36, Isodon47, Isodon64, and Isodon70; set3: Iso9, Iso16, Iso43, Iso46, and Iso47) using the Type-it Microsatellite PCR Kit (Qiagen). All PCR amplification reactions were performed following the protocol described by Yamashiro et al. (2021). The microsatellite alleles were resolved on a Beckman Coulter CEQ 8000 (AB SCIEX, Framingham, MA) with CEQ 8000 fragment analysis software (AB SCIEX). The presence of scoring errors and null alleles was checked using MICROCHECKER version 2.2.3 (Van Oosterhout et al. 2004) set for 9999 iterations and 95% confidence intervals.

We amplified the *trnH–psbA* intergenic region in chloroplast DNA using the primers developed by Sang et al. (1997) to identify the maternal parent. Polymerase chain reactions (PCRs) were conducted in 20 μL volumes containing 10 μL 2×Gflex PCR buffer (Takara Bio, Otsu, Japan), 1 μL of each primer, 0.3 μL Tks Gflex DNA polymerase (Takara Bio), 6.7 μL distilled water, and 1 μL template DNA. The PCR conditions were a 1 min pre-denaturation at 94˚C, followed by 30 cycles of 98˚C for 10s, 55˚C for 15s, and 68˚C for 30s, and a final extension of 10 min at 72˚C. PCR products were purified with ExoSAP-IT (Affymetrix, Cleveland, OH) and sequenced on a Beckman Coulter CEQ8000 (AB SCIEX) using a GenomeLab DTCS Quick Start Kit (AB SCIEX).

Data analysis

Based on the 13 microsatellite loci, we calculated standard genetic diversity parameters such as the mean number of detected alleles (N_A); observed (H_O) and unbiased expected (uH_E) heterozygosities, and the inbreeding coefficient (F_{IS}) for all populations using GenAlEX 6.15b2 software (Peakall and Smouse 2012). We also calculated allelic richness (A_R: El Mousadik and Petit 1996) for each population using FSTAT (Goudet 2001). For each population, we examined the significance of deviations from the Hardy-Weinberg equilibrium (HWE) at each locus and the linkage disequilibrium (LD) for pairwise combinations of the loci using GENEPOP ver. 3.3 software (Raymond and Rousset 1995). To evaluate allele differences between *I. inflexus* and *I. longitubus*, we examined numbers of private alleles for the pooled reference populations using GenAlEX 6.15b2 software (Peakall and Smouse 2012).

The genetic structure of the samples was estimated using the Bayesian clustering algorithms implemented in STRUCTURE version 2.3.4 (Pritchard et al. 2000). For running STRUCTURE, we employed an admixture model with correlated allele frequencies (Falush et al. 2003). All runs were repeated 10 times for each $K = 1–10$, with a burn-in of 50,000 Markov chain Monte Carlo (MCMC) iterations followed by an additional 100,000 iterations. The optimal K value was determined by plotting the average, across all iterations, of the estimated natural logarithm of the probability of the data (Ln $Pr(X|K)$), and by calculating the delta-K (Evanno et al. 2005) using STRUCTURE HARVESTER (Earl and vonHoldt 2012). Multiple runs of optimal K were averaged and graphed using the POPHELPER 2.2.1 package (Francis 2017) in R software (R Development Core Team 2013). To explore the genomic composition of each individual in the contact zone, individual ancestry (S) and interclass heterozygosity (H_I) were estimated using the *HIest* package (Fitzpatrick 2012). In this method, S is an index indicating the parental species from which each allele has originated, and H_I represents the proportion of the genome that is heterozygous for alleles derived from each parental species. Before analyzing the real data set, we simulated six different hybrid classes (the two pure populations, F_1, F_2, and backcrosses of F_1 with each pure population) from the genotype data of reference populations from Toon, Ehime prefecture using the freqbasedsim_AlleleSample function in the hybriddetective package (Wringe et al. 2017). We generated 19, 24, 46, 46, 19, and 24 simulated genotypes for *I. inflexus*, *I. longitubus*, F_1, F_2, the backcross to *I. inflexus* and the backcross to *I. longitubus*, respectively. These simulated data sets were analyzed using *HIest* to visualize the distribution of each hybrid category on the triangle plot. We used cluster allele frequencies estimated by STRUCTURE for $K = 2$ as an input for *HIest*. The *HIest* run, with simulated

annealing parameters, was set to 10000 iterations and a start grid of 20. For the analysis of 69 samples in the contact zone (47 samples and 22 seedlings), we employed the same simulated annealing parameters as those for the simulated data set. For this analysis, we also used cluster allele frequencies estimated by STRUCTURE for $K = 2$ as input for *HIest*.

All sequences of the *psbA–trnH* intergenic region were aligned using CLC Sequence Viewer 7.7.1 (Qiagen) to identify haplotypes.

Morphometric analysis

We used 41 individuals of *I. inflexus* (I1 and I2), 42 of *I. longitubus* (L1 and L2), and 47 from the contact zone for morphological measurements. The following eight characters were measured using a digital caliper and micrometer: corolla tube length and width (CTL and CTW, respectively), upper calyx lobe length and lower calyx lobe length (UCL and LCL, respectively), leaf blade length and width (BL and BW, respectively), leaf wing length (WL), and angle of the base of the leaf blade (ABB), were measured (Fig. 3). Principal component analysis (PCA) was conducted to examine the overall morphological variation using the stats package in R. Furthermore, we examined the correlation between the cluster membership coefficient *q*-values of STRUCTURE and corolla tube length to analyze the relationship between genome admixture and corolla length variation.

Results

Genetic diversity

The level of genetic diversity of *Isodon inflexus* (N_A = 5.9–7.0, A_R = 6.52–8.13, H_O = 0.63–0.67, uH_E = 0.66–0.70) was lower than that of *I. longitubus* (N_A = 9.1–9.5, A_R = 7.94–8.43, H_O = 0.71–0.73, uH_E = 0.74–0.78) (Table 1). The difference in the mean numbers of alleles was significant ($p = 0.04$, Mann–Whitney *U* test), although the differences in A_R, H_O, and uH_E were not ($p > 0.05$, Mann–Whitney *U* test). Plants in the contact zone exhibited greater genetic diversity than the parental species (N_A = 10.8, A_R = 9.10, H_O = 0.64, uH_E = 0.77). Scoring errors and null alleles were not found in the reference populations. The inbreeding coefficients (F_{IS}) of the reference populations did not deviate significantly from zero, implying that they were in Hardy-Weinberg Equilibrium (HWE). In contrast, the contact zone population displayed significant heterozygote deficiencies at eight loci: Isodon64, Isodon36, Isodon9, Isodon7, Iso37, Iso16, Iso46, and Iso43 ($p < 0.05$). While no pairs of loci exhibited linkage disequilibrium (LD) in the populations of the pure parental species after sequential Bonferroni correction (Rice 1989), 12 of 78 locus pairs showed significant LD ($p < 0.001$ after sequential Bonferroni correction) in the contact zone population.

Genetic structure and pattern of introgression

Both Ln Pr($X|K$) values and delta-K (Evanno et al. 2005: Fig. 4A and B) indicated that $K = 2$ is optimal for the 153 individuals in the Bayesian clustering analysis via STRUCTURE analysis. All

individuals from the reference populations of *I. inflexus* (I1 and I2) and *I. longitubus* (L1–L2) were assigned to Clusters I and II, respectively, each with a posterior probability exceeding 0.95 (Fig. 4C). Within the contact zone, two individuals were assigned to Cluster I ($q > 0.95$) and 32 to Cluster II ($q > 0.95$), while the remaining 13 individuals exhibited admixtures from both clusters. Of the 22 seedlings, three were allocated to Cluster I ($q > 0.95$) and 16 to Cluster II ($q > 0.95$), with the remaining three individuals showing admixtures of both clusters.

The plot of ancestry (S) and interclass heterozygosity (H_I), estimated from the simulated data using HIest, is shown in Online Resource 2. Most simulated pure *I. inflexus* (right bottom), *I. longitubus* (left bottom), and F_1 (top) individuals were correctly positioned on the triangle. F_2 individuals were widely scattered around the center of triangle, with some plotted as F_1, some as backcross to *I. longitubus*, and others as backcross to *I. inflexus*. Individuals backcrossed to either *I. inflexus* or *I. longitubus* were widely plotted on the right and left sides of the triangle, respectively, although some samples could not be distinguished from F1, F2, or pure parents. These results suggested that 13 microsatellite markers could distinguish both pure *I. inflexus* and *I. longitubus*, and F_1, although classification among F_2 and backcross to *I. inflexus* or *I. longitubus* was somewhat uncertain.

Referring to the results of the simulation plots, in the data from the contact zone population, 13 individuals allocated to admixed genotypes by the STRUCTURE results were plotted to the areas of F2-like (six individuals), backcross to *I. inflexus*-like (four individuals), and backcross to *I. longitubus*-like (three individuals) (Fig. 5). Among the seedlings, two and 12 individuals were assigned to pure *I. inflexus* and *I. longitubus*, respectively. The remaining eight seedlings were plotted to the F2 or BC to *I. longitubus* areas. The maternal plants of eight of the 12 seedlings plotted to pure *I. longitubus* were pure *I. longitubus*. The two seedlings plotted to pure *I. inflexus* originated from pure *I. inflexus*. Therefore these 10 seedlings were produced by conspecific crosses. Four seedlings plotted to pure *I. longitubus* were produced by crosses between hybrid and *I. longitubus* (dashed lines in Fig. 5). The eight seedlings plotted to F2 or BC to *I. longitubus* areas were presumed to be derived from crosses between hybrids individuals (dotted lines in Fig. 5). F_1 was not detected in all samples at all.

Appendix 1 summarizes the allele sizes and private alleles detected in pure *I. inflexus* and *I. longitubus*, eachassigned with more than a 95% posterior probability based on STRUCTURE analysis. A total of 24 and 69 private alleles were identified in pure *I. inflexus* and *I. longitubus*, respectively. Twenty alleles were shared between *I. longitubus* and hybrid individuals while four alleles were shared between *I. inflexus* and hybrid individuals.

cpDNA variations

We obtained 353 bp of unambiguous sequence of the *psbA-trnH* intergenic region of 153 individuals, identifying five distinct haplotypes (accession numbers LC764472–LC764476). In the pure *I. inflexus* populations, three haplotypes (A, C, and D) were observed (Table 2). Conversely, in the pure *I. longitubus* populations, four haplotypes (A, B, D, and E) were identified. Haplotypes C and D were

detected in both plant from the contact zone and their seedlings (Figs. 4 and 5). Notably, haplotype C was found in most individuals exhibiting admixture via STRUCTURE analysis, with one exception (Fig. 4). Additionally, seven individuals in the contact zone that were assigned to pure *I. longitubus*, also possessed haplotype C.

Morphometric analysis

The principal component loadings on the first and second axes are listed in Online Resource 3. The portions of the variance responsible for PC1 and PC2 were 0.93 and 0.03, respectively. Scatterplots of the scores for each sample are displayed in Fig.6. ABB, CTW, UCL, and LCL were positively correlated with PC1, while BL and BW negatively correlated with PC1. For PC2, CTL showed positive correlation, whereas BW was negatively correlated. The scatter plot clearly separated the pure *I. inflexus* and *I. longitubus* populations. Individuals from the contact zone displayed wide variation: four hybrid individuals exhibited intermediate morphology. Interestingly, five and four hybrids were plotted within the space of *I. longitubus* and *I. inflexus* spaces, respectively.

Corolla length variations in hybrid individuals

The correlation between corolla tube length and *q*-value derived from STRUCTURE is depicted in Fig. 7. Six hybrid individuals exhibited intermediate corolla length, while five had short and two had long corolla lengths, respectively. F2-like or backcross to *I. longitubus*-like individuals tended to have intermediate corolla length.

Discussion

Absence of F1 generation and reproductive isolation

Population clustering analysis using STRUCTURE revealed that 13 individuals in the contact zone of the two species are likely of hybrid-origin. Based on the result of HIest these individuals were classified as F_2-like or backcross-like genotypes, while no individuals were categorized as F_1. Because we sampled all individuals with intermediate corolla lengths, F_1 individuals must have already disappeared from the contact zone, as reported in previous studies on interspecific hybrid zones (Cruzan and Arnold 1993; Arnold et al. 2006; Yan et al. 2017). The absence of F_1 individuals in the hybrid population also suggests that hybridization between the parental species is very rare with this species combination. One possible reason for this rarity is the existence of strong prezygotic isolation between the species (Arnold et al. 2010; Yan et al. 2017). Seeds collected from hybrid individuals germinated (T. Yamashiro unpublished data), indicating that postzygotic isolation might be weak for this species combination. Although the flowering periods of *I. inflexus* and *I. longitubus* overlap around the contact zone (from late September to mid-October), the two species generally inhabit different areas. *Isodon inflexus* is found in grasslands or sunny forest margins in lowlands while *I. longitubus* is found in the somewhat shady forest margins situated at or above 500 m asl in Shikoku, Japan. The contact zone between the species is located along

a forest road in an artificial forest of *Cryptomeria japonica*, suggesting that human disturbance might have disrupted the habitat isolation that typically separated these species.

The corolla tube length of *I. longitubus* (mean and SD = 11.7 ± 1.4 mm) is approximately threefold that of *I. inflexus* (mean and SD = 3.3 ± 0.4 mm) (Fig. 7) and these two species are pollinated by different groups of insects (Suzuki 1992), suggesting that prezygotic isolation between the two species is likely strong. In the contact zone, the bumble bee species *Bombus diversus* primarly visited *I. longitubus* (Fig. 1C), while *I. inflexus* was predominantly visited by the honeybee *Apis creana japonica* (Fig. 1A). However, *B. diversus* was rarely observed visiting the flowers of *I. inflexus* (Fig. 1B). Such infrequent visitation of *I. inflexus* by *B. diversus* is likely to facilitate gene flow between the species. *Bombus diversus*, which possesses a long proboscis has been reported to be as effective as *B. honshuensis*, which has a short proboscis, in pollinating the short-corolla species *Isodon umbrosus* (with a corolla tube length of 6–8 mm) (Suzuki and Akazone 2000). In the contact zone, *B. diversus* visited hybrid individuals with intermediate corolla length (Fig. 7) as frequently as it visited *I. longitubus* (T. Yamashiro personal observation). Although the formation of F_1 hybrids between *I. inflexus* and *I. longitubus* seems to be infrequent, once a hybrid is formed, genes exchange between these species is likely to occur through introgression (Arnold and Hodges 1995; Arnold et al. 1999, 2001, 2003).

Direction of introgression

Ogishima et al. (2022) demonstrated that plants in secondary contact zones of short- and long- corolla tube populations of *I. shikokianus* had intermediate corolla tube length. While they did not describe asymmetric hybridization in the intermediate populations, we found a tendency of asymmetric introgression between short- and long- corolla tube species. A total of five haplotypes were found in pure *I. inflexus* and *I. longitubus* populations, of which haplotype C, which was only found in *I. inflexus*, was shared by all the hybrids detected by STRUCTURE, with the exception of one individual. The high frequency of *I. inflexus* cpDNA in the hybrid individuals suggests that this species tended to be the maternal parent for the initial F_1 formation. Furthermore later generation hybrids have haplotype C, suggesting that the hybrids tend to be maternal plants. Although both backcross to *I. inflexus*-like and backcross to *I. longitubus*-like genotypes were found in the contact zone, the genetic constitution of seedlings derived from hybrid individuals leaned toward *I. longitubus*, indicating that backcrossing events involving hybrid individuals and *I. longitubus* occur more often.

In pollen competition experiments conducted on *Iris* and *Helianthus*, pollen from a species that is quantitative dominant was more likely to be transferred to a plant in the minority species within a hybrid population, with the minority group often serving as the female parent (Carney et al. 1994; Rieseberg et al. 1995). Asymmetric introgression resulting from differences in the numbers of each parental species has been reported in several hybrid zone studies (Nason et al. 1992; Carney et al. 2000; Burgess et al. 2005). In the contact zone examined in this study, the number of *I. inflexus* individuals was significantly lower than that of *I. longitubus* (with counts of two and 33, respectively). It is plausible that *I. inflexus*, being the minority species, acted as the maternal parent during the formation of F_1 progeny. The

tendency of backcrossing between hybrids and *I. longitubus* may also stem from the discrepancy in the numbers of the two parental species. Furthermore, when hybridization occurs between *I. inflexus*, which has short corolla tubes and *I. longitubus*, with its long corolla tubes, their floral morphological differences might influence both the formation of F_1 progeny and the direction of introgression. Several studies have demonstrated that pollen from species with long styles and large pollen grains can easily pollinate species with short styles and smaller pollen grains (e.g. Carney et al. 1994; Rahmé et al. 2009). The average and SD of the pollen diameter of *I. longitubus* (43.1 ± 2.8 μm) were larger than those of *I. inflexus* (33.2 ± 2.4 μm) and hybrids (35.8 ± 4.0) ($P < 0.001$, Mann–Whitney *U* test; Yamashiro, T. unpublished data). Therefore, it is likely that *I. inflexus* and hybrid individuals, having shorter styles than *I. longitubus*, tend to act as maternal parents. However, this should be verified in the future through interspecific pollen competition experiments using artificial crosses.

Ogishima et al. (2019) concluded that chloroplast captures in Japanese *Isodon* species resulted from introgression between the species. Maki et al. (2010) suggested that Japanese *Isodon* species might have experienced ancient introgressions. In Japanese *Isodon*, contemporary interspecific hybridization seems to occur frequently, as evidenced by the records of eight hybrid species, including those between short- and long- corolla tube species (Murata and Yamazaki 1993). Taking into account that morphological differences are preserved among Japanese *Isodon* species and that no species of hybrid origin have been identified in previous phylogenetic analyses (Ogishima et al. 2019), it seems that even when interspecific hybridization occurs, the resultant hybrid zones are maintained only for a short time and do not yield any new taxa originating from hybridization. Signs of hybridization in nuclear DNA might be diluted due to repeated unidirectional backcrossing, as hypothesized for cytoplasmic introgression in a *Helianthus* population by Rieseberg (1995). Lavretsky et al. (2016) demonstrated that evidence of admixed histories in the nuclear genome was effectively lost after the fourth generation of unidirectional backcrossing, based on the simulation of 3448 SNP_S loci. In the contact zone examined in this study, signs of contemporary hybridization in nuclear DNA may have been diluted by repeated introgression to *I. longitubus*. Thus, the present study could illustrate the process of unidirectional introgression leading to chloroplast capture, a phenomenon frequently observed in *Isodon* species in Japan.

Conclusions

We found F_2-like and backcross like hybrids in a contact zone of long corolla *I. longitubus* and short corolla *I. inflexus*. Chloroplast DNA sequences suggested that initial F_1 hybrids were formed by the short corolla species, i.e. *I. inflexus* was the maternal parent. Genetic analyses on seedlings from the contact zone revealed a tendency of asymmetrical backcrosses toward *I. longitubus*. This asymmetrical hybridization might be caused by the discrepancy in the numbers of the two parental species. Differences in style length and pollen size might also be factors in asymmetrical hybridization between these species, although artificial crossing experiments are required to test this idea. Asymmetrical introgression likely to be a factor contributing to the chloroplast capture seen in Japanese *Isodon* species.

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Information on Electronic Supplementary Material

Online Resource 1. Information for 13 loci used in this study. Allele sizes and numbers of private alleles were estimated from pooled genetically pure individuals of *I. inflexus* and *I. longitubus*, respectively.

Online Resource 2. Triangle plot of ancestry (*S)* and interspecies heterozygosity (H_I) simulated for the first two generations of admixture (each parental, F_1, F_2 and each backcross) between *I. inflexus* and *I. longitubus*.

Online Resource 3. Principal component loading on the first and second axes.

Author contributions T.Y. and M.M contributed to the design of the study, and the proof outline. T.Y. and A.Y. collected the data and conducted data analyses. I.D. and M.M. aided in interpreting the results and worked on the manuscript. The draft of the manuscript was written by T.Y. and M.M. All authors discussed the results and commented on the manuscript.

Declarations

Author Contribution

T.Y. and M.M contributed to the design of the study, and the proof outline. T.Y. and A.Y. collected the data and conducted data analyses. I.D. and M.M. aided in interpreting the results and worked on the manuscript. The draft of the manuscript was written by T.Y. and M.M. All authors discussed the results and commented on the manuscript.

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Tables

Table1 Genetic variation at 13 microsatellite loci in *I. inflexu*s (I), *I. longitubus* (L), and plants in the contact zone (including seedlings) (C)

Population	N	N_A	A_R	H_O	uH_E	F_{IS}
I1	16	5.85	6.52	0.63	0.66	0.01
I2	21	7.00	8.13	0.67	0.70	0.03
L1	18	9.08	7.94	0.71	0.78	0.06
L2	29	9.46	8.43	0.73	0.74	-0.02
C	69	10.77	9.10	0.64	0.77	0.17

N = number of individuals, N_A = number of alleles, A_R = allelic richness, H_O = observed heterozygosity, uH_E = unbiased expected heterozygosity, F_{IS} = inbreeding coefficient.

Table 2 Haplotypes found in *I. inflexus*, *I. longitubus*, and plants in the contact zone

	haplotype				
Population ID	A	B	C	D	E
I1	-	-	-	16	-
I2	2		16	3	-
L1	-	-	-	13	5
L2	27	2	-	-	-
C	-	-	19	28	-
seedlings	-	-	9	12	-

Figures

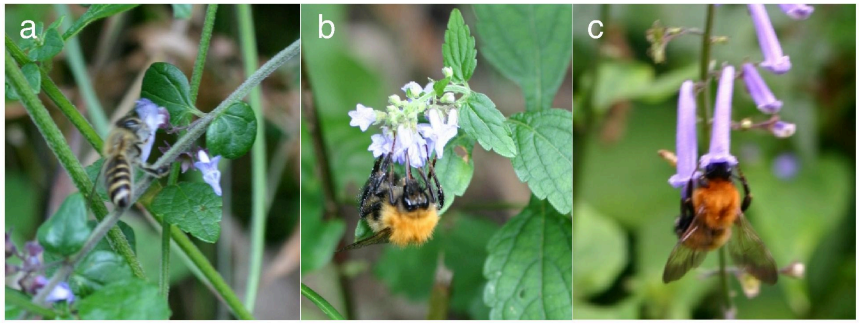

Figure 1

Flowers of *Isodon inflexus* and *I. longitubus*, and their pollinators. **a** *I. inflexus*visited by *Apis cerana japonica*; **b** *I. inflexus* visited by *Bombus diversus*; **c** *I. longitubus* visited by *Bombus diversus*

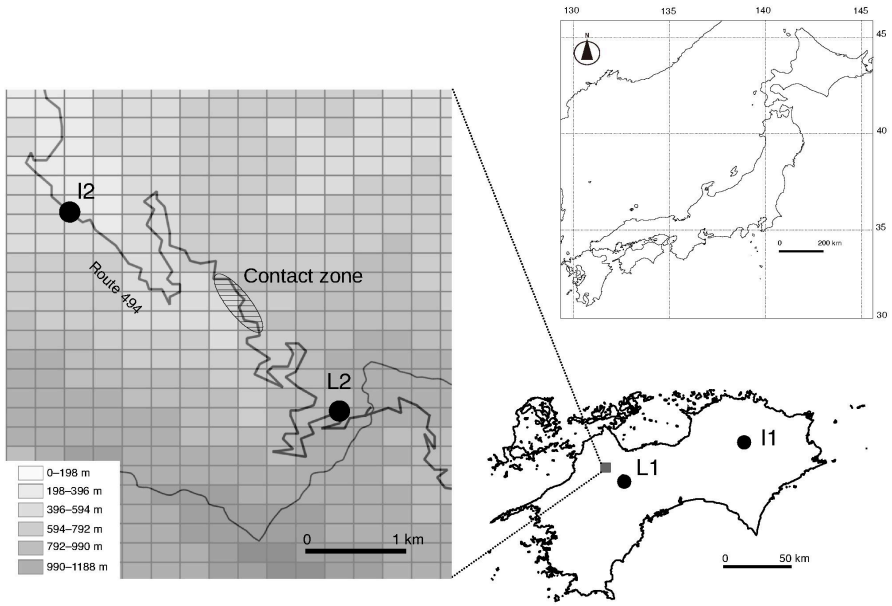

Figure 2

Map of the sampling sites of *Isodon inflexus* (I) and *I. longitubus* (L), and the contact zone of both species

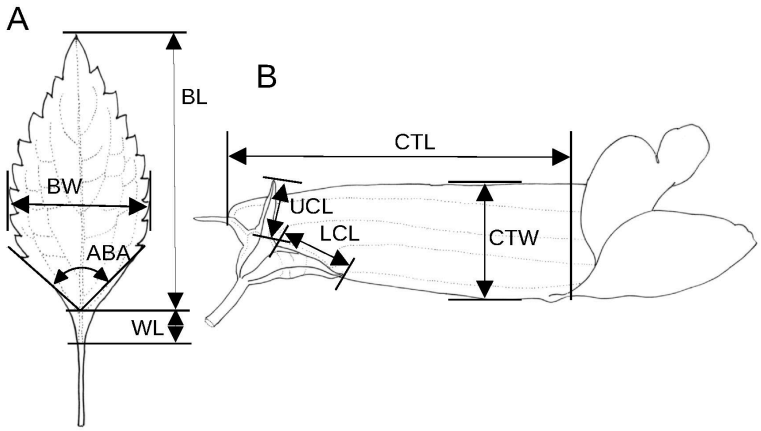

Figure 3

Leaf and floral characters used in morphometric analysis. **a** leaf; **b** whole flower. Symbols: *BL*leaf blade length, *BW* leaf blade width, *WL* wing length, *ABA*angle of leaf blade base, *CTL* corolla tube length, *CTW* corolla tube width, *UCL* upper calyx lobe length, *LCL* lower calyx lobe length

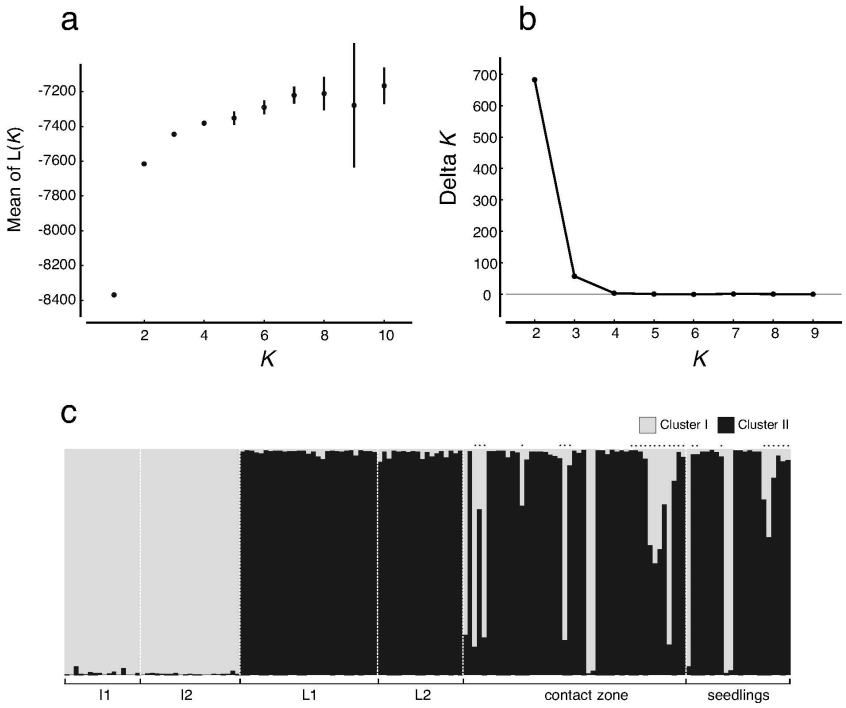

Figure 4

Results of Bayesian cluster analysis of *Isodon inflexus*, *I. longitubus*, and putative hybrids. **a** Mean L(*K*) ± SD over ten runs on STRUCTURE; **b** Plots of delta-*K* values against *K* = 2–10 on STRUCTURE; **c** Individual probabilities of assignment to *K* = 2. Dots above the bars for the

contact zone and seedlings indicate individuals with haplotype C

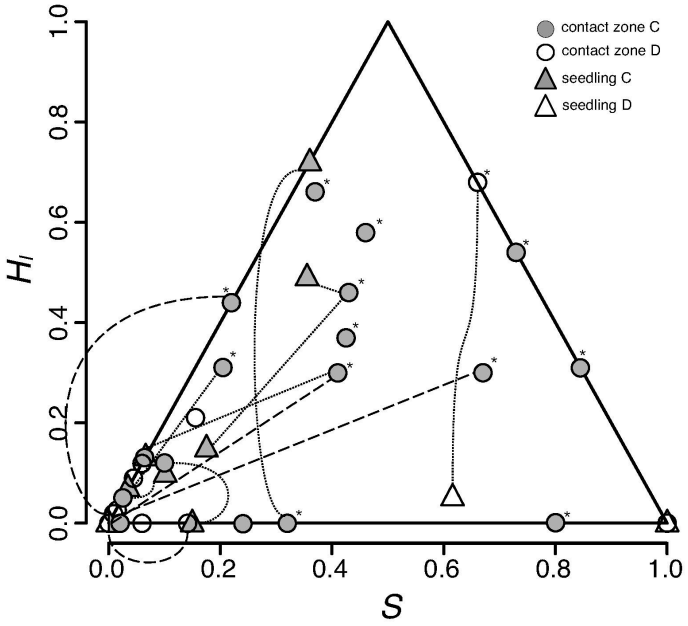

Figure 5

Triangle plot of ancestry (*S*) and interspecies heterozygosity (*HI*) between *I. inflexus*, *I. longitubus* and plants in the contact zone. Dotted and dashed lines connect maternal plants and their seedlings. Asterisks indicate hybrids detected by STRUCTURE. C and D indicate cpDNA haplotypes. The lower right

vertex of the triangle consists of two contact zone samples and two seedlings. The lower left vertex of the triangle consists of 22 contact zone samples and 12 seedlings

Genetic structure in an Isodon hybrid zone Fig. 6

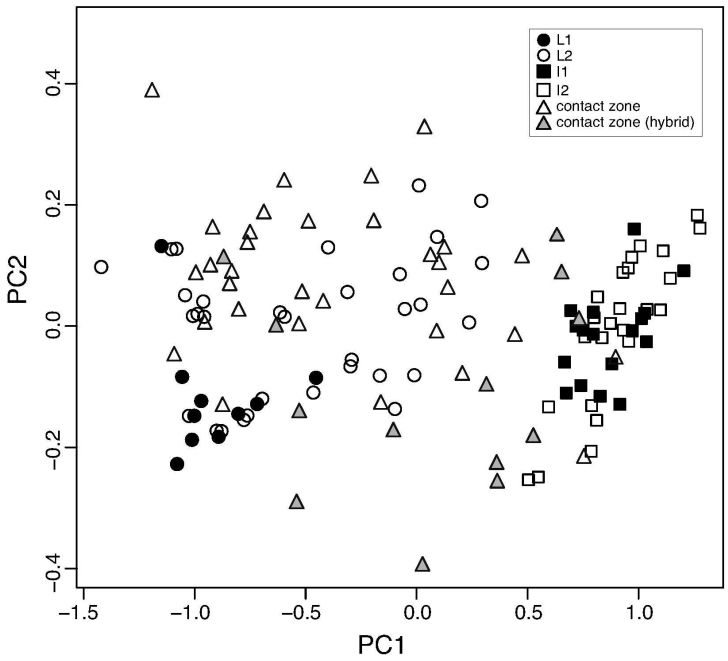

Figure 6

Principal component analysis (PCA) ordination based on 8 morphological characters

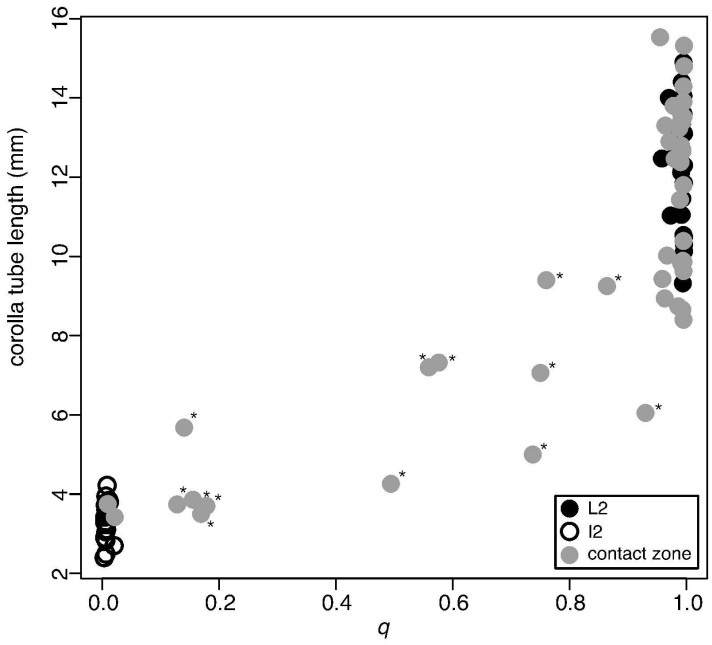

Figure 7

Relationship between corolla tube length (CTL) and q value derived from STRUCTURE. Asterisks indicate hybrid individuals

Supplementary Files

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