Origin of the disjunct distribution of flower colour polymorphism within *Limonium wrightii* (Plumbaginaceae) in the Ryukyu Archipelago

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The sea lavender, *Limonium wrightii*, has six morphs of flower colour variation. The geographical distribution of flower colour morphs is disjunct; the distribution of the pink flower morph is divided into two subregions, and that of the yellow flower morph intervenes between them. The present study aimed to examine the origin of this apparent distribution pattern of flower colour in *L. wrightii*. Two main hypotheses (i.e. past dispersal events and phenotypic changes by natural selection and/or stochastic processes) have been proposed to account for the origin of leapfrog distribution patterns. To determine which hypothesis was applicable, we conducted a molecular phylogenetic analysis using sequence variation in chloroplast DNA (three regions of intergenic spacers, *trnG-trnfM*, *trnV-trnM*, and *psbA-trnH*). We sequenced 58 accessions of *L. wrightii* frin 28 islands in the Ryukyu Archipelago and the Izu-Ogasawara Islands, located south of the Japanese mainland, and 12 accessions of four congeneric species. Within *L. wrightii*, we obtained four lineages of ten haplotypes. These lineages and haplotypes did not correlate with the different flower colours. These results indicate that the formation processes of populations are complex. The haplotypes of the pink flower morph did not show a sister relationship between the two disjunct subregions, indicating that the disjunct populations of the pink flower morphs are unlikely to share the pink flower colour as a result of common ancestry. We conclude that the observed leapfrog distribution pattern is caused by natural selection and/or stochastic processes. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **97**, 709–717.

ADDITIONAL KEYWORDS: allopatric – geographic structure – insular plant – leapfrog pattern – north-western Pacific – subtropical.

INTRODUCTION

Islands have fascinated biologists since the days of Darwin and Wallace. Their distinct biotas are considered to be natural laboratories for the evolution of organisms (Mayr, 1963; Barrett, 1998; Whittaker & Fernandez-Palacios, 2006) because they feature defined oceanic boundaries, reduced gene flow between islands, small geographical sizes, and other pertinent characteristics (Emerson, 2002). Organisms on islands show very attractive evolutionary phenomena; for example, adaptive radiation, loss of dispersibility, and the evolution of woodiness in plants (Carlquist, 1974; Williamson, 1981; Grant, 1998; Stuessy & Ono, 1998). Numerous studies using molecular methods have been carried out to examine the origins and the

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evolution of diverged groups on oceanic islands (reviewed in Emerson, 2002; see also Baldwin, 1997; Ballard & Sytsma, 2000; Gillespie, 2004; Hollanda & Hadfield, 2004; Howarth & Baum, 2005). However, apart from several phylogeographical studies of plants reported from the Caribbean and Mediterranean regions (Fritsch, 2003; Bittkau & Comes, 2005), there are few investigations of plant groups on land-bridge islands once connected to the mainland in times of lower ocean levels associated with Pleistocene glaciation. Despite the lack of drastic phenotypic radiation, land-bridge islands offer good study fields for the investigation of species divergence. In particular, interactions between plants and animals on islands, such as pollination systems, are key factor in the evolution of insular organisms (Inoue, 1988; Bowen & Van Vuren, 1997; Pérez-Bañón *et al.*, 2003; Traveset & Richardson, 2006).

The Ryukyu Archipelago is an arc of islands situated in a subtropical region in the north-western Pacific. On the one hand, this region belongs to the Holarctic Floral Kingdom (Takhtajan, 1986), and its flora is related to the evergreen forests of the Sino-Japanese Region, although numerous plants originated in tropics. On the other hand, the Ryukyu Archipelago is zoogeographically regarded as a palaeotropical region. The fauna is related to the tropics of south-east Asia (Gressitt, 1961), or Taiwan and continental China (Ota, 1998). Thus, the biota is compounded from tropical to temperate, and the species compositions of both flora and fauna vary between the islands (Walker, 1976; Yamane, Ikudome & Terayama, 1999). Such different species compositions would be expected to influence plant–animal interactions (Thompson, 1994, 2005; Herrera, Castellanos & Medrano, 2006). Because ecogeographical factors often contribute to the reproductive isolation of flowering plants (Schemske, 2000), different compositions of species in the Ryukyu Archipelago could mediate plant speciation by affecting pollination processes.

Limonium wrightii (Hance) Kuntze, a member of Plumbaginaceae, is distributed from the south-eastern offshore region of Taiwan throughout the Ryukyu Archipelago, reaching to the Daito (Borodino) and Izu-Ogasawara (Bonin) Islands of Japan (Fig. 1). The habitats of this species are restricted to coastal areas, mostly on limestone rocks. Fruits of *L. wrightii* are dispersed primarily by ocean currents because they float in seawater and germinate after being soaked (Matsumura, 2004). Polymorphism of flower colour has been taxonomically reported (Hara, 1947), and we have distinguished six morphs of flower colour variation (Matsumura *et al.*, 2006). There is no difference other than flower colour among the morphs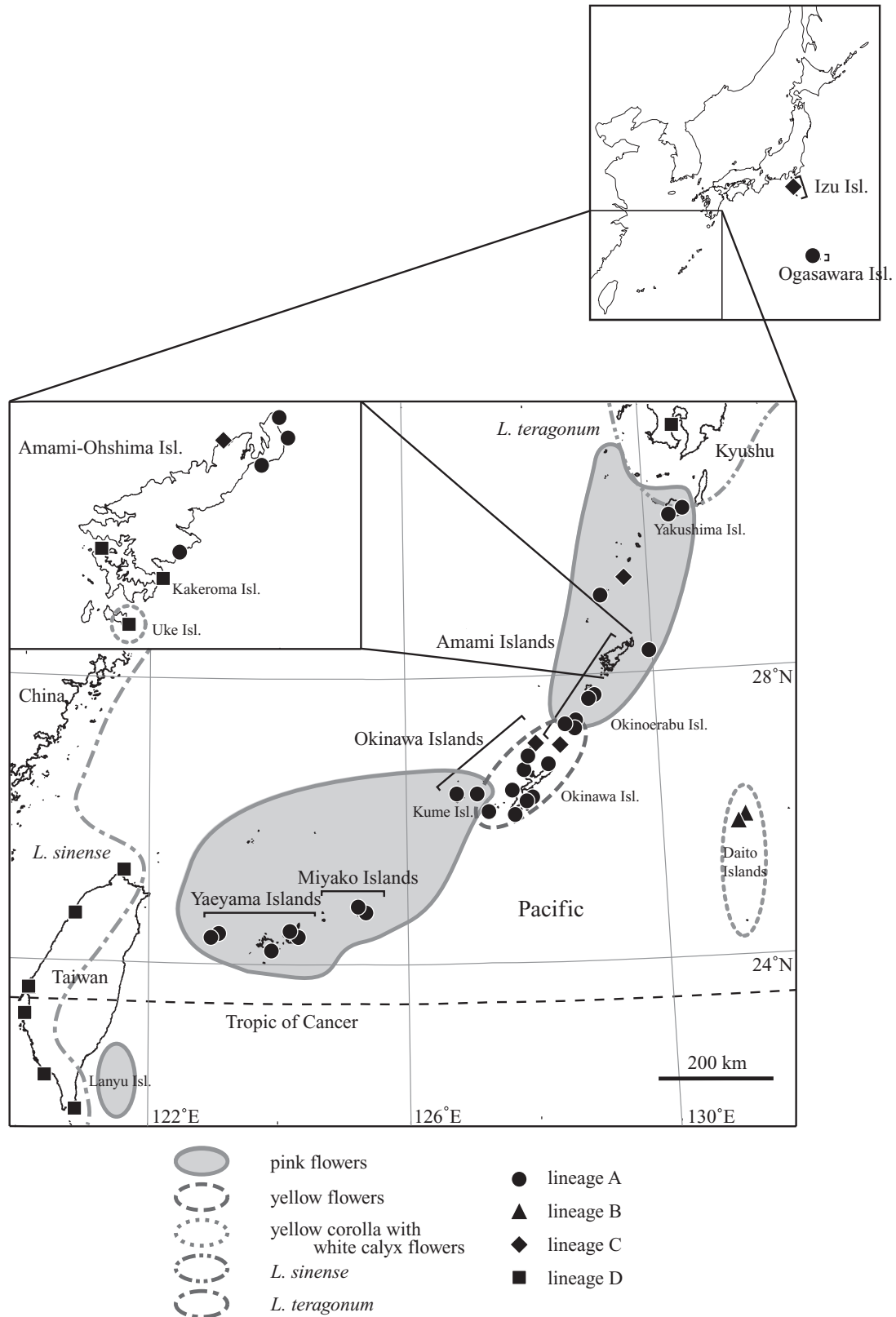. Two morphs (i.e. those with pink and yellow flowers) are most frequently observed, and the geographical distributions of these types are allopatric.

The distribution of the pink flower morph is divided into two subregions, and that of the yellow flower morph intervenes between them (Fig. 1); thus, the geographical distributions of the two flower colour morphs of *L. wrightii* show a so-called 'leapfrog pattern'. This pattern is one in which two populations very similar in appearance are geographically separated from each other by a different, intervening population of the same species (Remsen, 1984). This distinctive pattern of geographical distribution has also been reported in several bird species from humid Andean forests and rainforests in eastern Australia and New Guinea (Remsen, 1984; Norman *et al.*, 2002; Newton, 2003).

Of the remaining four flower colour types, yellow corolla with white calyx flowers is mainly found on the Daito Islands (Fig. 1), whereas only a single plant on Uke Island (Fig. 1), where the pink flower morph dominates, has this colour flowers (Matsumura *et al.*, 2006). Because three other colour types (i.e. orange, white, and ivory) are found relatively frequently on Okinoerabu Island, the only island where the pink and the yellow morphs co-occur, these types are suspected to have originated from hybridization between the pink and the yellow flower morphs, although these types are very rarely found on islands other than Okinoerabu Island (Matsumura *et al.*, 2006). At present, the genetic control of flower colour in *L. wrightii* and its relatives is unknown.

Norman *et al.* (2002) divided the hypotheses to explain the origin of leapfrog distribution (Remsen, 1984) into two main hypotheses. The first, which includes dispersal and vicariance hypotheses, assumes that the disjunct populations on one side originated from those on other side. The dispersal hypothesis presumes that disjunct populations have similar phenotypes resulting from colonization through long-distance dispersal from the other side. However, no such corridor connecting the disjunct populations has been reported in geological studies of the Ryukyu Archipelago (Hanzawa, 1935; Ujiie, 1994). Furthermore, even if dispersal occurs between disjunct populations, it is unlikely to be a main factor leading the leapfrog pattern because some dispersal between the southern populations of the pink flower morph and the central populations of the yellow flower morph will occur as a result of the Kuroshio Current going north along the Ryukyu Archipelago.

The second major hypothesis, the phenotypic change hypothesis, assumes unequal rates of phenotypic change among populations. This situation could arise through natural selection and/or stochastic processes. In such processes, there is a possibility that phenotypic characteristics changed rapidly in the

Izu Isl.

Ogasawara Isl.

L. teragonum

Kyushu

Amami-Ohshima Isl.

Yakushima Isl.

Kakeroma Isl.

Uke Isl.

Amami Islands

China

28°N

Okinoerabu Isl.

Okinawa Islands

Kume Isl.

Okinawa Isl.

Daito
Islands

L. sinense

Miyako Islands

Pacific

Yaeyama Islands

24°N

Taiwan

Tropic of Cancer

200 km

Lanyu Isl.

122°E

126°E

130°E

	pink flowers		lineage A
	yellow flowers		lineage B
	yellow corolla with white calyx flowers		lineage C
	L. sinense		lineage D
	L. teragonum		

Figure 1. Geographical pattern of the distribution of flower colour variations within *Limonium wrightii* and its closely-related species in the vicinity of the Ryukyu Archipelago. The distribution of each flower colour variation within *L. wrightii* and the related species *Limonium sinense* and *Limonium tetragonum* is indicated by line pattern. The locations of the haplotypes of lineages A, B, C, and D in Fig. 2 are indicated by a circle, triangle, diamond, and square, respectively.

central populations, or that convergent evolution occurred in the disjunct populations. Under this hypotheses, a lack of correlation between genetic similarities detected by neutral markers and phenotypic similarities would be expected.

These two main hypotheses, dispersal origin versus natural selection and/or stochastic forces, presume different genetic contexts. One clue to resolving the origin of the leapfrog distribution is to clarify whether the populations show a concordance of phenotypic and genetic differentiation.

To reveal which hypothesis correctly explains the origin of the leapfrog distribution pattern in *L. wrightii*, we conducted a molecular analysis based on sequences of chloroplast DNA (cpDNA). Because of their maternally inherited nature, it is worth analysing cpDNA sequences to estimate the gene flow occurring by seed migration (McCauley, 1995). In the present study, we use cpDNA variations to examine the cause of the leapfrog pattern of the flower colour morphs in *L. wrightii*.

MATERIAL AND METHODS

PLANT MATERIALS

We collected plant materials in the field and analysed 58 samples from 28 islands (Table 1). Almost all of the islands were land-bridge islands, although the Daito and Izu-Ogasawara Islands were oceanic islands. The samples covered the whole distribution area of *L. wrightii* to reduce the risk of an artefactual pattern caused by sampling error. Of the six flower colour types reported by Matsumura *et al.* (2006), we did not sample three types that putatively originated from hybridization between the pink and yellow flower morphs because their exclusion was unlikely to influence our investigation. We also collected and analysed seven samples of *Limonium sinense* (Girard) Kuntze in Taiwan and three samples of *Limonium tetragonum* (Thunb.) Bullock in Japan. For the outgroup, we added one sample each of *Limonium bicolor* (Bunge) Kuntze from continental China and *Limonium australe* (R. Br.) Kuntze from Australia using herbarium specimens. All five of these species belong to the same subsection *Chrysantheae* of section *Plathymenium* (Boissier, 1848). Voucher specimens have been deposited in the herbaria of Tohoku University (TUS).

DNA ISOLATION, AMPLIFICATION, AND SEQUENCING

The collected samples were quickly dried with silica gel and stored as herbarium specimens. Total genomic DNA was isolated from 200–300 µg of dried leaf tissues based on the 2× CTAB procedure of Doyle & Doyle (1987). Isolated DNA was resuspended in 100 µl of TE until use.

Two regions of intergenic spacers, *trnG* (GCC)-*trnfM* (CAU) and *trnV* (UAC)-*trnM* (CAU), were amplified with primers designed by Nishizawa & Watano (2000), and the *psbA-trnH* intergenic spacer was amplified with primers designed by Sang, Crawford & Stuessy (1997). Polymerase chain reaction (PCR) was performed in 50-µL reaction mixes containing 10–20 ng of total DNA, 1 µM primers, 1 mM dNTP, 50 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris HCl (pH 8.3) and 1.25 U of TAKARA rTaq (Takara Biomedicals). The amplification reaction began with an initial cycle at 94 °C for 2 min, and then 30 cycles of 1.5 min at 94 °C, 2 min at 48 °C, and 3 min at 60 °C, with a final extension of 1.5 min at 72 °C. After amplification, the reaction mixes were subjected to electrophoresis in 1% low-melting-point agarose gels to purify the amplified products. We sequenced the purified PCR products using a DYEnamic ET-Terminator Cycle Sequencing Kit (GE Healthcare) and an ABI 373A automated sequencer (Applied BioSystems). For sequencing, we used the same primers used for the amplification. All sequences in this report have been deposited in the DDBJ database.

STATISTICAL ANALYSIS

The sequences of the three intergenic spacer regions of cpDNA were combined into a single data set. All sequences were aligned using Se-Al, version 2.0a11 (Rambaut, 1996). An unrooted cladogram of haplotypes was constructed based on three regions of cpDNA using TCS, version 1.13 (Clement, Posada & Crandall, 2000). This software can be used to implement the statistical parsimony algorithm described by Templeton, Crandall & Sing (1992). By this method, an unrooted cladogram that has a high probability (more than 95%) of being true, based on a finite-site model of DNA evolution was identified. We analysed gaps as a fifth state, and considered gaps with a length greater than 1 as a single mutation.

Table 1. List of analysed samples and the localities in *Limonium wrightii* and its closely-related species in the north-western Pacific for chloroplast DNA sequences

Island	Number of localities	Flower colour	Haplotype (*N*)
Izu-Ogasawara Islands			
Shikine	2	Pink	8 (2)
Chichi-jima	1	PinK	1 (1)
Subtotal	3		
Ryukyu Archipelago			
Yaku-shima	2	Pink	2 (2)
Akuseki	1	Unknown*	9 (1)
Takara	1	Pink	1 (1)
Kikai	1	Pink	1 (1)
Amami-ohshima	6	Pink	1 (3), 2 (1), 8 (1), 11 (1)
Kakeroma	1	Pink	11 (1)
Uke	1	Yellow (white calyx)	11 (1)
Tokunoshima	2	Pink	1 (2)
Okinoerabu	2	Pink	1 (2)
Okinoerabu	2	Yellow	1 (2)
Okinoerabu	1	Orange†	1 (1)
Yoron	3	Yellow	1 (1), 7(2)
Iheya	3	Yellow	3 (1), 7 (2)
Izena	1	Yellow	3 (1)
Islet. Yanoshita	1	Yellow	1 (1)
Ie-jima	1	Yellow	1 (1)
Okinawa	5	Yellow	1 (2), 2 (2), 4 (1)
Islet. Oh-u	1	Yellow	4 (1)
Ikei	1	Yellow	2 (1)
Yabuchi	1	Yellow	4 (1)
Aka-jima	1	Yellow	1 (1)
Aguni	1	Pink	2 (1)
Kume	1	Pink	2 (1)
Kita-Daito	4	Yellow (white calyx)	6 (3)
Minami-Daito	4	Yellow (white calyx)	6 (3)
Miyako	2	Pink	5 (2)
Ishigaki	2	Pink	1 (1), 2 (1)
Kuroshima	1	Pink	2 (1)
Yonaguni	4	Pink	1 (4)
Subtotal	57		
Limonium sinense			
Taiwan			
Chunan	1	Yellow (white calyx)	11 (1)
Peimen	1	Yellow (white calyx)	13 (1)
Longkeng	1	Yellow (white calyx)	10 (1)
Yuwengtao	1	Yellow (white calyx)	11 (1)
Tongtunyu	1	Yellow (white calyx)	11 (1)
Shiao-liuchiu	1	Yellow (white calyx)	10 (1)
Subtotal	6		
Limonium tetragonum			
Japan			
Isl. Tashiro, Miyagi	1	Yellow (white calyx)	12 (1)
Akoh, Hyogo	1	Yellow (white calyx)	11 (1)
Kiire, Kagoshima	1	Yellow (white calyx)	11 (1)
Subtotal	3		
Limonium bicolor‡		Yellow (white calyx)§	
China			
Mt. Dachinshan, Nei-Mongole	1	Y.-C. Ma 2-87 in TUS	10 (1)
Limonium australe‡		Yellow (white calyx)§	
Australia			
New South Wales	1	Yonekura K., *et al.* 99334 in TUS	11 (1)
Total	71		

*This is most likely the pink type because the calyx was white, although it was old.
†Putative hybrid origin of the pink and yellow morphs.
‡Herbarium specimens.
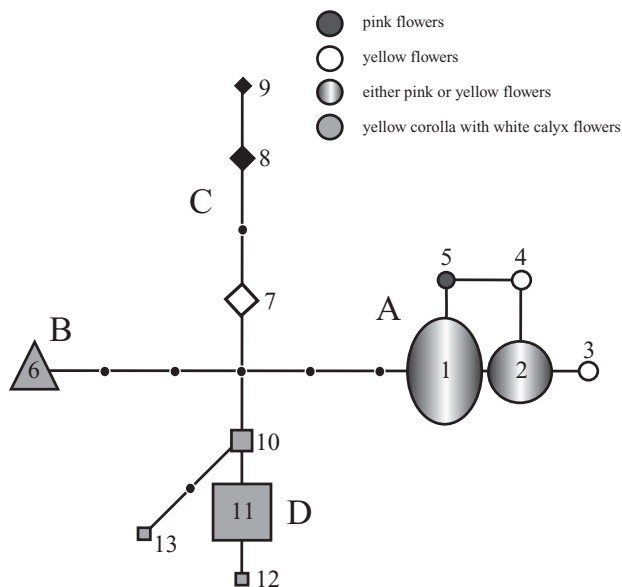§General traits of flower colour in the species.
TUS, Tohoku University.

RESULTS

NETWORKS OF CHLOROPLAST HAPLOTYPES

The lengths of the analysed intergenic spacers, *trnG* (GCC)-*trnfM* (CAU), *trnV* (UAC)-*trnM* (CAU), and *psbA*-*trnH* were 216, 200, and 316–324 bp, respectively (accession numbers AB450046–AB450222). A total of 11 variable nucleotide sites and two indels were observed within these regions (see Supporting information, Table S1). The length of the *psbA*-*trnH* intergenic spacer was stretched by including a highly variable poly-T (6–14 bp) site. We excluded the poly-T site from the data analyses because of possible homoplasy. Of the two indels, only one was phylogenetically informative, and the result did not change whether it was included or excluded. We distinguished 14 haplotypes and four lineages (labeled lineages A–D and haplotypes 1–14) from the cpDNA sequences (Fig. 2). Ten haplotypes belonged to *L. wrightii* and formed three lineages (lineages A, B, and C). These lineages did not correlate with the different of the flower colour polymorphisms in *L. wrightii*. The remaining four haplotypes were generally composed of the other species and formed one lineage (lineage D). The most common haplotype, haplotype 1, neighboured the second most common haplotype, haplotype 2. Including these haplotypes, five haplotypes formed in lineage A. Lineage B consisted of a sole haplotype,

haplotype 9. Lineage C was composed of three haplotypes that were unique to one island. Although the haplotypes of *L. wrightii* were generally discrete from those of other species, haplotype 11 was shared among three closely-related species, *L. sinense*, *L. tetragonum*, and *L. australe*, and was included in lineage D. This may suggest gene flow between these species and *L. wrightii* in and around the study region, although we have no other evidence of this at present.

GEOGRAPHIC DISTRIBUTION OF THE HAPLOTYPES

Lineage A, which included the most common haplotype, haplotype 1, was distributed widely throughout the geographical distribution of *L. wrightii*, including the Izu-Ogasawara Islands, whereas the other three lineages were restricted to scattered and relatively isolated sites (Fig. 2). Lineage B was restricted to the Daito Islands, oceanic islands located 380 km to the east of the Ryukyu Archipelago. The geographical distribution of lineage C was restricted to several sites in the Okinawa and Amami Islands. The populations of the lineage C were relatively isolated and consisted of smaller numbers of individuals than those of lineage A. The localities of the haplotypes of lineage C shifted northward as the haplotypes passed from the interior to tip. Haplotype 11, included in lineage D, was observed in three populations on Amami-Ohshima Island and its offshore islands (Fig. 2).

DISCUSSION

The lineages of cpDNA sequences did not correlate with the differences in flower colour. The two most common haplotypes were distributed across a boundary of flower colour morphs. Because the haplotypes of pink flowers did not show a sister relationship between northern and southern populations and no corridor(s) connecting the disjunct populations have been reported in this region as described above, it appears unlikely that the disjunct populations have a common ancestor and that the leapfrog distribution pattern was driven by long-dispersal events bypassing the central populations. Thus, it is probable that the leapfrog distribution pattern of the flower colour morphs is caused by natural selection and/or stochastic processes rather than a dispersal factor by leap colonization.

In the case of Andean birds, 28 of 416 species and species complexes of birds showed leapfrog distribution patterns in the Andean Cordillera. Remsen (1984) suggested that stochastic forces were likely responsible for generating the leapfrog patterns because of the lack of a geographical concordance in

Figure 2. Statistical parsimony network of chloroplast DNA haplotypes in *Limonium wrightii* and its closely-related species. A line and small black dots between haplotypes indicate mutational steps between them. The relative frequency of each haplotype is represented by the size of the symbol. The state of flower colour is indicated by shading pattern.

the position of the central populations and the fragmented life habitats near deep rivers. However, the geographical discordance in the distribution range of the central populations of 28 species only refutes the sharing of a corresponding selective factor. It is unclear whether some selective mechanism led to the evolution of plumage colour in each species. Concerning two groups of the birds in the Australo-Papuan rainforests, Norman *et al.* (2002) implied that, by promoting unequal rates of phenotypic evolution, both selection and stochastic processes were the mechanisms underlying the leapfrog distribution pattern because of unclear evidence for a sister relationship between disjunct populations in mitochondrial DNA sequences.

It is often difficult to discriminate between natural selection and stochastic factors because stochastic processes may also affect the frequencies of flower colour morphs, even if natural selection has mediated the evolution of flower colour polymorphisms (Gray & McKinnon, 2006). For example, evolutionary biologists have long debated the relative contributions of natural selection and random genetic drift to the evolution of flower colour in *Linanthus parryae* (Epling, Lewis & Ball, 1960; Wright, 1978). Schemske & Bierzychudek (2001) suggested that the frequency of each flower colour morph was largely affected by local selection pressures. They showed that natural selection of flower colour in *L. parryae* was strong (Schemske & Bierzychudek, 2007) and that the direction and intensity of selection varied both in time and space.

Regarding *L. wrightii*, the details of how natural selection affects flower colour in both direction and intensity have not yet been elucidated. Thus, we cannot determine the relative importance of natural selection and stochastic factors in causing the geographical pattern of flower colour in *L. wrightii*. However, we can at least suggest that it is not just stochastic processes that play an important role. In a previous study, we surveyed the flower colours of 137 populations on 36 islands (Matsumura *et al.*, 2006), and the geographical distributions of intraspecific variations were divided into three major subregions. In each subregion composed of more than 30 populations, all populations exhibited a single flower colour (i.e. pink or yellow) (Matsumura *et al.*, 2006). Accordingly, it is unlikely that stochastic processes alone lead all plants to take one flower colour on a larger geographical scale, such as a subregion. Thus, we suspect that natural selection works as a primary factor in the formation of the flower colour polymorphism.

In our observations of *L. wrightii*, the flowers of both the pink and yellow morphs were visited primarily by several species of small bees, *Ceratina* spp.

and *Lasioglossum* spp., whereas pink flowers were also visited by various medium- to large- bees, wasps, and butterflies (Matsumura, 2004). Thus, although the pollinators of both flower colour morphs might overlap, the selective preferences of the pollinators are apparently different. Unfortunately, it is not obvious how differences in pollinators affect the evolution of flower characteristics and whether the small bees discriminate flower colour in their foraging bouts. However, many studies have shown that variation in flower colour generally accounts for an adaptive response to divergent selective pressures generated by pollinators, even if pollinator visitations differ only quantitatively (Stanton, Snow & Handel, 1986; Levin & Brack, 1995; Meléndez-Ackerman, Campbell & Waser, 1997; Niovi Jones & Reithel, 2001). To examine this further, examinations involving manipulation should be conducted (e.g. transplantation into populations with a different flower colour). By examining the behavioural responses of pollinators and the relative effectiveness of each pollinator species, we could determine the evolutionary causes of the differentiation of flower colour.

In conclusion, the disjunct populations with the pink flower morph do not comprise cpDNA haplotypes that are distinct from those of the yellow flower morph. The leapfrog pattern of the flower colour morphs in *L. wrightii* is thus unlikely to be explained by the dispersal or vicariance hypotheses. Natural selection, probably by pollinator preferences and/or stochastic processes, appears to play a major role in the distribution patterns of the flower colour morphs in *L. wrightii*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Nucleotide site variations in *Limonium wrightii* and its related species for three intergenetic spacer regions of chloroplast DNA. The above haplotype 1 is the most common one in *L. wrightii*. Intergenic spacer regions: (I) *trnG* (GCC)-*trnfM* (CAU), (II) *trnV* (UAC)-*trnM* (CAU), and (III) *psbA-trnH*.

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