

Population Genetic Structure of *Titanotrichum oldhamii* (Gesneriaceae), a Subtropical Bulbiliferous Plant with Mixed Sexual and Asexual Reproduction

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Received: 7 April 2003 Returned for revision: 29 August 2003 Accepted: 4 November 2003 Published electronically: 5 January 2004

• **Background and Aims** *Titanotrichum oldhamii* is a monotypic genus distributed in Taiwan, adjacent regions of China and the Ryukyu Islands of Japan. Its conservation status is vulnerable as most populations are small and widely scattered. *Titanotrichum* has a mixed system of reproduction with vegetative bulbils and seeds. The aim of this study was to understand the population genetic structure of *Titanotrichum* in relation to its specific reproductive behaviour and to determine possible implications for conservation strategies.

• **Methods** After an extensive inventory of most wild populations of *Titanotrichum* in East Asia, samples from 25 populations within its major distribution were carried out utilizing RAPD and inter-SSR molecular fingerprinting analysis.

• **Key Results** The findings support the conclusion that many populations reproduce predominantly asexually but that some genetic variation still exists within populations. However, significant amounts of variation exist between populations, perhaps reflecting population differentiation by drift. This partitioning of genetic diversity indicates that the level of inter-population gene exchange is extremely low. These findings are consistent with field observations of very limited seed production. The Chinese populations are similar to those of Northern Taiwan, while the Ryukyu populations fall within the range of variation of the north-central Taiwan populations. The Taiwanese populations are relatively variable and differentiation between north, east and south Taiwan is evident.

• **Conclusions** The distribution of *Titanotrichum* seems to be consistent with a former land connection between China, Taiwan and the Ryukyu Islands at a glacial maximum during the Quaternary, followed by progressive fragmentation of the populations. North-central Taiwan is the centre of genetic diversity, possibly due to the proximity of the former land bridge between the regions, together with the variety of suitable habitats in north Taiwan. The significance of these findings for conservation is discussed. © 2004 Annals of Botany Company

Key words: Facultative vegetative apomixis, sea-level change, Pleistocene biogeography, bulbil, vivipary, propagules, clonal plant.

INTRODUCTION

Many plant species can reproduce clonally by creeping roots or stems, propagules such as bulbils and tubers, or agamous seeds. Bulbiliferous species have developed specialized vegetative organs for reproduction and the genetic and ecological significance of bulbiliferous reproduction is of evolutionary interest.

Bulbiliferous plants usually continue to produce flowers, thus retaining the potential for sexual reproduction (Arizaga and Ezcurra, 1995). Under extreme environmental conditions, as in Arctic or montane regions, clonal reproduction by pseudo-vivipary (*sensu* Kerner) may become dominant as suitable conditions for sexual reproduction are limited (Kerner, 1904; Klimeš *et al.*, 1997). *Polygonum viviparum* has been observed to initiate more bulbils in the lower part of the inflorescence when growing in Arctic-alpine regions as compared with warmer regions (Diggle, 1997). In other Arctic clonal plants, such as *Saxifraga cernua* and *Poa*

alpina, seed set has only very rarely been observed (Briggs and Walters, 1997; Gabrielsen and Brochmann, 1998).

In tropical and subtropical plants, bulbils are fairly unusual (with a few exceptions such as *Remusatia vivipara* and *Globba* spp.), and bulbiliferous reproduction is seldom documented in tropical environments where pseudo-vivipary is not a reaction to extreme conditions (Moody *et al.*, 1999). Clonal plants in the tropics, with certain notable exceptions such as *Eichhornia paniculata* (Glover and Barrett, 1987) and *Aechmea magdalenae* (Murawski and Hamrick, 1990), have rarely been investigated for genetic diversity and population structure.

Titanotrichum oldhamii has evolved a mixed reproductive strategy of bulbils, rhizomes and seeds in its habitat of temperate to subtropical monsoon rain forest. The rhizomes are important for winter survival. It produces showy, tubular flowers in summer and is able to set seed if pollinators are available (Wang, 2003). The family Gesneriaceae, to which *Titanotrichum oldhamii* belongs, exhibits a wide range of morphological variation (Jong and Burt, 1975; Möller and Cronk, 2001). Variable meristem behaviour is well known in vegetative parts of the Gesneriaceae (Burt, 1970;

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TABLE 1. Locations of all populations of *Titanotrichum oldhamii* in this study

Population				
Code	Country	Locality	Latitude	Longitude
A	China	Fujian (Northern): Nanping	26°38'N	118°10'E
B		Fujian (Southern): Yungchun	25°19'N	118°17'E
C		Fujian (SW): Niumuling	24°31'N	117°21'E
D	Taiwan (northern)	Taipei: Huangtitian	24°59'N	121°40'E
E		Taipei: Wulai	24°52'N	121°33'E
F		Taipei: Juansi waterfall	25°10'N	121°33'E
G	Taiwan (north-central)	Taipei: Datun waterfall	25°09'N	121°32'E
H		Taoyuan: Ronhua	24°48'N	121°20'E
I		Taoyuan: Paling	24°43'N	121°22'E
J		Taoyuan: Tawan	24°36'N	121°19'E
K		Taoyuan: Manueuan waterfall	24°43'N	121°25'E
L		Taoyuan: Peichatien shan	24°42'N	121°27'E
M		Taiwan (eastern)	Hualien: Tairoko	24°09'N
N	Taiwan (central)	Hualien: Chinsui shan	24°12'N	121°39'E
O		Hualien: Hopping logging trail	24°17'N	121°41'E
P		Hualien: Leewu chi	24°10'N	121°33'E
Q	Taiwan (southern)	Hualien: Shi-Pao	24°11'N	121°29'E
R		Taichung: Pahsien shan	24°10'N	121°01'E
S		Kaohsiung: Shou shan	22°39'N	120°15'E
T		Pingtung: Chuyun shan	23°04'N	120°45'E
U		Taitung: Patunkuan trail	23°21'N	121°11'E
V	Japan	Taitung: Tienlung waterfall	23°13'N	120°59'E
W		Taitung: Luye	22°55'N	121°08'E
X		Irimote (Eastern): Funawula	24°20'N	123°46'E
Y		Irimote (Western): Urauchi	24°23'N	123°51'E

Tsukaya, 1997; Imaichi *et al.*, 2000), but variable floral meristem behaviour is unusual. However, in late season, inflorescence-borne bulbils replace all the floral meristems at the top of the racemes of *Titanotrichum*, forming hundreds of bulbil clusters for vegetative reproduction (Wang and Cronk, 2003).

In *Titanotrichum*, the division between bulbil propagation and sexual reproduction appears to vary between populations, possibly due to environmental factors. Some populations set more seeds than others, suggesting that the availability of pollinators between populations may differ (Wang, 2003). In general, however, seed set in *Titanotrichum* is rare, as can be observed in the wild and from herbarium material. This suggests that bulbil propagation in *Titanotrichum* is the major mode of reproduction and spread in the wild. However, the plant still produces functional flowers, and pollination experiments in *Titanotrichum* have shown that artificial pollination can readily produce seeds (Wang, 2003).

Titanotrichum oldhamii grows in shady habitats along creeks, particularly on wet, dripping cliffs. This favours water-mediated dispersal by fragments of rhizomes, bulbils and, rarely, seeds or leaf pieces (which can root and produce plantlets; C.-N. Wang, pers. obs.). From field and glass-house observations, it is evident that it can regenerate from its tiny bulbils into 10 cm high plants in 2 months. However, *Titanotrichum* is not common in the wild and continues to decline (Walker, 1976; Wang *et al.*, 1998). It has a scattered distribution in Taiwan, adjacent areas of China and the south Ryukyu Islands of Japan. Populations are often small and isolated and population bottlenecks are likely to occur. In small populations ($N < 100$), genetic drift and inbreeding

may cause a loss of genetic diversity (Ellstrand and Elam, 1993). Moreover, insufficient gene flow between and within populations due to fragmentation may affect its fitness to adapt to current and future environmental changes.

In this study, both inter-SSR and RAPD markers have been used to explore the genetic structure of *Titanotrichum*, as these markers require no prior genomic information. The ready availability of data and the ease of use make them highly suitable for surveying endangered species of clonal reproduction (e.g. Stiller and Denton, 1995; Hollingsworth and Bailey, 2000). Inter-SSR markers require high PCR annealing temperatures and consequently have good reproducibility. Although the assumptions of RAPD analysis are slightly different from those of inter-SSR analysis, recent studies suggest results from these two data types are consistent with each other (Hollingsworth *et al.*, 1998).

The primary aim of this study was to investigate clonal diversity at different levels in *Titanotrichum oldhamii* (within and among populations), and to link these patterns to geographical distribution. A detailed field survey and assessment of genetic diversity was therefore carried out using RAPDs and inter-SSRs to underpin its future conservation. The results presented here may also be suggestive of general patterns and processes in the history and origin of the Taiwan flora.

MATERIALS AND METHODS

Field survey

As no detailed field survey of *Titanotrichum* had previously been carried out, information from herbarium specimen

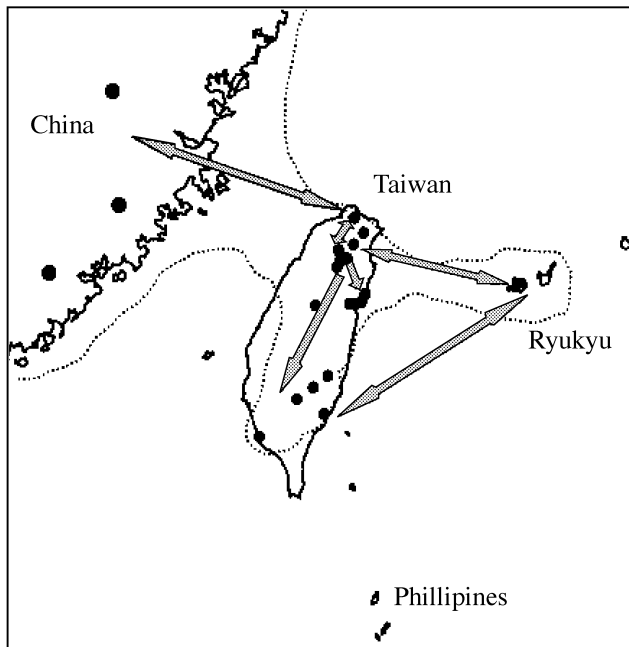


FIG. 1. Map of East Asia showing the distribution of *Titanotrichum oldhamii* (circles). Twenty-five populations analysed are plotted. Arrows indicate the hypothesized directions of migration at the glacial maximum. Dashed lines indicate the hypothetical coast line during the Pleistocene glacial maximum (after Ota, 1998). This simplified relationship among populations of *Titanotrichum oldhamii* in different geographic regions is proposed according to the clustering results given in this paper.

sheets, regional floras and local botanists was assembled prior to two extensive field trips. During the summer of 1999 and 2000, 25 natural populations of *Titanotrichum oldhamii* (Hemsl.) Soler in Taiwan, China and the Ryukyu Islands of Japan were located and investigated. In the field, the phenology of each population, population size, associated species, vegetation type and possible pollinators were recorded and identified. The geographical location of each population is summarized in Table 1 and Fig. 1.

Collection of plant material and genomic DNA extraction

To assess the genetic diversity of *Titanotrichum*, DNA material for RAPDs and inter-SSRs were collected during the field survey. Leaf samples from up to 20 randomly chosen individuals per population were collected, but in southern Taiwan and the Ryukyu Islands only between five and ten individuals were sampled, reflecting the small size of these populations. For each individual, two medium-sized healthy leaves were chosen and dried rapidly in a sealed plastic bag using a 20 times greater volume or more of silica gel. The leaf tissue was then stored at ambient temperature until DNA extraction. In addition, living plants, as well as bulbils and seeds, were brought back to the Royal Botanic Garden Edinburgh for pollination experiments and progeny analysis. Voucher specimens were deposited in the herbarium of the Royal Botanic Garden Edinburgh (E).

Genomic DNA was extracted using a modified CTAB-based protocol (Doyle and Doyle, 1987). Isolated DNA was

TABLE 2. Sequence of primers used in this analysis and number of loci detected with these 11 primers for populations of *Titanotrichum oldhamii* analysed

Primer	Sequence 5' to 3'	Amplified loci	Proportion of polymorphism
OPA-12	TCGGCGATAG	17	0.59
OPA-15	TTCCGAACCC	10	0.90
OPA-16	AGCCAGCGAA	11	0.73
OPA-19	CAAACGTCGG	15	0.80
OPF-6	GGGAATTCGG	17	0.88
OPF-7	CCGATATCCC	12	0.25
OPF-12	ACGGTACCAG	18	0.70
OPP-5	CCCCGGTAAC	18	0.94
SSR-818	CACACACACACACACAG	14	1
SSR-835	AGAGAGAGAGAGAGAGYA	5	1
SSR-846	CACACACACACACACART	16	0.81
Total loci found (total polymorphism)		153	(0.77)

quantified by electrophoresis on a 1 % agarose gel along with a 1-kb DNA molecular weight marker (NEB Biolabs, Herts, UK) as a standard. The DNA concentration was adjusted to 1 ng μl^{-1} for all samples. This quantification is essential as different amounts of starting template may cause template competition in PCR amplification of RAPD and inter-SSR analysis (Halldén *et al.*, 1996).

Primer selection and PCR conditions

One hundred 10-mer RAPD primers (Operon Technologies, Surrey, UK) and 30 inter-SSR primers (NAPS Unit, University of British Columbia, Canada) were screened for performance and allelic polymorphism among populations. Only eight RAPD primers and three inter-SSR primers showed the required polymorphism, suggesting low levels of genetic diversity within *Titanotrichum oldhamii*. The primer sequences are listed in Table 2.

The PCR conditions for RAPD and inter-SSR followed Hollingsworth *et al.* (1998). For RAPD, 5–10 ng template DNA was incorporated in 15 μl reactions, containing 0.5 μM primer, 100 μM each dNTPs (Roche, Indianapolis, USA), 2.5 mM MgCl_2 , and 0.5 U Taq polymerase (Bioline, London, UK) and 1 \times Taq buffer (16 mM $(\text{NH}_4)_2\text{SO}_4$, 67 mM Tris-HCl (pH 8.8), 0.01 % Tween20). The PCR amplification profile was as follows: 2 min at 95 $^{\circ}\text{C}$, two cycles of 30 s at 95 $^{\circ}\text{C}$, 1 min at 37 $^{\circ}\text{C}$, 2 min at 72 $^{\circ}\text{C}$, and two cycles of 30 s at 95 $^{\circ}\text{C}$, 1 min at 35 $^{\circ}\text{C}$, 2 min at 72 $^{\circ}\text{C}$, followed by 41 cycles of 30 s at 94 $^{\circ}\text{C}$, 1 min at 35 $^{\circ}\text{C}$, 2 min at 72 $^{\circ}\text{C}$, then a final extension at 72 $^{\circ}\text{C}$ for 5 min. For inter-SSR analysis, PCR reactions were carried out as above except using 0.2 μM primer. The PCR conditions for inter-SSR were more stringent because the primers are longer: 95 $^{\circ}\text{C}$ for 3 min, then 40 cycles of 20 s at 93 $^{\circ}\text{C}$, 1 min at 55 $^{\circ}\text{C}$, 20s at 72 $^{\circ}\text{C}$, with a final 6 min extension at 72 $^{\circ}\text{C}$.

All PCR reactions for RAPD and inter-SSR were performed with a Perkin-Elmer GeneAmp 9600 Thermal Cycler or a DNA engine (Peltier Thermal Cycler PTC-100). PCR products were separated on 1.6 % agarose gels in 1 \times

TBE buffer with medium field strength [voltage/gel length (cm) ratio equal to 4, i.e. 20 V on 80 cm gel] and visualized under UV after ethidium bromide staining (0.1 µg ml⁻¹). Negative controls (no template DNA) were also included in each PCR. To ensure RAPD reproducibility, most PCR reactions were repeated for reliability in data scoring.

Data analysis

RAPD and inter-SSR amplified DNA bands were scored conservatively and, to avoid co-migrating band errors only positions of bright bands sharing similar intensity, fragment size above 0.4 kb and exact position were scored for presence/absence (1/0). A pair-wise distance matrix ($D_{ist} = 1 - J$) based on RAPD and inter-SSR data was generated, using Jaccard's similarity coefficient: $J = S_{ij}/T_{ij}$, where S is the total number of shared bands and T is the total number of bands between the i th and j th individuals (Sneath and Sokal, 1973). The computer package R (Legendre and Vaudor, 1991) and the statistical program NT-SYS (Rohlf, 1996) were used to perform a principal coordinate analysis (PCO). The same distance matrix was imported to the program package Arlequin (Schneider *et al.*, 2000) for AMOVA (analysis of molecular variance; Excoffier *et al.*, 1992), to analyse the population structure within and among populations (percentage of variance, Φ_{st}). For diversity measurement, two indices were used to estimate genetic diversity within and between populations. These are Shannon's index (S) and Simpson's index (D). Shannon's index of diversity was computed using POPGENE (Yeh *et al.*, 1999) using the formula corrected for binary RAPD and inter-SSR data: $S = -\sum p_i \log_2 p_i$, where p_i is the frequency of presence of each RAPD or inter-SSR band to provide an estimate of degree of variation within each population. Although Shannon's index provides less genetic information than other diversity indices, it is less biased simply because it does not rely on a Hardy-Weinberg equilibrium (Chalmers *et al.*, 1992). Many recent studies favour this index for comparison across different species (Bussell, 1999; Allnutt *et al.*, 2001). For comparison with other studies on clonal species, the proportion (G/N) of genets (G) in the total population (N) and the unbiased Simpson's index of diversity (D) were also calculated, using the formula: $D = 1 - \sum [n_i(n_i - 1)]/[N(N - 1)]$, where n_i is the number of individuals with RAPD and inter-SSR genotype in clone i , and N is the sample size. Finally a UPGMA tree connecting 25 populations was constructed to interpret population relationships. To test the possible correlation between geographic distance and genetic distance, a Mantel test provided by the R package was used.

RESULTS

Field observations

In this survey, 25 natural populations of *Titanotrichum oldhamii* were investigated (20 from Taiwan, three from China and two from Japan). Usually, *Titanotrichum* can be found around the headwaters of streams under subtropical broadleaved rain forest dominated by Fagaceae and

Lauraceae. It is often associated with other herbaceous plants such as *Rhynchosyris* and *Begonia* species but has a far more restricted distribution. *Titanotrichum* favours habitats such as wet limestone areas where roots are semi-exposed to dripping water. This habitat type has been affected by habitat change and human disturbance, although some populations in Taiwan and Japan grow within the protection of national parks. Most populations of *Titanotrichum* are small, scattered and isolated from each other. Seed set was only observed in medium to large populations (more than 25 individuals) in open habitats. Pollinators like bumble-bees are more frequent visitors to these populations than to those in dense shade. It was also observed that many individuals remained in a vegetative phase during the flowering season. This is especially true for individuals growing in dense shade, where they seldom reach the reproductive phase before the above-ground parts die at the end of the flowering season, while the rhizome perennates.

Population differentiation from combined RAPD and inter-SSR data

A total of 118 reproducible bands were amplified from eight RAPD primers and 35 bands from three inter-SSR primers. Similar conclusions were drawn from each data set independently and therefore data were combined to further explore our findings. In the combined data set of 153 RAPD and inter-SSR amplified 'loci', 119 bands (77.78 %) were polymorphic (Table 2). Two hundred and seven RAPD and inter-SSR banding phenotypes were identified from 283 individuals. Individuals that shared the same phenotypes were only found within populations and not between populations. One small population ('Urauchi' from Japan) consisted entirely of individuals sharing the same banding phenotype (Table 3).

PCO analysis

The first three coordinates of the PCO analysis for all genotypes describe 15.2 %, 8.4 % and 8.2 % of the total variance (31.8 % cumulative). Individuals from China, southern Taiwan and eastern Taiwan formed three distinctive groups, while populations from northern and 'north-central' Taiwan and Japan showed some degree of overlap (Fig. 2). Generally, Taiwanese populations covered most of the PCO space. Chinese populations were closest to northern Taiwan populations. The former grouped in two clusters: North Fujien Province versus south and west Fujien, but the two groups are placed together at one end of the PCO space (Fig. 2). The two Japanese populations exhibited considerable differences, despite their close proximity on the small island of Iriomote (arrow indicated in Fig. 2). From the UPGMA tree, considerable genetic differences can also be seen between populations in northern and north-central Taiwan (Fig. 3). Populations 'Manueuan waterfall' (K) and 'Peichatien shan' (L), which are situated at different altitudes on Peichatien mountain, were more dissimilar than north (A) and south Fujien

TABLE 3. Diversity measures of 25 populations of *Titanotrichum oldhamii* in this study

Populations	Locality	<i>N</i>	<i>G/N</i>	Simpson's <i>D</i>	Shannon's <i>S</i>	<i>P</i> (%)
A	Nanping	20	0.85	0.88	0.06	13.73
B	Yungchun	9	0.56	0.86	0.05	11.11
C	Niumuling	8	0.75	0.93	0.12	24.18
Chinese samples				0.89	0.16	30.72
D	Huangtitian	11	0.82	0.89	0.05	8.5
E	Wulai	17	0.59	0.95	0.26	50.98
F	Juansi waterfall	14	0.86	0.98	0.07	12.42
G	Datun waterfall	13	0.77	0.96	0.14	24.18
Northern Taiwan samples				0.96	0.29	54.90
H	Ronhua	9	0.67	0.92	0.12	23.53
I	Paling	8	0.75	0.93	0.11	22.88
J	Tawan	14	0.64	0.95	0.20	37.91
K	Manueuan waterfall	17	0.76	0.97	0.17	33.99
L	Peichatien shan	9	0.78	0.94	0.13	26.14
North-central Taiwan samples				0.94	0.26	49.67
M	Tairoko	20	0.85	0.90	0.10	21.57
N	Chinsui shan	9	0.89	0.93	0.09	20.92
O	Hoping logging trail	19	0.84	0.98	0.08	18.95
Eastern Taiwan samples				0.94	0.17	33.33
P	Leewu chi	10	0.70	0.93	0.05	11.11
Q	Shi-Pao	9	0.89	0.97	0.05	13.73
R	Pahsien shan	4	0.75	0.83	0.02	3.92
Central Taiwan samples				0.90	0.13	22.22
S	Shou shan	11	0.64	0.93	0.03	7.19
T	Chuyun shan	5	0.60	0.70	0.01	3.27
U	Patunkuan trail	16	0.63	0.95	0.07	15.03
V	Tienlung waterfall	12	0.58	0.92	0.06	12.42
W	Luye	5	0.60	0.80	0.02	5.23
Southern Taiwan samples				0.85	0.16	33.33
X	Funawula	9	0.63	0.87	0.03	7.84
Y	Urauchi	5	0.20	0.00	0	0
Japanese samples				0.44	0.13	21.57
Total for <i>Titanotrichum</i>		283	0.73	0.933	$S'_{sp} = 0.314$	77.78

N = sample size; *G/N* = numbers of genets/numbers of individuals; *D* = Simpson's index; *S* = Shannon's index; *P* % is percentage of RAPD + inter-SSR band polymorphism.

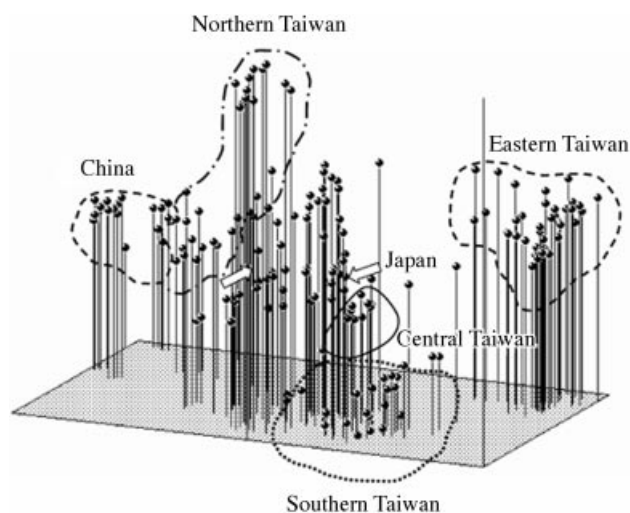


FIG. 2. Three-dimensional representation of a principal coordinate analysis of the genetic relationships among 283 individuals (207 genotypes) of *Titanotrichum oldhamii*, inferred from a distance matrix using the Jaccard index. Arrows indicate individuals in two Japanese populations. Scattered dots without grouping identify individuals in 'north-central' Taiwan.

(China) populations (B and C) in China (Fig. 3). Fujian Province is about the same size as Taiwan.

Genetic diversity indices

Shannon's diversity index (*S*) and Simpson diversity index (*D*) both indicated that populations in northern Taiwan ($S = 0.29$ and $D = 0.96$) and north-central Taiwan ($S = 0.26$ and $D = 0.97$) were most diverse. This is confirmed by the percentages of RAPD and inter-SSR band polymorphisms (Table 3). Populations in Japan, China, central and southern Taiwan are comparatively low in diversity, i.e. their *S* values are below 0.17 and the percentage of polymorphic bands is below 33.33 % (Table 3). The overall diversity for *Titanotrichum* was not high ($S'_{sp} = 0.314$) but was higher than expected for a clonal plant.

The Mantel test showed no significant correlation between genetic and geographic distance ($r = 0.417$, $P = 0.12$).

Composition of genetic variation

The partitioning of the genetic variation was further examined by analysis of molecular variance (AMOVA).

The majority of variation was found among populations, with genetic differences among geographic regions (Taiwan, China and Japan) less marked (14.5 %, Table 4). Half of the variation (50.0 %) was found among all populations rather than their geographic locations ($P <$

0.001, Table 4). It is also notable that only 35.5 % of the variance occurred within populations.

DISCUSSION

Population structure and genetic diversity

The PCO distribution of the 25 *Titanotrichum* populations suggests that the Chinese populations, populations in eastern Taiwan and populations from southern Taiwan form three distinct groups (Fig. 2). In contrast, individuals from north-central Taiwan are scattered across the PCO space and partly overlap with these three groups. The two Japanese populations were quite different from each other, one with affinity to north-central Taiwan populations and the other to central and southern Taiwan populations (Fig. 2). It is tempting to speculate that these results represent at least two independent migration events from Taiwan to the tiny island Iriomote of Japan.

The AMOVA showed that the majority of the genetic variation was among populations within regions rather than within populations or among geographic regions (Table 4). Genetic distances among all the populations, as indicated in the UPGMA tree (Fig. 3), were similar, with little correlation with geographic distance, possibly suggesting that the populations represent fragments from a wider distribution in the past (see discussion below). In no case was the same clonal genotype found within different populations, although they were sometimes observed within populations.

The populations in southern Taiwan and Japan were small and relatively clonal (i.e. $G/N < 0.63$ in Table 3), which could be due in part to a founder effect at the edge of the range. On the other hand, populations in northern and north Taiwan are quite diverse (Table 3 and Figs 2 and 3). Field observation and the diversity measures indicate that these populations may have a higher level of sexual reproduction (or a lower recruitment from bulbils). Considerable seed set, a high proportion of sexually mature individuals and pollinator visits were recorded in populations of northern and north-central Taiwan (C.-N. Wang, pers. obs.). These

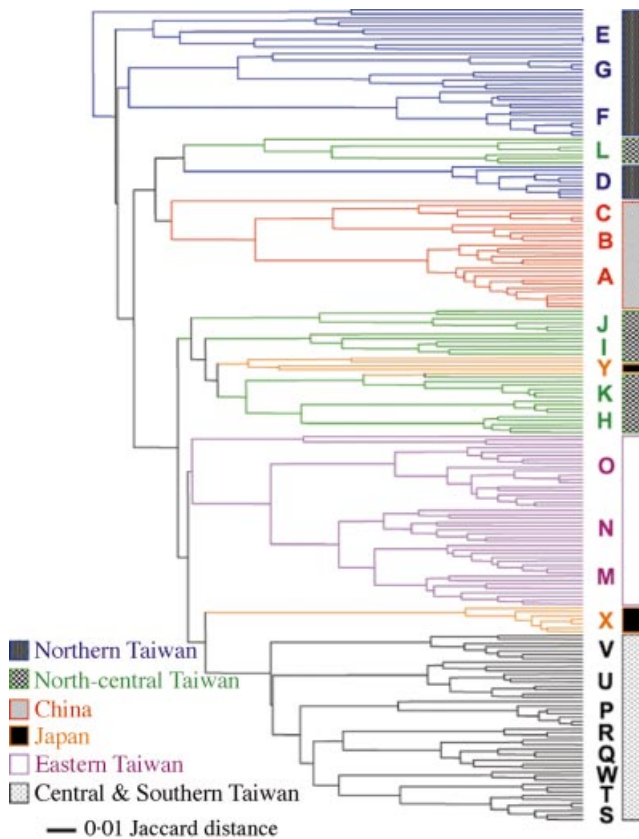


FIG. 3. UPGMA phylogram constructed using pair-wise Jaccard distances based on combined RAPD and inter-SSR data among 283 individuals of 25 *Titanotrichum oldhamii* populations from Taiwan, China and Japan.

TABLE 4. Summary of analyses of molecular variance (AMOVA) at different hierarchical levels for 283 *Titanotrichum oldhamii* individuals, based on combined RAPD and inter-SSR data

Source of variation	Variance components			Fixation index	
	Absolute	%	d.f.	$\Phi_{ct, st, sc}$	P value
Among Taiwan, China and Japan	0.028	14.5	2	0.14	$P < 0.001$
Among populations within Taiwan, China and Japan	0.096	50	22	0.64	$P < 0.001$
Within all populations of <i>Titanotrichum oldhamii</i>	0.069	35.5	258	0.58	$P < 0.001$
Among northern, central, eastern and southern Taiwan	0.028	15.91	3	0.16	$P < 0.001$
Among populations within Taiwan	0.074	42.16	15	0.58	$P < 0.001$
Within all populations in Taiwan	0.074	41.93	212	0.50	$P < 0.001$
Among populations within China	0.057	41.53	1	0.59	$P < 0.001$
Within all populations in China	0.055	40.61	34	0.51	$P < 0.001$
Among populations within Iriomote, Japan	0.135	87.24	1	0.87	$P < 0.005$
Within populations in Iriomote, Japan	0.02	12.76	12	n/a	

factors probably contribute to their higher diversity index than populations in China, Japan and south Taiwan ($S = 0.26\text{--}0.29$; Table 3). The relative high estimate of genotypic diversity given by the Simpson index ($D = 0.933$) is in line with the observation that there is relatively little redundancy of genotypes even within populations.

Population differentiation in relation to paleogeography and glaciation

Species history and former distribution can have a major effect on population genetic differentiation. In *Titanotrichum*, it was found that the genetic distance among populations did not fully correlate with the geographic distance between Taiwan, China and the Ryukyu Islands. The lack of congruence suggests that there is no simple 'isolation by distance' explanation for the population differentiation. Other factors, such as sea-level changes at the glacial maximum, are likely to be involved in structuring the populations, as the local flora (south-east China–Taiwan–Ryukyu) was greatly affected by Quaternary glacial periods (1.6 Myrs to present) (Sheng, 1995). The four most recent glaciations apparently facilitated a huge amount of lowland (altitude below 1000 m) species redistribution and migration between China, Taiwan and south Ryukyu (Sheng, 1995). Wang and Zhang (1994) note that 30–40 % of the extant flora of Taiwan is shared with south-east China, emphasizing the importance of the land bridge. Similarly, a common Ryukyu–Taiwan–south-east China fauna is also evident from reptile and amphibian assemblages (Ota, 1998). The depth of the sea between Taiwan, mainland China and the Ryukyu Islands (the Taiwan Strait) is about 100 m on average, and during a glacial maximum the sea level is likely to have been 120–140 m lower, thus connecting the islands to the mainland (Zeng, 1993). Taiwan has long been thought to be a refugium for the south-east Chinese flora as it contains a variety of habitats.

It should also be noted that the north and central Taiwan Strait is shallower (only 40 m deep) but the south Taiwan Strait is deeper, up to 400 m. Therefore any land bridge was likely to have first formed between north-central Taiwan, the southern Ryukyu Islands and China (Zeng, 1993). This is consistent with our finding that north and central Taiwan appears to be the centre of *Titanotrichum*'s genetic diversity, possibly because a Pleistocene land bridge formed here between China and Ryukyu (Fig. 1). The extreme rarity of *Titanotrichum* in south-east China may be due to extinction during glacial periods and human activity, as south-east China is highly populated. This is evident in our UPGMA analysis as the Chinese samples are not as deeply separated as those in northern Taiwan (Fig. 3). It is even possible that they have migrated relatively recently from Taiwan.

Diversity measures in mixed reproduction plants

The diversity measures in *Titanotrichum* indicate considerable total variation ($S'_{sp} = 0.314$ and $D = 0.933$). This is similar to other findings for mixed-reproduction plants, which indicates that their diversity is comparable to that of

outcrossing plants (Nybom and Bartish, 2000). From separate pollination experiments conducted both in the field and glasshouse, it has been shown that *Titanotrichum* benefits from outbreeding, as outcrossing between populations results in significantly higher seed set (Wang, 2003). Thus, it is likely that sexual outcrossing still plays an important role in maintaining the genetic diversity of *Titanotrichum*. However, in *Titanotrichum* most of the variation was found among all populations (64.5 % of AMOVA) rather than within (35.5 %, Table 4). Therefore, it is possible that sexual reproduction has mainly happened in the past whilst vegetative reproduction via bulbils has become increasingly dominant within their isolated habitat. The small population sizes and scattered distribution indicate the gene flow between populations is infrequent or even absent altogether. The high estimate of Φ_{st} in *Titanotrichum oldhamii* perhaps indicates genetic drift, as has been reported from *Allium vineale* (Ceplitis, 2001).

The continuing isolation of populations and limited mating could lead to the loss of sexual reproduction. A study of the clonal plant *Decodon verticillatus* indicates that complex traits like sex can be degraded by mutation when they no longer increase fitness (Eckert *et al.*, 1999). A pilot study on fertility in individuals from different populations of *Titanotrichum oldhamii* also found different levels of fertility among individuals and populations (Wang, 2003).

Mixed-reproduction bulbiliferous plants seem to have higher genetic variation than obligate apomicts due to the contribution of more frequent sexual reproduction (Diggle *et al.*, 1998). Although several papers have attempted to summarize the diversity measures of clonal plant species, general conclusions are extremely difficult to draw because of different sampling strategies and variation in plant life history (Ellstrand and Roose, 1987; Widén *et al.*, 1994; Diggle *et al.*, 1998). Some species show high levels of clonal diversity, e.g. *Populus tremuloides* (Jelinski and Cheliak, 1992; Yeh *et al.*, 1995), while others show low levels, e.g. *Saxifraga cernua* (Bauert *et al.*, 1998).

One general phenomenon in clonal species, and particularly in bulbiliferous plants, is polyploidy. *Titanotrichum* has a high chromosome number ($2n = 40$) for the Gesneriaceae. Duplicated chromosomes buffer polyploids from somatic point mutations, deletions and translocations. Polyploidy may also arise from natural hybridization, which facilitates fixed heterozygosity (Stebbins, 1984). It is possible that polyploid clonal plants contain a high amount of marker polymorphism compared with diploid species, simply because of their large genome size and hybrid origin.

Analysis of genetic variation using dominant markers

One criticism of RAPDs is that they are prone to PCR artefacts. During DNA amplification, nested primer annealing and intrastrand interactions at the first few PCR cycles could accumulate a significant amount of rearranged DNA PCR products (Rabouam *et al.*, 1999; Caetano-Anollés, 2001). Using inter-SSR data in parallel to RAPDs seems to be a good way for testing the reliability of RAPD data. The results presented here showed that the banding patterns obtained from inter-SSR primers are congruent with the

results obtained from the RAPDs data, and therefore it can be assumed that RAPD artefacts in our data are not marked. The first two cycles of our RAPD profile used a higher annealing temperature (37 °C) than the remaining cycles to eliminate false amplification. Also fragments shorter than 400 base pairs were excluded, as short fragments are preferentially falsely amplified in PCR.

Co-migration is another potential problem in RAPD and inter-SSR analysis. However, for intraspecific comparison (as in our study), the co-migration error is likely to be low (Wu *et al.*, 1999; Nybom and Bartish, 2000). Several studies have used techniques such as Southern blot and restriction digestion for checking RAPD band homology, and come to the conclusion that more than 91 % of RAPD bands are homologous, especially within species (Rieseberg, 1996; Wu *et al.*, 1999). Although we did not check our RAPD band homology, due to the large data set, we have no evidence that our results are biased.

For rare or endangered species, a small sample size is unavoidable. This could lead to a significant bias in the population genetic estimates (Fischer *et al.*, 2000). However, simulation results show that a sample size of 10–15 individuals is adequate for largely unbiased results (Isabel *et al.*, 1999). In the study reported here 10–20 individuals from each population included in the analysis unless the population size was less than ten.

Conservation implications

The genetic differences between populations of *Titanotrichum oldhamii* are significant, which is reflected by its high Ph_{ist} value. This may be due in part to genetic drift in small populations. As *Titanotrichum* populations undergo year-to-year fluctuations in numbers of mature individuals (populations in southern Taiwan and Ryukyu Islands have less than 15 mature individuals in each population) and there are an estimated total of fewer than 10 000 mature individuals, it is categorized as ‘vulnerable’ according to IUCN red list criteria (IUCN, 2001). Moreover, as further decline is likely with increasing human disturbance, it may become endangered in the future. The red list of the Japan flora and the flora of China includes *Titanotrichum oldhamii* as endangered (Walker, 1976; Wang *et al.*, 1998).

The Japanese populations and those in eastern Taiwan are protected as they are located within the range of national parks or natural reserves. However, there is no protection as yet for the populations in north Taiwan, which is the centre of diversity. These populations are often found along highways where regular human disturbance may cause a significant reduction in population size. Low seed set in the wild, together with observations of infrequent visits of non-specialized pollinators, indicated that sexual reproduction has been impaired. Despite the numerous bulbils they produce, the recruitment rate is low and they are restricted by their limestone habitat requirement. Artificial transplanting of individuals in different populations may be advantageous to promote gene flow, since most populations are so isolated. Monitoring of populations to determine the effects of inbreeding depression and genetic drift is advisable in the

future (Ellstrand and Elam, 1993). The results presented in this paper form a baseline from which future changes can be monitored.

ACKNOWLEDGEMENTS

We thank Zhen-yu Li, from the Institutum Botanicum, Academiae Sinicae of China and Tokushiro Takaso from the Tropical Biosphere Research Center of Ryukyu University, Japan for their kind assistance on fieldwork, and Goro Kokubugata from Tsukuba Botanical Garden, Tokyo for providing DNA samples. Staff at the Royal Botanic Gardens Edinburgh, particularly Dr Michelle Hollingsworth and Will Goodall-Copestake, are greatly thanked for their assistance with molecular work. Financial support for the fieldwork was kindly provided by the Ministry of Education, Taiwan, the A. G. Side Fund of the Linnean Society and the Davis Expedition Fund of the University of Edinburgh.

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