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Genetic Evidence that *Lomatia tasmanica* (Proteaceae) Is an Ancient Clone

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Abstract

Lomatia tasmanica W.M.Curtis is an endangered species with only one population. The population occurs over a distance of 1.2 km and consists of several hundred stems. Although it flowers occasionally, fruit production has never been observed, and it propagates vegetatively. The genetic diversity in *L. tasmanica,* and its relationship with the other species of this genus in Tasmania was investigated using allozyme analysis and chromosome counts. Sixteen isozyme loci were scored on 78 *L. tasmanica* plants collected from throughout the range of the species. No genetic diversity was found in *L. tasmanica*. *Lomatia tinctoria* possessed 22 ($2n = 22$) chromosomes, like other *Lomatia* species previously counted, while *L. tasmanica* had 33 to 29 chromosomes, which makes it an unstable triploid. The triploid nature of *L. tasmanica* would explain its lack of genetic diversity and its apparent sterility. This suggests that the entire species may be one genet, one of the largest plant clone ever found. Fossilised leaves identified as *L. tasmanica* by Jordan *et al*. (1991) and dated as at least 43 600 years old may indicate the minimum age of this genet. This clone maybe one the world's oldest known living plant individual.

Introduction

Lomatia tasmanica W.M.Curtis is an endangered shrub species, restricted in distribution to south-western Tasmania. It was first collected by D. King in May 1934. However, this population appears to have since become extinct. A flowering specimen from a second population was later collected by D. King in 1965. This specimen was sent to J. H. Willis for identification and the plant was described by W. M. Curtis in 1967. Only one population is currently known. The species is found along creek gullies in rainforest in south-western Tasmania.

Interestingly, the species has never been observed to form mature fruit and seeds. It has been observed to flower but not annually. Regeneration appears to be vegetative by rhizome (Brown and Gray 1985). W. M. Curtis suggested that the species may be of hybrid origin (A. M. Gray, pers. comm., to Brown and Gray 1985). Brown and Gray (1985) further speculated that *L. polymorpha* may be a parent, since it grows in the vicinity. *Lomatia tinctoria* was considered a less likely parent because it does not presently grow in the vicinity of *L. tasmanica*. The objectives of this study were firstly to estimate the level of genetic diversity within *L. tasmanica* using allozyme analysis and, secondly, to determine the genetic distinctiveness of *L. tasmanica* from the other Tasmanian *Lomatia* species, *L. tinctoria* and *L. polymorpha,* using allozyme analysis and chromosome counts.

Materials and Methods

Leafy shoots were collected from the extant population of *Lomatia tasmanica*: 32 out of the eastern creek, 30 out of the western creek and 16 from the middle gully. Care was taken not to sample stems that appeared connected to one another. *Lomatia tinctoria* was sampled from seven sites (Table 1); however,

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as some of the collections were very small, the sites were grouped into four regional 'populations': Tin–1, with sites from the Hobart region (n = 16); Tin–2, with sites from the Bronte Park region (n = 13); Tin–3, with sites from the Tasman Peninsula (n = 14); and Tin–4, with sites from Bruny Island (n = 11). *Lomatia polymorpha* samples were collected from Goring Creek in south-western Tasmania (near the *L. tasmanica* population), and the Hobart region (total, n = 8). It was outside the scope of this study to sample the entire geographical ranges of *L. tinctoria* and *L. polymorpha*.

Young leaf tissue was assayed for allozyme polymorphisms using starch gel electrophoresis (Wendel and Weeden 1989; Lynch and Vaillancourt 1995). Leaf tissue from two samples of each species was utilised to determine the stains to be used in the study. The following enzyme stains were used: AAT, ACP, ADH, DIA, alphaEST, GPI, PER, 6PGDH, PGM, SKDH and TPI (see Lynch and Vaillancourt 1995 for list of abbreviations). Buffer systems used are as indicated by Lynch and Vaillancourt (1995). Enzyme-specific staining was undertaken according to recipes described by Wendel and Weeden (1989). Only stains that showed repeatable results were used. Enzyme loci were numbered sequentially starting with the most anodal, called 1, and proceeding cathodally with increasing numbers. Each zone of enzyme activity was assumed to represent a single locus. Allozymes were named with a lowercase letter following the name of the locus. Sixteen loci were scored in *L. tasmanica,* and 11 of these loci were also scored in *L. tinctoria* and *L. polymorpha*.

Genetic diversity, genetic distance between plants, and cluster analysis (UPGMA) were performed using BIOSYS-1 (Swofford and Selander 1981). The proportion of polymorphic loci (P) was calculated by dividing the number of polymorphic loci by the total number of loci analysed using the 0.95 criterion. The mean number of alleles per locus (A) was calculated by summing the number of alleles at each locus and dividing by the total number of loci analysed. Genetic diversity analysis was carried out using all loci, for each species, then separately for each population of *L. tinctoria*.

Chromosome counts were undertaken on root tips harvested from freshly germinated seeds of *L. tinctoria* collected on the Tasman Peninsula. Root tips 0.5–1 cm long were pretreated in saturated bromonaphthalene for 2 h at 23°C. Root tips of *L. tasmanica* were excised from 1-year-old cuttings grown in perlite and pretreated with either 0.002 % colchicine or a saturated bromonaphthalene solution in the same conditions. Root tips from both species were then fixed in 3 : 1 ethanol : glacial acetic acid for 24 h at 4°C, hydrolysed in 1M HCl at 60°C for 15 min, stained according to the Feulgen method and squashed in a drop of 0.5% acetocarmine. Pressed specimens of *L. tasmanica* and herbarium specimens (Tasmanian Herbarium and personal collection of Robert Hill, University of Tasmania) of *L. tinctoria*, *L. polymorpha*, *L. fraseri* and other *Lomatia* species were examined for gross morphological characters.

Table 1. Collection sites of *Lomatia tasmanica, L. polymorpha* and *L. tinctoria*
The location of *L. tasmanica* is not given to protect this endangered species

Species	Site code	No. of stems	Site name	Latitude (S) and longitude (E)
L. tasmanica	Tas–1	32	Eastern Creek	N/A
	Tas–2	30	Western Creek	N/A
	Tas–3	16	Central Creek	N/A
L. polymorpha	Pol	5	Goring Creek	43°30′ 146°15′
	Pol	3	Mount Wellington	42°53′ 147°13′
L. tinctoria	Tin–1	5	Mount Nelson	42°56′ 147°21′
	Tin–1	6	Sandfly	43°00′ 147°10′
	Tin–1	5	Collinsvale	42°52′ 147°8′
	Tin–2	13	Bronte Park	42°11′ 146°30′
	Tin–3	6	Cape Hauy	43°9′ 147°58′
	Tin–3	8	Cape Pillar	43°12′ 147°58′
	Tin–4	11	Taylor Bay, Bruny Is.	43°22′ 147°13′

Results

Electrophoresis of *L. tasmanica* leaf samples and enzyme-specific staining gave repeatable results with 10 stains: AAT, ACP, ADH, DIA, GPI, PER, 6PGDH, PGM, SKDH and TPI. Sixteen loci were scored in *L. tasmanica*; however, no genetic diversity was detected. Electrophoresis of *L. tinctoria* and *L. polymorpha* leaf samples gave repeatable results with eight stains: AAT, ACP, ADH, DIA, GPI, 6PGDH, PGM and SKDH. Eleven loci were scored in all three species. Table 2 shows allozyme frequencies at polymorphic loci for all three species. Three loci, *Adh-2*, *Gpi-1* and *Pgm-2* were monomorphic within and between species. Five isozymes (AAT-2, AAT-3, PER-1, TPI-1 and TPI-2) which were scored on *L. tasmanica* were not scored with the other species because staining was not as reliable in these other species. The banding pattern for SKDH in *L. tasmanica* was difficult to interpret. All *L. tasmanica* samples were two-banded, with a fainter band above a dark one. This result could be interpreted as a case of fixed heterozygosity, or the fainter band may be a breakdown product of $SKDH^c$. The fainter band was of a mobility between allozyme $SKDH^c$ and $SKDH^b$. The second hypothesis was retained, as it is more conservative. Genetic diversity within *L. tinctoria* and *L. polymorpha* was higher than that in *L. tasmanica* (Table 3). The least variable *L. tinctoria* population (Tasman Peninsula, Tin–3), with 1.4 alleles per locus, 27% polymorphic loci and an expected heterozygosity of $H_e = 0.082$, was more variable than *L. tasmanica*.

Lomatia tasmanica was fixed for 3 out of 11 allozymes ($ACP\text{-}2^a$, $SKDH^c$, $DIA\text{-}3^b$) not shared by *L. polymorpha* and *L. tinctoria*. *L. tinctoria* and *L. polymorpha* shared the same set of allozymes at all sampled loci. Nei's unbiased genetic distance (Nei 1978), calculated using population frequencies, was used for the cluster analysis (Fig. 1). Nei's distance coefficients between: *L. tasmanica* and *L. polymorpha* was 0.729; *L. tasmanica* and *L. tinctoria,* 0.755 to 0.967; *L. polymorpha* and *L. tinctoria,* 0.100 to 0.204; and within *L. tinctoria* populations 0.014 to 0.099. The cluster analysis (Fig. 1) summarises the distance matrix and clearly shows that all populations of *L. tinctoria* are more closely related to one another than to the other two species, *L. tinctoria* and *L. polymorpha* are closely related, and that neither *L. tinctoria* or *L. polymorpha* are closely related to *L. tasmanica*.

The chromosome number was found to be $2n = 22$ in *L. tinctoria* (from over 100 counts). The chromosome number in *L. tasmanica* (counted on six different plants from two sites, 2–10 counts per plant) varied from 29 to 33 (Fig. 2). The attempts to germinate seeds of *L. polymorpha* for chromosome counts failed.

Discussion

The chromosome number of *L. tinctoria* is in accordance with the unpublished result of Rao (1957) and four other *Lomatia* species, where all species were $2n = 22$ (*L. silaifolia*, Ramsay 1963; *L. fraxinifolia*, Johnson and Briggs 1963; *L. hirsuta*, Johnson and Briggs 1975; and *L. polymorpha*, Rao 1957). The tribe Embothrieae, which includes the genus *Lomatia*, appears to be very stable in chromosome number with all published chromosome counts agreeing with $2n = 22$ (Johnson and Briggs 1975). *Lomatia tasmanica* appears to be triploid ($2n = 33$). The variation in chromosome number in *L. tasmanica* may be caused by measurement error, since the chromosomes are numerous and have a tendency to be poorly condensed. However, it is also possible that *L. tasmanica* is unstable in chromosome number and this requires more research. The triploid counts for *L. tasmanica* represent the discovery of the first triploid species in the Proteaceae. Chromosome number is normally very stable in the Proteaceae, with only one reported case of tetraploidy (Johnson and Briggs 1963; Helen M. Stace, pers. comm.).

An analysis of 16 isozyme loci using 78 stems collected from throughout the range of *L. tasmanica* revealed no polymorphism. *Lomatia tasmanica* may have a very low level of genetic diversity. There are few plant species with no isozyme diversity. The Torrey pine

Table 2. Allozyme frequencies at polymorphic isozymes in *Lomatia* populations

Isozyme		Population							
		Tas–1	Tas–2	Tas–3	Pol	Tin–1	Tin–2	Tin–3	Tin–4
AAT-1	(N)	32	30	16	8	15	12	12	10
	a	1.00	1.00	1.00	1.00	0.93	0.62	0.92	0.45
	b	0.00	0.00	0.00	0.00	0.07	0.38	0.08	0.55
ACP-2	(N)	32	30	16	8	16	13	14	11
	a	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00
	b	0.00	0.00	0.00	0.81	0.87	1.00	1.00	1.00
	c	0.00	0.00	0.00	0.19	0.12	0.00	0.00	0.00
DIA-1	(N)	32	30	16	5	15	13	14	11
	a	0.000	0.00	0.00	1.00	1.00	1.00	0.96	1.00
	b	1.00	1.00	1.00	0.00	0.00	0.00	0.04	0.00
DIA-3	(N)	32	30	16	8	16	13	14	11
	a	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00
	b	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00
GPI-2	(N)	5	12	16	8	16	13	14	11
	a	0.00	0.00	0.00	0.44	0.41	0.15	0.11	0.64
	b	1.00	1.00	1.00	0.12	0.00	0.00	0.00	0.00
	c	0.00	0.00	0.00	0.44	0.59	0.85	0.89	0.36
6PGDH	(N)	32	30	16	8	14	13	14	10
	a	0.00	0.00	0.00	1.00	0.54	0.08	0.00	0.60
	b	1.00	1.00	1.00	0.00	0.46	0.92	1.00	0.40
PGM-1	(N)	32	30	16	8	10	6	14	10
	a	1.00	1.00	1.00	0.75	0.00	0.17	0.00	0.00
	b	0.00	0.00	0.00	0.25	1.00	0.83	1.00	1.00
SKDH	(N)	32	30	16	8	16	13	14	11
	a	0.00	0.00	0.00	0.50	0.78	0.23	0.36	0.09
	b	0.00	0.00	0.00	0.50	0.22	0.77	0.64	0.91
	c	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00

Table 3. Proportion of polymorphic loci, number of alleles per locus and genetic diversity in three Tasmanian *Lomatia* species

Two separate analyses were carried out, the first, for each species by pooling all the samples within a species together, and the second for each population of *L. tinctoria* separately

Species	Number of populations sampled	Mean sample size per locus per population	Proportion of polymorphic loci (P)	Number of alleles per locus (A)	Genetic diversity (H_e)
L. tasmanica	3	23.8	0.00	1.00	0.000
L. tinctoria	4	12.6	0.36	1.6	0.173
Tin–1		13.5	0.45	1.5	0.156
Tin–3		12.2	0.46	1.5	0.144
Tin–3		13.8	0.27	1.4	0.082
Tin–4		10.7	0.36	1.4	0.153
L. polymorpha	1	7.2	0.36	1.50	0.173

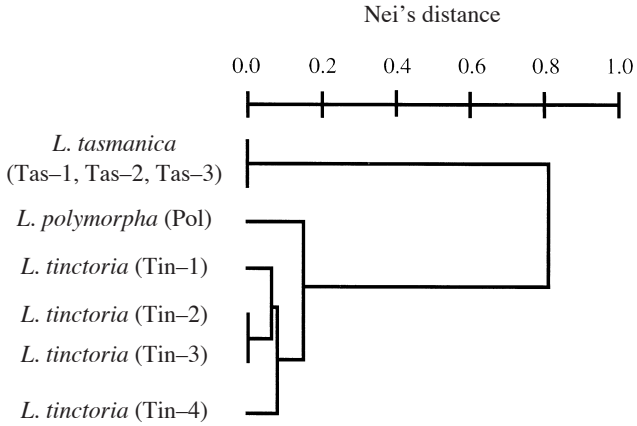

Fig. 1. UPGMA clustering using Nei's distance among populations of three Tasmanian species of *Lomatia*. For a key to the population codes, see Table 1.

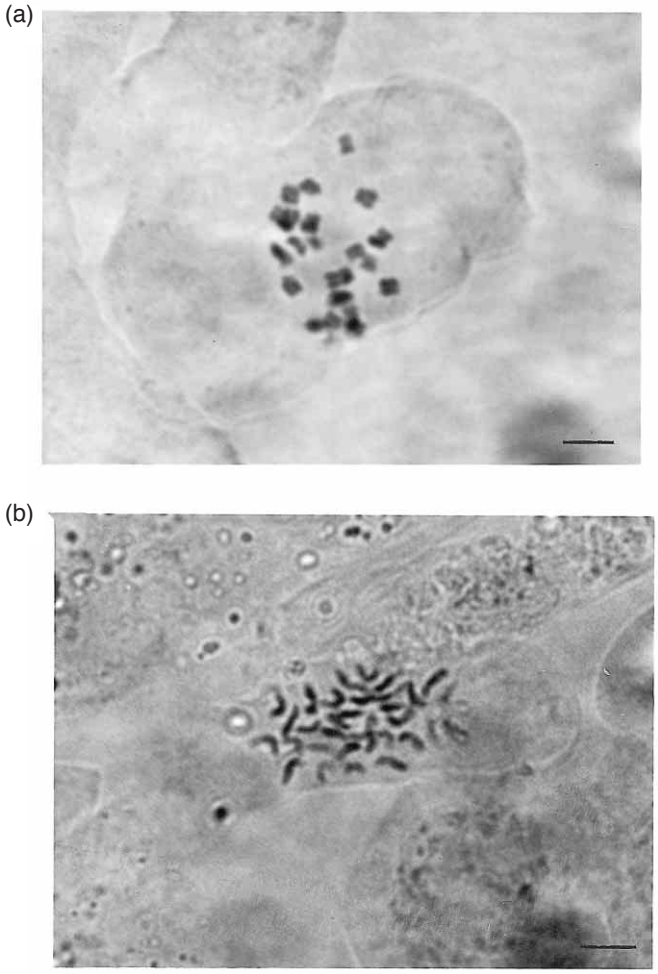

Fig. 2. Mitosis in root tips showing that *Lomatia tasmanica* is triploid while *L. tinctoria* is diploid: (*a) L. tinctoria* ($2n = 22$); (*b) L. tasmanica* ($2n = 33$).

(*Pinus torreyana*) of California is an example where no diversity was detected within two populations, although fixed differences were found between populations (Ledig and Conkle 1983). These authors theorised that a single founder effect coupled with genetic drift may have caused the loss of genetic diversity within populations. Another explanation for the lack of allozyme diversity in *L. tasmanica* is that it consists of a single clone (one genet). This hypothesis is consistent with the observations that: (*i*) *L. tasmanica* shows no morphological variation by comparison with *L. tinctoria*, *L. polymorpha* and *L. fraseri,* which are all extremely variable in leaf morphology; (*ii*) it propagates vegetatively; (*iii*) it has never been observed to form mature fruit; and (*iv*) it is triploid. The lack of genetic diversity in *L. tasmanica* can be contrasted to that in *L. tinctoria,* which also uses vegetative propagation and shows a normal level of genetic diversity within populations. Many clonal plants show a normal level of genetic diversity between clones (Ellstrand and Roose 1987; Mitton and Grant 1996). The probability that polymorphism was not detected because of sampling error was calculated, using the level of diversity in the least variable *L. tinctoria* population (H_e = 0.082) as the expected level of heterozygosity (2pq using Hardy–Weinberg theorem) and applying these to the sampling of 16 isozymes and 78 stems in *L. tasmanica*. Assuming Hardy–Weinberg equilibrium and diploid inheritance, the probability of not sampling at least one different allozyme in *L. tasmanica* due to chance is negligible ($P = 3.3 \times 10^{-35}$). It is possible that the ancestral diploid progenitor of *L. tasmanica* had a much lower level of diversity. If we assume that the expected heterozygosity was $H_e = 0.01$, then the probability of not finding at least one different allozyme is 3.46×10^{-6}. The average level of diversity within populations of endemic plant species is $H_e = 0.063$ (Hamrick *et al.* 1991). A level of diversity much below what is seen in endemic plants would have to be assumed to have a significant probability ($P = 0.082$ if H_e is dropped to 0.002).

Triploidy is rare in nature (Pienaar *et al.* 1989; Harmaja 1992; Kuta 1991), and is usually allied with the ability to propagate vegetatively (Chaboudez 1994). The rarity of triploids is due to the triploid block, which results in massive abortion of triploid seeds because of the failure of the endosperm to develop normally (Bretagnolle and Thompson 1995) and also in part due to their innate sterility (Burnham 1962; Schultz-Schaeffer 1980; Singh *et al.* 1992; Lee *et al.* 1994). Therefore, the production of a triploid plant is a rare event and its inability to reproduce sexually decreases its chance of persisting in nature. However, in some species the triploid block is weaker, allowing the rare production of triploid plants (Pringle and Murray 1992). Triploidy can arise in two ways: (*i*) the formation of a 2*n* gamete followed by successful fertilisation, which would tend to form autotriploids; or (*ii*) through crossing between a diploid and a tetraploid (Burnham 1962), if these are different species it results in allotriploidy. The formation of 2*n* gametes is now believed to be the dominant process in the origin of polyploid species (Bretagnolle and Thompson 1995). *Lomatia tasmanica* is most likely an autotriploid since no tetraploid species has so far been found in the genus *Lomatia*. Furthermore, *L. tasmanica* was fixed for three allozymes not present in either *L. polymorpha* or *L. tinctoria*, the two other members of the genus extant in Tasmania. The diploid ancestor of *L. tasmanica* is therefore likely to be a species that has disappeared from Tasmania.

Several hundred living stems of *L. tasmanica* are scattered amongst rainforest relicts along the margins of several creeks, which appear to be the last refuge of the species (Fig. 3). The surrounding region, including the area between the creeks is dominated by a buttongrass sedgeland. The disjunct distribution of *L. tasmanica* is likely to be the result of fire having fragmented a once continuous population, which may have covered the area between creeks now dominated by buttongrass. The total length of the putative clone is 1.2 km. A clone of this size would be very old, because under the cold climate of south-western Tasmania, it is likely to have a slow rate of vegetative dispersal and growth. The slow growth rate of *L. tasmanica* is exemplified by the fact that a stem 6.3 cm in diameter has been dated using dendrochronology to be approximately 240 years old (Brown and Gray 1985), which is only

Fig. 3. Aerial photograph showing the known range of *Lomatia tasmanica* (enclosed by dotted lines). Creeks are shown as solid lines. The darker vegetation is rainforest, and lighter areas are fire-induced sedgeland and scrub. The scale bar represents 200 m.

0.26 mm per year. Large clonal patches have been observed within other species, such as *Populus tremuloides* (Cook 1983), but are not common. The *L. tasmanica* clone would be the second longest in the world after the huckleberry (*Gaylussacia brachycera*) clone in North America which is reported to be 2 km in length (Wherry 1972). The size of the huckleberry clone has not been verified using molecular markers. It may, therefore, be made up of more than one genet.

The geographical range of *L. tasmanica* was probably more extensive in the past. Until about 30 years ago, *L. tasmanica* also occurred about 5 km west of the extant site, which increases the likely age. Fossil leaves of *L. tasmanica* with a minimum age of 43 600 ± 1100 years (NZA 6745) were found in a late Pleistocene fossiliferous deposit from Melaleuca Inlet by Greg Jordan, less than 8.5 km north-west of the extant population (Jordan *et al*. 1991). This new ^{14}C dating, undertaken on charcoal fragments extracted from the same layer as the fossil, is consistent with a previous dating of 39 000 years on a different layer (Jordan *et al*. 1991). The age is a minimum because it is likely that the charcoal pieces used for dating were contaminated with younger carbon from ground water. The sediments are likely to be Late Pleistocene (<~130 000 years old; Jordan *et al*. 1991). The fossil could originate from the diploid ancestor of *L. tasmanica*, from a triploid ancestor, or from the same clonal individual. It is possible that the extant *L. tasmanica* is part of the same individual that left fossil leaves 43 600 years ago, because: (*i*) despite thorough sampling, only one putative clone was detected; and (*ii*) triploidy is extremely rare, therefore it is unlikely to happen twice. The oldest previously reported plant clone is the huckleberry, which was aged at a minimum of 13 000 years (Wherry 1972), however this date is based on uncertain growth rate and size. The oldest living tree is believed to be a bristlecone pine (*Pinus aristata*) in Arizona which has been dated using dendrochronology at 4700 years. In conclusion, *L. tasmanica* may be the oldest living plant individual known to date.

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