

## Patterns of Cytoplasmic Variation in *Arabidopsis thaliana* (Brassicaceae) Revealed by Polymorphic Chloroplast Microsatellites

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**ABSTRACT.** Despite being the model organism for plant molecular genetic studies, little is known about the origins and evolutionary history of extant natural populations of *Arabidopsis thaliana*. We have analysed phylogenetic relationships between worldwide populations of *Arabidopsis* using polymorphic chloroplast microsatellites. These highly variable markers have revealed previously undetected levels of cytoplasmic variation and confirm previous hypotheses of a recent and rapid expansion of the species from its centre of origin. Furthermore, the results seem to verify previous nuclear analyses that call into question the true origin of several individual *Arabidopsis* ecotypes.

*Arabidopsis thaliana* is a member of the family Brassicaceae that grows wild as a winter annual in most temperate zones of the world. The species has become the scientific community's most common system for the study of physiology, genetics and development in higher plants. Reasons for the continued popularity of *A. thaliana* are its short seed-bearing cycle (~6 wk), small size, low chromosome number ( $n = 5$ ), small genome size (~120 Mb), non-repetitive DNA, numerous genetic markers and recent genomic sequencing (Meyerowitz and Pruitt 1985; Arabidopsis Genome Initiative 2001; Meyerowitz 2001). Studies on the evolution and phylogeny of *A. thaliana* have become common in recent years (Todokoro et al. 1995; Innan et al. 1997; Koch et al. 2000; Vander Zwan et al. 2000). Despite this work, there are still many questions that have not been answered about the evolution of *A. thaliana*. The geographic evolutionary origins of *A. thaliana* are still obscure. It was proposed by Berger (1965) that the genus finds its geographic origins in central Asia. This hypothesis was based on the diverse speciation of *Arabidopsis* taxa observed by Berger while on site in central Asia. Molecular evidence supporting this hypothesis for the origins of *A. thaliana* has been observed using nuclear microsatellite marker analysis (CampANELLA et al. 2002).

Since organellar (chloroplast and mitochondrial) markers are haploid and transmitted uniparentally, they are expected to show different levels of variation and population structure than those revealed by nuclear markers and thus provide complementary information on modes and patterns of gene flow (Ennos 1994; McCauley 1995). In particular, the seed-based transmission of the chloroplast genome in the majority of angiosperms coupled with the smaller effective population size of the haploid genome means that chloroplast-specific markers should be more geographically structured and thus represent good indicators of

historical gene flow. Unfortunately, the relatively low mutation rates associated with organelle genomes means that it has often been difficult to detect sufficient levels of variation below the species level in studies employing organelle-specific markers. However, the recent discovery of high levels of polymorphism at mononucleotide repeat loci in chloroplast genomes has facilitated the analysis of chloroplast variation at levels of resolution previously unattainable using traditional RFLP studies (for review see Provan et al. 2001). These chloroplast microsatellites are now being widely employed to study levels and patterns of cytoplasmic variation in natural plant populations (Provan et al. 1998; Clark et al. 2000; Mengoni et al. 2001; Palme and Vendramin 2002).

In this paper, we present the first report of large-scale cytoplasmic variation in *Arabidopsis thaliana*. In a previous study by Bergelson et al. (1998), RFLP analysis of the mitochondrial *nad5* gene revealed no variation. We have used chloroplast microsatellites to study genetic relationships between worldwide populations of the species. This analysis will allow comparison with previous results obtained using nuclear markers and may provide some novel insights into the possible geographic origin of the species.

### MATERIALS AND METHODS

**Plant Genetic Material.** Ecotypes studied are listed in Table 1. The Tanzania ecotype was donated by Roy Gereau (Missouri Botanical Garden, St. Louis, USA). New Zealand seeds were donated by Mary Skotnicki (Australian National University). Canary Islands, Cape Verde Islands and LaPalmas ecotypes were obtained from Nobuharu Goto (Miyagi University of Education, Sendai, Japan). All other ecotypes were obtained from Randy Scholl (Ohio State University, Columbus, Ohio, USA). Charles Langley (University of California at Davis, USA) donated the *Arabidopsis lyrata* seed.

All seedlings were germinated under sterile conditions and grown under the methods previously described in Vander Zwan et al. (2000). Eight plants from each of the 17 populations were examined, as well as eight plants from a population of *Arabidopsis*

TABLE 1. *Arabidopsis* populations used in this study.

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<i>A. thaliana</i> . <b>Europe:</b> Finland, Espoo (ESP). Germany, Bayreuth (BAY). Germany, Landsberg Erecta (LER). Poland, Lipowicz (LIP). Scotland, Aberdeen (ABD). Scotland, Loch Ness (LC). <b>Asia:</b> India, Kashmir (KAS). Japan, Tsu (TSU). Tajikistan, Con-dara (CON). <b>Africa:</b> Canary Islands (CAN). Cape Verde Islands (CVI). Cape Verde Islands, LaPalmas (LAP). Libya, Martu-ba (MT). Tanzania, Ketumbeine Forest (TAN). <b>America:</b> USA, Berkeley, California (BER). USA, Yosemite National Park (YO). <b>Australasia:</b> New Zealand (NZ).
<i>A. lyrata</i> . <b>America:</b> USA, North Carolina.

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*lyrata*. DNA was extracted from whole seedlings that typically weighed 0.1–0.7 gm. A modified CTAB method from Keller (1992) was used for DNA extraction. Plant tissue was frozen overnight at  $-70^{\circ}\text{C}$  and homogenised in an ice-cooled mortar and pestle.

**Polymerase Chain Reaction.** The primers used were designed from the published complete chloroplast genome sequence of *Arabidopsis thaliana* to amplify mononucleotide repeats and include the following primers as described in Provan (2000): ATCP112, ATCP7905, ATCP28673, ATCP30287, ATCP46615, ATCP66701. In addition, two further pairs of primers (ATCP42555 and ATCP68340) were designed (Table 2) for a total of eight primers employed. PCR was carried out in a total volume of 10  $\mu\text{l}$  containing 50 ng genomic DNA, 5 pmol  $^{32}\text{P}$  end-labelled forward primer, 5 pmol reverse primer, 1x PCR reaction buffer (10 mM Tris-HCl [pH 9.0], 50mM KCl, 0.1% Triton<sup>®</sup> X-100), 2.5 mM  $\text{MgCl}_2$ , 0.05 U Taq polymerase (Promega). Reactions were carried out on an MWG Primus thermal cycler using the following parameters: initial denaturation at  $94^{\circ}\text{C}$  for 3 min; 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 sec, annealing at  $55^{\circ}\text{C}$  for 60 sec, extension at  $72^{\circ}\text{C}$  for 60 sec; final extension at  $72^{\circ}\text{C}$  for 5 min. After addition of 10  $\mu\text{l}$  loading buffer (95% formamide), products were resolved on 6% denaturing polyacrylamide gels containing 1x TBE buffer and 8 M urea at 70 W constant power for 2 hr. Gels were transferred onto 3MM blotting paper (Whatman) and exposed to X-ray film overnight at  $-70^{\circ}\text{C}$ .

**Phylogenetic Analysis.** Genetic distances between haplotypes were calculated based on the absolute size difference of alleles ( $D_{\text{AD}}$ ) using the computer program MICROSAT (V1.5, Eric Minch, Stanford University, USA). This distance, like Goldstein's  $\delta\mu^2$  metric (Goldstein et al. 1995), is based on a stepwise mutation model (SMM) and is appropriate for microsatellite analysis of closely related species or studies below the species level. A neighbour-joining tree was constructed based on the  $D_{\text{AD}}$  distance matrix using the NEIGHBOR and DRAWTREE options in the PHYLIP package (V3.57c: Felsenstein 1995).

## RESULTS

The results presented here represent the first study to reveal substantial levels of cytoplasmic variation in natural populations of *Arabidopsis thaliana*. Over eight polymorphic chloroplast microsatellite loci, a total of 37 alleles were detected, ranging from two at locus ATCP68340 to seven at locus ATCP28673 (Table 3). Levels of within-population variation were extremely low: only the Tanzania population displayed any intrapopulation variation in the form of co-segregating alleles at loci ATCP112 and ATCP66700. Combining al-

lele data across the eight loci gave rise to a unique haplotype for each population, with two haplotypes present in the Tanzania population. A phylogenetic analysis of the relationships between haplotypes showed very little correlation with the geographical distribution of the haplotypes (Fig 1).

Although there was little phylogenetic structure, with the tree exhibiting a "star" topology where internal branch lengths were short relative to the lengths of terminal nodes, the tree did reveal some relationships among the ecotypes. Cape Verde Isles and LaPalmas are found in the same clade in this analysis as predicted with close, geographically isolated populations. Loch Ness and Kashmir show the same close relationship seen in Vander Zwan et al. (2000) where nuclear microsatellite markers were employed; this present result strengthens the hypothesis that the Kashmir population is not geographically native to India. The Tanzania populations are found on the same branch with *A. lyrata*; this relationship mirrors that detected in nuclear microsatellite analysis (Campanella et al. 2002).

## DISCUSSION

**Levels of Polymorphism Detected in Arabidopsis Using Chloroplast Microsatellites.** It is now well-known that the analysis of cytoplasmic variation in natural plant populations can provide novel information on the evolutionary history of such populations. While there have been several studies carried out to investigate levels and patterns of diversity in populations of *Arabidopsis thaliana* using nuclear markers (Todoroko et al. 1995; Vander Zwan et al. 2000; Bergelson et al. 1998), until now there had been no substantial studies carried out that reported any cytoplasmic variation in the species. Bergelson and co-workers (1998) carried out RFLP analysis of the mitochondrial *nad5* gene but detected no variation in 11 worldwide populations of *Arabidopsis*. Although they suggested that

TABLE 2. *Arabidopsis* primer pairs unique to this study.

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ATCP42555: repeat (A) <sub>11</sub> ; location <i>psaA/ycf3</i> (intergenic); size 125 bp; primers (5'–3') CGAGAGGACCAAGAAACCAA, GGTTGAC-GATCACGAGGC
ATCP68340: repeat (A) <sub>11</sub> ; location <i>rps18/rpl20</i> (intergenic); size 100 bp; primers (5'–3') TTTTGTTCAAAAATCCAATCA, AAC-GAGTTATGCTTTTCGACG

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TABLE 3. Allele sizes and haplotypes detected. Two haplotypes were detected in the Tanzania population.

Population	Locus							
	112	7905	28673	30287	42555	46615	66701	68340
LIP	101	140	139	102	120	111	154	100
LC	100	140	142	101	121	112	150	100
ABD	99	140	143	100	125	111	151	99
BAY	100	140	139	102	125	110	155	100
LER	100	140	141	102	125	112	150	100
ESP	100	141	142	101	125	111	154	100
CON	100	141	141	102	125	113	150	100
KAS	100	141	142	102	126	113	151	100
TSU	101	141	144	101	126	111	155	100
MT	100	141	140	102	126	112	149	100
TAN	101	143	140	98	125	111	152	100
	99	143	140	98	125	111	154	100
CAN	101	142	141	103	125	111	154	100
CVI	102	142	139	103	125	111	154	99
LAP	102	142	141	104	125	111	154	100
YO	102	141	138	103	125	111	154	100
BER	101	141	141	102	121	111	154	100
NZ	100	141	143	101	125	111	151	100
Alleles	4	4	7	6	4	4	6	2
<i>A. lyrata</i>	89	148	143	98	125	105	164	100

this could be indicative of a recent common ancestry of the *Arabidopsis* mitochondrial genome, it has also been well documented that mutation rates in plant mitochondrial genomes are extremely low (Wolfe et al. 1987) and previous studies in plant populations using markers specific to the mitochondrial genome have also revealed little or no variation (Soranzo et al. 2000).

Chloroplast genomes also exhibit low mutation rates, but the development of chloroplast microsatellites as cytoplasmic markers has provided new opportunities to study patterns of cytoplasmic variation in plant populations (Provan et al. 2001). An initial study by Provan (2000) revealed that chloroplast microsatellites could be used to detect variation in *Arabidopsis*, but only two individuals from each of 11 populations were studied. In the present study, larger sample sizes were used to elucidate levels and patterns of between- and within-population diversity, but although populations were found to be genetically distinct from each other, within-population variation was minimal. All populations, except that from Tanzania, were monomorphic. This may reflect the history of the seeds obtained from the *Arabidopsis* Biological Resource Center, as opposed to any within-population reproductive processes. Most likely, these have been propagated at some time through single seed descent, which would explain the within-population uniformity observed. Overall, levels of polymorphism at chloroplast microsatellite loci were lower than those reported using nuclear microsatellites, with an average of 4.6 alleles per chloroplast microsatellite locus compared with between 5.3 and 11.9 alleles per nuclear locus (Todokoro et al. 1996; Innan et al. 1997; Vander Zwan et al. 2000). This reflects the findings of other studies using chlo-

roplast microsatellites (Provan et al. 1996, 1999a) and is most likely due to a combination of the haploid nature of the chloroplast genome, lack of recombination and lower mutation rates at chloroplast microsatellite loci compared with their nuclear counterparts (Provan et al. 1999b).

**Genetic Relationships between Ecotypes.** The neighbour-joining tree based on  $D_{AD}$  genetic distances showed little phylogenetic structure and, on the whole, did not reflect any geographical associations between ecotypes. This most likely confirms the idea of a recent and rapid expansion of the species worldwide from an originally highly diverse gene pool. Miyashita et al. (1999) obtained similar results using AFLPs on individual ecotypes of *Arabidopsis*, as did Innan et al. (1997) and Vander Zwan et al. (2000) using microsatellites.

Previous studies (Vander Zwan et al. 2000; Campagna et al. 2002) analyzing *Arabidopsis* phylogeny using nuclear microsatellite polymorphisms have supported Robinson's (Gray and Watson 1895) and Redei's (1993) hypothesis that the North American ecotypes of the species originated in Eurasia. The stepwise mutation model (Valdes et al. 1993; Goldstein et al. 1995) suggests that the longer the physical separation in evolutionary time, the greater the relative changes in the allele sizes at microsatellite loci. As with the nuclear markers, the two "American" strains (Berkeley and Yosemite) examined in this present study, employing chloroplast markers, do not demonstrate enough microsatellite change in contrast to the European populations to indicate a long separation (Fig 1). The Yosemite ecotype, isolated from central North America, shares a clade and seems to have close phylogenetic

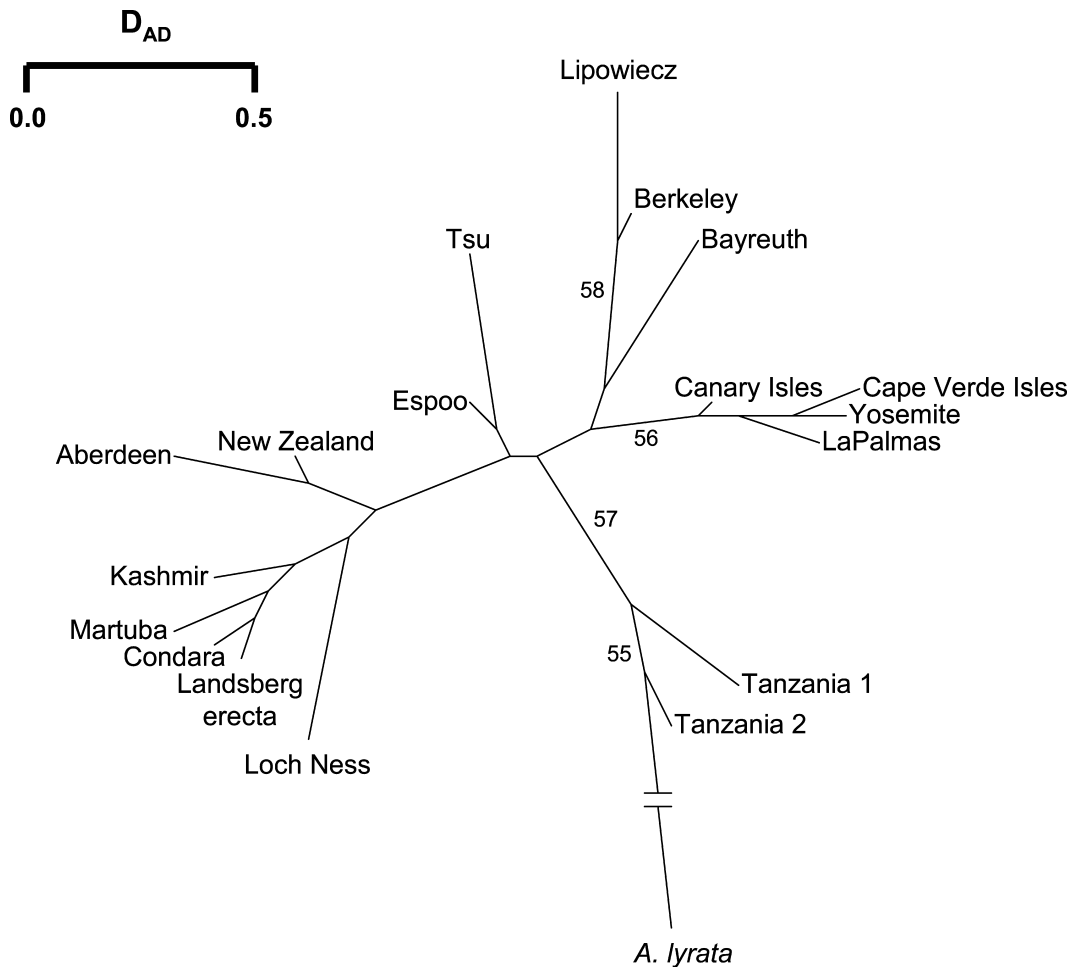


FIGURE 1. Phylogenetic tree based on  $D_{AD}$  genetic distances showing cytoplasmic relationships among populations of *Arabidopsis thaliana*. The actual length of the branch leading to *A. lyrata* is 4.05. Only bootstrap values above 50% are shown.

ties with ecotypes from two Atlantic Ocean island groups (the Canary and Cape Verde Isles); Yosemite is also phylogenetically close to LaPalmas, an additional ecotype from the Cape Verde Isles (Fig 1). This result may geographically illustrate where the penultimate homes of the Yosemite ecotype were as it migrated with humans from Europe, "leap-frogging" from island to island until it reached North America.

Additional comparisons of the chloroplast and nuclear marker data (Vander Zwan et al. 2000) show further parallels, where many of the same ecotypes fall into the same clades. Loch Ness, Kashmir, Aberdeen, and Tsu share clades in the nuclear and chloroplast analyses, as well as Bayreuth and Lipowiecz. The close placement of Kashmir to European ecotypes in both analyses supports the hypothesis that Kashmir's geographic origin is Europe and not Asia (Vander Zwan et al. 2000). The New Zealand ecotype also appears to have originated in Europe since the ecotype is a short

phylogenetic distance from the Scottish ecotype Aberdeen on the same branch.

It is unclear whether our chloroplast microsatellite data supports Berger's Asian Origin Hypothesis (Berger 1965; Vander Zwan et al. 2000; Campanella et al. 2002). While there is other experimental data published supporting this hypothesis (Berger 1965; Vander Zwan et al. 2000; Campanella et al. 2002), the presumably Asian ecotypes (Condara, Kashmir, Tsu) in this study do not demonstrate the large genetic distances that would be expected from long geographic separation.

The related species *A. lyrata* and the African ecotype Tanzania, one of the only wild ecotypes examined, demonstrate the largest phylogenetic distances in the tree (Fig 1). The large genetic distance observed here for Tanzania is mirrored in the nuclear microsatellite analysis (Campanella et al. 2002). This result strongly suggests that the Tanzania ecotype has been separated

from the Eurasian ecotypes for a long evolutionary period. The Tanzania population may have been one of the first to migrate out of Central Asia and thus became genetically separated from its relatives.

In summary, the use of chloroplast microsatellites has revealed previously undetected cytoplasmic variation in worldwide populations of *Arabidopsis*. This variation confirms earlier theories that populations of the species may have radiated fairly recently and provides further evidence in the debate over the true geographical origins of certain ecotypes (e.g. Kashmir). Despite this, however, there is still little molecular evidence, either from nuclear or chloroplast microsatellite studies, to support the hypothesis of an Asian origin for the species as a whole.

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