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Chloroplast microsatellites: measures of genetic diversity and the effect of homoplasy

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Abstract
Chloroplast microsatellites have been widely used in population genetic studies of conifers in recent years. However, their haplotype configurations suggest that they could have high levels of homoplasy, thus limiting the power of these molecular markers. A coalescent-based computer simulation was used to explore the influence of homoplasy on measures of genetic diversity based on chloroplast microsatellites. The conditions of the simulation were defined to fit isolated populations originating from the colonization of one single haplotype into an area left available after a glacial retreat. Simulated data were compared with empirical data available from the literature for a species of *Pinus* that has expanded north after the last glacial maximum. In the evaluation of genetic diversity, homoplasy was found to have little influence on Nei’s unbiased haplotype diversity ($H_e$) while Goldstein’s genetic distance estimates ($D_{sh}^2$) were much more affected. The effect of the number of chloroplast microsatellite loci for evaluation of genetic diversity is also discussed.
**Introduction**

Microsatellites, or simple sequence repeats (SSRs), are sequences of repetitive DNA where a single motif consisting of one to six base pairs is repeated tandemly a number of times. Microsatellite sequences have been identified in the three eukaryote genomes: nucleus, chloroplast (Powell *et al.*, 1995) and mitochondria (Soranzo *et al.*, 1999).

Nuclear microsatellite loci are usually highly polymorphic with alleles varying in the number of repeat units; they are codominant and inherited in a mendelian mode. These characteristics, plus being considered selectively neutral, have made them a popular marker for population genetic studies (Sunnucks, 2000). With respect to the organelle genomes, mitochondrial microsatellites have had little impact so far, but chloroplast microsatellites have been increasingly used in population genetics since their discovery.

Conserved primers for the amplification of chloroplast microsatellites (cpSSRs) have been reported for conifers (Vendramin *et al.*, 1996), gramineae (Provan *et al.*, 2004) and dicotyledons (Weising & Gardner, 1999), but it is among conifers, for studies of population genetics, that chloroplast microsatellite markers have mainly been used (e.g. Cuenca *et al.*, 2003; Fady *et al.*, 2003; Gómez *et al.*, 2003).

Chloroplast microsatellites typically consist of mononucleotide motifs that are repeated eight to fifteen times. Levels of polymorphism in cpSSRs are quite variable across loci and across species, and some loci have been found to be monomorphic in all species studied. There are two important features that differentiate chloroplast from nuclear microsatellites. First, chloroplasts are uniparentally inherited. Some species have maternal inheritance of the chloroplast, others paternal. This means that cpSSRs provide
information for the lineages of only one of the sexes. Also, the chloroplast chromosome is a non-recombinant molecule and, therefore, all cpSSR loci are linked. The genotyping of cpSSRs will result in haplotypes that will be composed of the combination of alleles found at each cpSSR locus.

Mutation rates for length variation in microsatellites have been found to be higher ($10^{-2}$ to $10^{-6}$) than point mutations rates (Li et al., 2002). In order to explain this difference two kinds of mutational mechanism have been proposed: replication slippage (Tachida & Iizuka, 1992) and recombination with out-of-phase aligning (Harding et al., 1992). Both processes result in changes in the number of repeat units which is compatible with the observed size polymorphism of microsatellites. One consequence of these mutational mechanisms is that the same genetic state (i.e. number of repeats) may evolve in two different microsatellite lineages through independent mutational events, a phenomenon known as homoplasy.

Homoplasy may cause problems in population genetic analysis as it can affect measures of genetic diversity, gene flow, genetic distances (both between individuals and populations), neighborhood size, assignment methods and phylogenetic analysis (see Estoup et al., 2002 for a review). Homoplasy within cpSSRs is considered as a potential limitation for its use as a genetic marker (Provan et al., 2001), however the problem has only been addressed at the genus level (Doyle et al., 1998; Hale et al., 2004). Researchers have generally considered homoplasy levels low enough to allow population genetic analysis, and even when homoplasy has been evident (i.e. a haplotype network with up to
nine loops) it has been considered as “moderate” and its potential for confounding results disregarded (Cuenca et al., 2003).

In the present study, simulation analysis was used to investigate the evolution of cpSSRs in conifers and how homoplasy may influence the informativeness of these markers. Instead of a more traditional simulation approach where the whole population is considered, only the genetic makeup of a sample of individuals was studied, following a coalescence-based approach. The strategy consists of: first, generating a genealogy for a sample of individuals from the last generation; and second, placing random mutations on the genealogy to generate the genetic state of the sample (Hudson, 1990). This approach has proved useful for the study of levels of homoplasy in nuclear microsatellites under different mutational models (Estoup et al., 2002).
Modelling chloroplast microsatellite evolution

Simulation of Coalescent Events

The probability of a coalescent event for any two lineages in a given generation depends on the population size, the population structure and the mating system. Thus, it became necessary to establish the biological scenario of the simulation, which would determine the shape of the genealogy.

After the last glacial maximum most conifer distributions shifted northward leaving their refugia (Jackson et al., 2000). When populations were established, they had a period of expansion followed by a period of approximately constant population density until the present time (MacDonald & Cwynar, 1991). Our simulations reproduced populations under such conditions. A range of population ages (i.e. coalescence times) were investigated with population origins at 50, 100, 150, 200 or 250 generations before the present to simulate colonization events at different stages of the glacial retreat [assuming: 1 generation=100 years (as in Provan et al., 1999) and the last glacial maximum=20,000YBP (Hewitt, 1996)]. Population growth was determined by the logistic equation:

\[ N_{t+1} = N_t e^{\left(r \frac{N_t}{K} \right)} \]  

where \( N_t \) is the population size in the generation \( t \) (\( N_0=1 \)), \( r \) is the population growth rate and \( K \) is the carrying capacity of the population. This model has been widely employed to describe population growth following colonization events (Shigesada & Kawasaki, 1997). The growth rate was set at \( r=0.7 \), producing a period of expansion with a duration of 22 generations (~2200 years), which is within the range observed for Pinus (MacDonald & Cwynar, 1991). Then, population size remained constant at carrying capacity for the
remaining 28, 78, 128, 178 or 228 generations. Because effective population sizes are considered to be “large” in forest trees (Muona & Harju, 1989), carrying capacity was arbitrarily set at $K=10,000$; a size big enough to avoid the effects of genetic drift (Savolainen & Kuittinen, 2000).

Generally, chloroplasts are considered paternally inherited in conifers, although there is a possibility that low levels of maternal leakage and heteroplasmy may be present (Cato & Richardson, 1996). Thus, the coalescent process influencing the genealogy of chloroplast haplotypes was considered to be the simplest case possible: a neutral, haploid, non-recombinant genome with every individual having the same probability of being the parent of any individual in the following generation. With the demographic history determined, the genealogy was constructed using a generation-by-generation algorithm. This type of algorithm allows the simulation of complex demographic and dispersal models, contrasting with other coalescence algorithms which are faster for computational time (Leblois et al., 2003).

The algorithm worked backward in time, starting with the last generation and finishing in the first one ($t=0$). In every generation the coalescent events were generated by an algorithm that assigned to every individual, $x$, from the sample ($x \in [1, \ldots, n_t]$) in generation $t$, its ancestor, $y$, in the previous generation $t-1$ (Fig. 1). Every individual $x$ from the sample in generation $t$ had a probability $P=n_{t-1}/N_{t-1}$ (where $n_{t-1}$ is the number of ancestors already assigned and $N_{t-1}$ is the population size in generation $t-1$) to share its ancestor $y$ with any of the individuals from the sample which ancestors had already been
assigned. A random number, $0 < R < 1$, drawn with a uniform probability distribution function, is used to determine the occurrence of a coalescent event. When $R < n_{t-1}/N_{t-1}$, the ancestor $y$ of the individual $x$ is within the $n_{t-1}$ previously assigned ancestors ($y \in [1, \ldots, n_{t-1}]$). In order to determine which ancestor, $y$ took the value of the integer part of $1 + R \cdot N_{t-1}$ (int$(1 + R \cdot N_{t-1}) \in [1, \ldots, n_{t-1}]$ when $R < n_{t-1}/N_{t-1}$). The generation and lineages involved in this coalescent event were recorded to construct the genealogy of the sample. When $R > n_{t-1}/N_{t-1}$, the ancestor $y$ was a new individual and $y$ took the value $n_{t-1} + 1$; for the next individual $x+1$ the value of $n_{t-1}$ increased by one unit.

Coalescent events were simulated using this algorithm on every generation until all lineages converged to a single lineage. Because the population size in the first generation ($t=0$) is only one individual ($N_0=1$) the probability for any number of individuals to share their ancestors was $P = n_{t-1}/N_{t-1} = 1$, i.e. all the lineages coalesced at least at the first generation. This allowed controlling the coalescence time within the range of time for the phenomenon simulated (i.e. colonization after glacial retreat). It is important to note that due to the non-recombinant nature of the chloroplast genome all cpSSR loci were linked and shared the same genealogical history.

**Simulation of Mutational Events**

Several theoretical mutational models have been proposed to describe microsatellite evolution, each one of them with recognized weaknesses and strengths (Estoup & Cornuet, 1999). All of them refer to, and have been tested against, nuclear microsatellites, where mutational events are believed to occur by two mechanisms: replication slippage
and recombination (Li et al., 2002). To our knowledge, no specific model has been
developed for cpSSRs (where no recombination occurs) so the stepwise mutation model
(SMM) was chosen since it is the simplest realistic model for microsatellites. Mutation
rate estimates for cpSSRs are scarce and vary from $10^{-3}$ (Marshall et al., 2002) to $10^{-5}$
(Provan et al., 1999) per locus per generation, so simulations were run under four
different mutation rates: $10^{-3}$, $5 \times 10^{-4}$, $10^{-4}$, $10^{-5}$ per locus, per generation.

The genetic state of nine cpSSR loci in samples of 25 individuals was simulated under 20
different combinations of population ages and mutation rates (Table 1). For each of these
simulations 20 replicates were run. The output of every replicate consisted of the
genotypic information of all the individuals from the sample and the genealogical tree
that describe their relationships, with information on the number of mutations and
number of generations for every branch. The raw data obtained was analyzed as
described in the following section.
Genetic diversity analysis

The effect of homoplasy was studied on a number of standard measures of genetic diversity for cpSSRs: total number of haplotypes, $N$ (direct count of different haplotypes); effective number of haplotypes, $N_e$ (reciprocal of the chance that two randomly chosen alleles are identical); unbiased haplotype diversity, $H_e$ (Nei, 1978), and average genetic distances among individuals, $D_{sh}^2$ (Goldstein et al., 1995) applied to cpSSRs by Morgante et al. (1998):

\[ N_e = \frac{1}{\sum_{h=1}^{N} p_h^2} \]  

\[ H_e = \left[ \frac{n}{n-1} \left(1 - \sum_{h=1}^{N} p_h^2\right) \right] \]  

\[ D_{sh}^2 = \frac{2}{n(n-1)} \cdot \frac{1}{L} \sum_{i=1}^{n} \sum_{j=i+1}^{n} d_{ij}^2 \]  

\[ d_{ij} = \sum_{k=1}^{L} |a_{ik} - a_{jk}| \]

where $n$ is the number of individuals in the simulated sample, $p_h$ is the relative frequency of the $h^{th}$ haplotype, $N$ is the number of different haplotypes in the simulated sample, $L$ is the number of loci simulated, $a_{ik}$ is the size (measured in repeat units) of the allele for the $i^{th}$ individual and at the $k^{th}$ locus, and $a_{jk}$ is the size of the allele for the $j^{th}$ individual and at the $k^{th}$ locus.

Indices $N$, $N_e$ and $H_e$ were calculated both for the stepwise mutation model (SMM; where haplotypes were defined by their genetic state) and for the infinite allele model (IAM; where every mutation defined a new haplotype, even if the haplotype produced was
already present in the sample). The difference between the SMM and IAM values represents information about genetic diversity that is lost due to homoplasy.

Estoup *et al.* (2002) defined an index of homoplasy, $P$, to quantify theoretically the effects of mutational and population variables on homoplasy. This index of homoplasy is the probability that two haplotypes sharing the same genetic state are not identical by descent. We have calculated a similar index generated from Nei’s genetic diversity for the SMM and IAM:

$$
P = 1 - \left( \frac{1 - H_{IAM}}{1 - H_{SMM}} \right) \quad (6)$$

The number of mutations occurring between every pair of lineages was scored and the average genetic distance, based on number of mutations ($D^2_M$), was calculated following Equation (4) where $d_{ij}$ is substituted for the number of mutations scored between the individual $i$ and $j$. Due to the possibility of recurrent mutation and back mutations under the SMM, the genetic distance estimate is expected to be incongruent to some degree with the actual number of mutations between lineages. The differences between the absolute values $D^2_{sh}$ and $D^2_M$ were compared. In addition, the correlation of the matrices of actual and estimated genetic distances was analyzed with a Mantel test (Mantel, 1967) when the difference between $D^2_{sh}$ and $D^2_M$ was large.
Results and discussion

The results for all the simulations are presented in Figures 2 and 3. Each of the 20 replicates performed for each simulation is equivalent to an independent random sampling from the same population. Hence, for any of the genetic diversity indices, the mean value for the 20 replicates is interpreted as an estimate for the actual population value of that statistic and the standard deviation as the error associated to the sample size used. The effect of sample size was assessed by performing some simulations with larger sample sizes. Not surprisingly this resulted in a reduction of the variance for the different measures; for instance, gene diversity in simulation 16 \( \overline{H_{eIAM}}=0.552\pm0.178; \overline{H_{eSMM}}=0.542\pm0.178 \) for 25 individuals, as shown in Figure 3G; \( \overline{H_{eIAM}}=0.561\pm0.037; \overline{H_{eSMM}}=0.553\pm0.036 \) for 1000 individuals) and average genetic distance in simulation 20 \( \overline{D^2_{sh}}=1.665\pm0.382; \overline{D^2_{M}}=2.746\pm0.706 \) for 25 individuals, as shown in Figure 3H; \( \overline{D^2_{sh}}=1.696\pm0.185; \overline{D^2_{M}}=2.589\pm0.292 \) for 1000 individuals). The effect of the sample size could be eliminated by simulating the coalescent history for the whole population. However that would require excessive computational time, and it does not appear to be a problem warranting this.

As was expected, the simulations with parameters that produced higher genetic diversity also showed higher levels of homoplasy (see Table 1 and Fig. 4). For simulations with mutation rates higher than \( 10^{-4} \), homoplasy caused an underestimation, to different degrees, for the four diversity indices. However, Nei’s haplotypic diversity values for SMM and IAM were very close in all simulations.
In order to understand the effect of homoplasy on Goldstein’s genetic distance estimates, a more complex approach is necessary. A difference between the values of $D_{sh}^2$ and $D_{M}^2$ will lead to an underestimation of the absolute time of coalescence for that sample. However, the actual and estimated distances between individuals could be correlated, and if that were the case, then the distance estimates could be used, or even corrected, to study relative genetic distances. In order to test that correlation, simulation 20 was chosen as it presents the biggest differences between estimated and actual distances. The Mantel test performed for the correlation of the actual and estimated genetic distance resulted in a significant correlation (p-value < 0.025) for all the replicates, with the correlation coefficient, $r$, ranging from 0.52 to 0.82. Since the correlation with the actual genetic distances is significant but not predictable, we conclude that, for populations with high genetic diversity, the Goldstein’s genetic distance estimates can be misleading, and any attempt to apply a correction to these estimates would be prone to error.

The different effect of homoplasy on the various indices is explained by the nature of these indices. The three indices based on the number of haplotypes ($N$, $N_e$ and $H_e$) seem to perform better for assessing levels of genetic diversity than the one based on distance estimates ($D_{sh}^2$). Estimated distances are influenced by every parallel mutation and back mutation; however, these mutations may have no influence in the number of haplotypes in the sample. For example, two lineages with unique and parallel mutations will have two distinct haplotypes at the final generation, thus the number of haplotypes will not be reduced. However, the genetic distance will be underestimated because of the parallel mutations. Within the indices based on number of haplotypes, the indices that consider
their frequencies ($N_e$ and $H_e$) were less affected by homoplasy. This result is due to the low frequency that most of the homoplasic haplotypes had within the simulated populations.

In the present work, methods for phylogenetic reconstruction have not been assessed. However, our results discourage the use of cpSSR to infer phylogenetic relationships. For instance, in simulation 20, the high occurrence of homoplasic mutations (78.8% parallel mutations and 6.4% back mutations) would make parsimony method useless. There would be an effect on distance methods too, because distance estimates are affected by homoplasy. However, further work would be necessary to estimate rates of error.

The number of cpSSR loci studied also influenced levels of homoplasy. A set of simulations performed for four loci produced higher values for the homoplasy index, $P$, and higher differences between expected and actual average distances than the equivalent simulations performed for nine loci (Fig. 4). Thus, the linkage of cpSSRs can be seen as beneficial for the analysis of genetic diversity. With a greater number of loci analyzed one has more power to distinguish haplotypes with homoplastic alleles at a given cpSSR loci through the polymorphism of linked loci.

Mutation rate and time of coalescence are the two factors influencing the levels of genetic diversity our simulations. The different combinations of these parameters produced a broad range of genetic diversity, from simulation 01 with null diversity to simulation 20 with the highest diversity (see Fig. 2 and 3). This set of simulations reproduces the
simplest scenario that could describe the recent history of a conifer population: an isolated population originating from a single colonization event, followed by a population expansion. The recent population history of *Pinus resinosa* would seem to be consistent with such conditions. During the last glaciation *P. resinosa* was restricted to southern refugial populations and has colonized northern areas after the glacial retreat (Fowler & Morris, 1977). A study of the population genetics of *P. resinosa* with cpSSRs also supports a metapopulation with restricted gene flow between populations (Echt et al., 1998). This case provides us with an empirical study of a conifer with isolated populations and with different colonization ages (either because of the metapopulation dynamics or colonization after glacial retreat). Also, the number of cpSSR loci and sample sizes (nine loci, 21-24 individuals) were similar to the simulations presented here, providing an appropriate combination of conditions for comparison.

The patterns of genetic diversity found in the empirical study of *P. resinosa* with cpSSRs (Echt et al., 1998) can be best compared with simulations 11-15. In both cases the populations are composed of one high frequency haplotype (the ancestral haplotype in the simulations) plus several low frequency haplotypes. The diversity levels for the different indices, $N$, $N_e$, $H_e$ and $D^2_{sh}$, are also comparable and consistent. Therefore, we can argue that the genetic diversity and distances were unlikely to have been underestimated within *P. resinosa* (see Fig. 2E, 2F, 3E and 3F).

Any further comparison of our simulations with other conifer species studied with cpSSRs has to be done with caution. The simulations we have performed do not take into
account a number of additional factors that may influence homoplasy. Thus the current
set of simulations will represent the minimum amount of homoplasy that could be present
within a given population. Demographic scenarios including migration or more ancient
coalescent events (where colonization events were produced by more than one
haplotype), would result in increased levels of homoplasy. Higher mutation rates and size
constraints in the mutational model will also increase the levels of homoplasy (Estoup et
al., 2002).

To conclude, further simulation studies would be beneficial for the understanding of the
homoplasy in the analysis of cpSSRs. In particular, we are now working in the
implementation of the generation-by-generation algorithm for the simulation of multi-
population scenarios with dispersal that will allow us to understand the effects of
homoplasy in the measurement of gene flow and genetic distances among populations.
Regarding future empirical studies with cpSSRs, it is strongly recommended that studies
use as many cpSSR loci as are available in order to reduce the negative consequences of
homoplasy on estimations of genetic diversity. In order to assess levels of genetic
diversity Nei’s index seems to perform the best, being least affected by homoplasy. In
contrast conclusions made with Goldstein’s genetic distances should be regarded with
cautions, as these can underestimate absolute distances.
References


Acknowledgments

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Fig. 1 Coalescence events were simulated with a generation by generation algorithm. This algorithm assigned to every individual, $x$, of the generation $t$, its ancestor in the immediately previous generation, $t-1$. The probability for this ancestor to be shared with another individual was calculated from the population size in the previous generation, $N_{t-1}$, and the number of ancestors already assigned, $n_{t-1}$ (see text for details).
**Fig. 2** Number of haplotypes, $N$, and effective number of haplotypes, $N_e$, for the 20 simulations. Each graph represents the values for one of the indices, $N$ or $N_e$, for the five simulations with the mutation rate, $\mu$, shown on the left and the coalescence time (in number of generations) shown on the abscissa axis. The mean and standard deviation (from 20 replicates) is shown for each simulation. Both indices were calculated under the stepwise mutation model (SMM) and the infinite allele model (IAM). The difference between both values represents the extent to which information is lost due to homoplasy.
Fig. 3 Unbiased haplotype diversity, $H_e$, and average genetic distances among individuals, $D^2$, for the 20 simulations. Each graph represents the values for one of the indices, $H_e$ or $D^2$, for the five simulations with the mutation rate, $\mu$, shown on the left and the coalescence time (in number of generations) shown on the abscissa axis. The mean and standard deviation (from 20 replicates) is represented for each simulation. $H_e$ was calculated for the stepwise mutation model (SMM) and the infinite allele model (IAM). $D^2$ was calculated for estimated distances ($D^2_{sh}$) based on the number of observed mutations and for the true distances based on the actual number of mutations ($D^2_M$). The difference between both values represents the extent to which information is lost due to homoplasy.
Fig. 4 Levels of homoplasy plotted against genetic diversity for every replicate of the 20 simulations (see Table 1 for simulation conditions). Levels of homoplasy are represented: (A) with the homoplasy index, $P$, and (B) with the difference between the actual average genetic distance ($D^2_M$) and the estimated genetic distance ($D^2_{sh}$). Filled circles represent simulations with nine loci and empty circles represent equivalent simulations with only four loci.
Table 1 Combinations of parameters for coalescence time and mutation rate used in different simulations, and the index of homoplasy found for each.

<table>
<thead>
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<th>Simulation</th>
<th>Coalescence time</th>
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<th>Homoplasy index, $P$</th>
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</tr>
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coalescence time in number of generations; \textsuperscript{b} mean from 20 replicates
$N(\text{coalescence}) = n_x / N_{t+1}$

$R < s_{t+1} / N_{t+1}$

$R > s_{t+1} / N_{t+1}$

COALESCE

$X = x + 1$

$int(x, N_{t+1})$

NO COALESCE

$X = x + 1$

$n_{t+1} = n_{t+1} + 1$