Towards conservation strategies for forest tree endangered species: the meaning of population genetic statistics

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Abstract

Forest tree species normally hold high levels of genetic diversity. Nevertheless, these ecosystems have been seriously threatened by fragmentation and intensive exploitation as well with the phenomenon of global warming. It has created a huge demand for the development of conservation strategies for endangered species. Population genetic statistics are very useful indicators of the genetic status of a species and can be applied for conservation, sustainable management and breeding. Thereby, here are reviewed and discussed how estimates such as mean number of alleles (A), heterozigosity (Ho and He), fixation index (F), outcrossing rate (t), gene flow (Nm) and genetic differentiation (F_{ST}, G_{ST}, and genetic distance) can assist in the development of a conservation program for tree species. The molecular techniques which can be employed to accurately determine such parameters are also highlighted. Furthermore, the most advanced techniques regarding molecular characterization are pointed out. Finally, some hypothetical situations are listed and the applicability of each population genetic statistics for trying to give a solution for each problem is described.

Key words: Molecular markers; Conservation genetics; Global warming; Forest fragmentation.

Delineamento de estratégias para conservação de espécies arbóreas ameaçadas: o significado de parâmetros genéticos populacionais

Resumo

As espécies florestais normalmente apresentam elevados níveis de diversidade genética. No entanto, os ecossistemas florestais têm sido severamente ameaçados por intensa exploração e fragmentação, além de fenômenos ambientais tais como o aquecimento global. Disso decorre a necessidade de delinear estratégias para a conservação de espécies ameaçadas. Parâmetros genéticos populacionais são informativos e importantes indicadores do *status* genético de uma espécie e podem ser adequadamente aplicados em estratégias que visam à conservação, manejo sustentável e melhoramento genético. Desse modo, neste trabalho foram revisados e discutidos parâmetros genéticos populacionais clássicos, como número médio de alelos (A), heterozigosidade (Ho and He), índice de fixação (F), taxas de cruzamento (t), fluxo gênico (Nm) e estimativas de divergência genética (F_{ST}, G_{ST} e distâncias genéticas), e como estes parâmetros podem auxiliar no desenvolvimento de um programa de conservação de espécies arbóreas florestais. Adicionalmente, são discutidas as técnicas moleculares empregadas para obter valores acurados de tais parâmetros. Além disso, os mais avançados métodos para tais análises são brevemente discutidos. Finalmente, foram listadas situações sobre a aplicabilidade de cada uma das estatísticas para possíveis soluções dos problemas.

Palavras-chave: Marcadores moleculares; Conservação genética; Aquecimento global; Fragmentação florestal.

Introduction

Forest ecosystems hold high levels of biodiversity and intra-specific genetic diversity. Genetic variability is extremely important for species evolution since it is related to adaptation to the environment (Reusch et al. 2005; Pauls et al. 2013). Genotypes with high fitness have better chances of survival, conferring better reproductive success to individuals. Therefore, high genetic variability is preponderant to give plasticity for a population to overcome perturbations in its habitat (Ridley 2004; Schaberg et al. 2008; Futuyma 2013).

Forests have been strongly threatened by fragmentation, leading to reduction in size of extensive areas and resulting in landscape mosaics (Bourlegat 2003). Changes in shape, size and distribution of forests, caused by fragmentation, affect the extinction rate and the size of local populations as well as the patterns of spatial distribution of individuals among these populations (Schneider et al. 2003; Ezard and Travis 2006). Forest fragmentation has potential genetic consequences, including reduction of genetic variability, increasing inbreeding and differentiation among populations. These factors enhance the possibility of extinction of a species (Young and Boyle 2000; Costa and Scariot 2003; Ezard and Travis 2006).

Recent evidences pointed out increasing temperatures on Earth surface, revealing an intensive and fast global warming process (Reusch et al. 2005; Pauls et al. 2013). Whether these changes are due to anthropogenic action or not, as has been strongly discussed, all forest environments may have changes in their rainfall balances, affecting survival and perpetuation of species. Some modeling analyses predict, for example, that covering area of Amazon forests will be reduced and replaced by savannas (Salazar et al. 2007). A major concern is for endemic species, which are frequently restricted to small areas. If climate conditions are changed and unfavorable to their adaptation, they may be seriously threatened to extinction (Malcolm et al. 2005).

All these issues highlight the importance of designing strategies aiming at conservation and sustainable management of forest tree species. Forest genetic conservation focuses management of current and future conditions of genes in forest species with the purpose of influence adaptability of individuals, populations and species. Besides maintenance of biological diversity and restoration of forest ecosystems, breeding methods are also very important (Millar 1999).

The primary information necessary in designing a conservation strategy for a species is the level of genetic variability which it presents. Currently, molecular markers are the most suitable sources for variability discovery. Originally, polymorphisms were traced with enzyme-based markers (allozymes) (Hamrick and Godt 1990) and Restriction Fragment Length Polymorphisms (RFLPs). The development of PCR procedure (Saiki et al. 1985; Mullis et al. 1986) gave rise to a set of new marker-analysis techniques such as Random Amplified Polymorphic DNA

(RAPD) (Williams et al. 1990), Simple Sequence Repeats (SSR) (Litt and Luty 1989), Inter-Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995) and their conversion into gene-specific markers as Allele-Specific (AS-PCR), Sequence Tag Sites (STS) and Sequence Characterized Amplified Regions (SCAR), among others. In the last decade, studies have been focused in SSR and also Single Nucleotide Polymorphisms (SNP), the latter based on sequence variation at a single base position, as markers for studying genetic structure, gene/genetic mapping and association of genes to phenotypes. New trends post also Functional Molecular Markers as a way to discover polymorphisms involved in certain genes expressed along different developmental stages, organs, exposition to environmental stresses and within and among populations (Andersen and Lubberstedt 2003; González-Martinez et al. 2006; Varshney et al. 2007; Wong et al. 2012).

Here are discussed population genetic statistics that are obtained through molecular marker analyses. A brief description of molecular techniques used for obtaining these statistics is presented but the main focus is given on the genetic parameters and how such variables can be applied in conservation approaches. Case studies are emphasized from the literature regarding the evolutionary aspects underlying values of genetic parameters and possible anthropogenic influence on changes on genetic structure and diversity of populations of forest trees. Some highlights are given to the most recent methods to study diversity in populations. The interpretation of population genetic estimates certainly has to be given more attention since they can reveal aspects from populations that are beyond any other statistics attempting goals equally important.

The most suitable and cost-effective techniques for population genetic analyses

Although the recent advances in molecular fingerprinting, which are mostly based in sequencing technologies, as genotyping by sequencing (GBS), several questions regarding population genetic diversity and structure of tree species can be answered by using traditional molecular markers. This may also be justified since most tree species do not have a single sequence published in databases (as the GeneBank - http://www.ncbi.nlm.nih.gov/genbank/), which turns really difficult an in dept genomic analysis.

The original RFLP, isozyme and RAPD markers analyses have been mostly abandoned in recent years. Isozymes and RAPD markers have been rarely used for tree species since low polymorphism and genomic coverage are obtained (Ferreira and Grattapaglia 1998). RAPD reproducibility has also been questioned (Ferreira and Grattapaglia 1998) but if well optimized, can also be quite reliable (Skoric et al. 2012).

Currently, microsatellite markers represent one of the most suitable and cost-effective techniques to study population statistics for species that are at initial stages of genetic characterization. Microsatellite markers are co-dominant and composed by DNA motifs repeated in *tandem*. These markers allow the detection of high levels of polymorphism within and among populations. At the present time, several studies are still being conducted in order to discover new microsatellites across tree species (Guidugli et al. 2010; Ferreira-Ramos et al. 2011; Guidugli et al. 2012; Misiewicz et al. 2012; Tnah et al. 2012; Moraes et al. 2013; Tambarussi et al. 2013). Some studies also unraveled the genetic diversity of endangered tree species based on chroloplast microsatellites (Lira et al. 2003; Andrianoelina et al. 2009), which may be more sensitive to population

fragmentation since they are uniparentally inherited (cytoplasmic) and therefore they suffer seed-mediated gene dispersal, which is more limited than by nuclear inheritance (Lira et al. 2003).

AFLP markers can be successfully employed for those studies which aim to determine the genetic structure of populations (Vos et al. 1995; Cardoso et al. 2005), since hundreds of *loci* can be analyzed easily in one single gel or with an automated method. The accuracy of these markers can be extended to phylogenetic studies, aiming to differentiate at the species or genera level (Koopman et al. 2008). On the contrary, microsatellite fingerprinting usually is performed for a single locus or for just a few in a multiplex system by using gel or automated methods (sequencers). Jump and Peñuelas (2006) detected more robust results for genetic structuring using AFLP in *Fagus sylvatica* populations than compared to SSR markers.

Nevertheless, AFLP markers present dominant inheritance and therefore do not allow accurate studies about intra-population diversity, such as allele richness, heterozigosity and inbreeding levels, which are better and precisely determined by microsatellites.

SNP markers have recently been studied for many tree species, however, they require sequencing information, which is not available for most of trees. Also, they are mostly applied for genotype-phenotype association analyses (Sexton et al. 2011; Khan and Korban 2012).

Genetic diversity and genetic structure

Genetic diversity can be measured by several statistics such as the mean number of alleles (A), percentage of polymorphic loci (P), heterozigosity (observed Ho and expected He under Hardy-Weinberg Equilibrium) among others. These statistics give an idea of the amount of diversity in populations. Examples can be given based on studies that performed comparisons among populations that are in continuous and fragmented forests, aiming to show if human pressures were responsible for reduction in genetic variability (Jump and Peñuelas 2006; Martins-Corder et al. 2009) or not (Fuchs and Hamrick 2010). Also, such estimates can explain evolutionary events that led to changes in genetic variability (Bodare et al. 2013).

Genetic structure encompasses the concept of genetic diversity but can be further understood as the way how genetic diversity in distributed through space and time. It can be analyzed based on fixation index (F_{IS}), differentiation among populations (F_{ST}, G_{ST}), genetic distances. Also, it is frequently studied by the partition of total genetic diversity in the genetic diversity within and among populations through Analysis of Molecular Variance (AMOVA - Excoffier et al. 1992). As an example for this case, increased genetic differentiation maybe expected for fragmented populations of a species; therefore these estimates can be used combined with those of genetic diversity to define forest restoration strategies (Siqueira et al. 2013) or in the design of wildlife corridors to reconnect fragmented spots.

The meaning of allele number

One of the primary estimates regarding the genetic status of a species is the mean allele number per locus (A), which can be better measured with co-dominant markers such as microsatellites. It is the initial step to understand how much diversity can be counted at a single or at multiple *loci*. It can be further used to calculate the allelic richness of a population, which takes into account the size of the population (Foulley and Ollivier 2006). Many molecular studies have shown variation for A in several species and a few studies demonstrated reduction in allelic richness or A due to forest fragmentation (Jump and Peñuelas 2006) or

intensive exploitation of species in their natural environment (Farwig et al. 2008; Martins-Corder et al. 2009). This can result in loss of rare alleles (Jump and Peñuelas 2006), which on one hand may play important roles in adaptation of species to different and changing environments (Martins-Corder et al. 2009), although they could be just excluded from populations because of non-adaptive value.

One example to be cited is a species widely distributed in Brazil, *Euterpe edulis*, known as heart-of-palm, which is seriously threatened to extinction. Studies have detected rare alleles in several populations of the species and some populations from south Brazil revealed lower allele number which may be possible explained by anthropogenic action (Martins-Corder et al. 2009).

Heterozigosity level as a main indicator of genetic diversity

Heterozigosity levels (observed *Ho* and expected *He*) are good indicators of the genetic diversity level of populations and are precisely determined with co-dominant markers. Microsatellite markers allow precise estimates for such variables based on their co-dominant nature. Jump and Peñuelas (2006) detected reduction of *Ho* from continuous to fragmented populations of the European tree *Fagus sylvatica*, which was reflected in lower allelic richness as previously discussed.

Farwig et al. (2008) also found reduction of *Ho* when comparing adults and seedlings of a population of *Prunus africana*, a species that naturally grows in Kenya, Africa. The authors suggested that human exploitation led to the decline of population genetic diversity. The latter may be true since exploitation continues to these populations, compromising even the survival of juvenile plants. However, cautious should be taken in such conclusion, since seedling populations for many species may be larger at initial stages and maybe naturally selected and reduced upon adult phase (Conte et al. 2003), which leads to changes in *Ho* levels between the two stages (consider sampling all individuals in seedling stage and resample the population for all those which achieved reproductive phase).

Another study, with the tropical tree *Guaiacum sanctum* revealed no difference between *Ho* of fragmented and continuous forest populations (Fuchs and Hamrick 2010).

These tree situations show the potential of heterozigosity to reveal differences between disturbed and non-disturbed forests, however it relies on the species ecology and system of mating, since some species have better perpetuation strategies and are capable to maintain higher levels of diversity rather than others.

For dominant markers such as RAPD and AFLP, measures of heterozigosity are not precise, so it is more suitable the partition of genetic variability in within (H_S) and among populations (D_{ST}), giving the total genetic diversity (H_T), according to Nei (1973, 1977, 1978).

In this case, however, G_{ST} as the proportion of genetic diversity among populations, is the most important statistics since it gives an idea of the level of differentiation among population, comparative to F_{ST} better discussed later.

Outcrossing rates and inbreeding levels

Forest species are predominantly allogamous (outcrossing rate *t* is at least 95%), although many species also present a mixed system (*t* less than 95% but still mostly outcrossing). The mating system has several implications on the genetic diversity and structure of tree populations. In a broader sense, allogamous species present high intra-population genetic diversity and low divergence among populations (Hamrick and Godt 1990). This also implies in low inbreeding levels, commonly measured as the relative

deviation of *Ho* from *He* and expressed as a fixation index (*F*). The higher the *F* values the more pronounced are inbred crosses, which can expose several deleterious alleles over generations, compromising the perpetuation of species.

Some researchers reported high inbreeding levels for forest species and related these values to increased disturbance to the natural environments where these species occur (Jump and Peñuelas 2006; Martins-Corder et al. 2009). Moreover, a research with the conifer *Picea sitchensis* showed that population position within the range of the species interferes significantly in inbreeding levels and the latter is even higher when the population is geographically isolated (Mimura and Aitken 2007). The authors showed inbreeding increased from around 7% in populations located in central areas of distribution while an isolated population presented up to 35% (Mimura and Aitken 2007).

However, inbreeding levels should also be interpreted cautiously since some species occur in low densities in their original forests and also possess low intrapopulation genetic diversity. In this case, the presence of natural spatial genetic structure in a population can lead to increasing inbreeding, this not being associated to human exploitation, as for *Theobroma grandiflorum* (Alves et al. 2007). Similarly, some studies also report that even when disturbed, populations of some species remain with no significant increase in inbreeding levels, as for *Caryocar brasiliense* (Collevatti et al. 2001).

Estimates of genetic differentiation and gene flow

One of the primary consequences of habitat fragmentation is the loss of connection among fragments with similar characteristics. Many species are therefore disconnected and this may lead to genetic isolation, minimizing the possibility of exchanging alleles, which means a low gene flow (*Nm*) among populations. This creates local population structure and tends to increase genetic divergence among fragments. Divergence can be measured by the concept that populations are subdivided and they present a total level of genetic diversity when pooled (H_T), which is partitioned in the genetic diversity within (H_S) and among populations (D_{ST}), giving rise to the proportion of genetic diversity that is due the difference among populations (G_{ST}) (Nei 1973). This is analogous to the prior *F*-statistics developed and improved by Wright (1949, 1965) which introduced the formula: $(1 - F_{IT}) = (1 - F_{IS})(1 - F_{ST})$. F_{IT} and F_{IS} define fixation indexes, so they can be used for the interpretation of inbreeding levels in populations. F_{ST} designates the genetic differentiation among populations, being similar to the later developed G_{ST}. Currently, however, is also very common to perform Analysis of Molecular Variance (AMOVA - Excoffier et al. 1992), which is based on the *F*-statistics and shows how genetic variance is distributed within and among populations, determining the so called phi-statistics.

Using these statistics, a couple of studies have shown increased genetic differentiation due to human perturbation. One example is with the endangered tropical species *Caesalpinea echinata*, typical from Atlantic Rain Forest which has been reduced to less than 7% of its original covering area. AFLP markers data showed increased correlation between genetic differentiation and geographical distance, suggesting high levels of isolation by distance due to fragmentation (Cardoso et al. 2005).

For *F. sylvatica*, as previously stated, not only within population genetic diversity was reduced, but also higher genetic divergence among fragmented populations than compared to continuous forests was detected (Jump and Peñuelas 2006).

Genetic drift and effective population size

Populations naturally undergo alterations in their genetic diversity over generations. This can be explained on the basis of genetic drift which is defined as a random change in the frequency of two or more alleles or genotypes in a population (Futuyma 2013). In larger populations, gamete resampling tends not to affect significantly allele frequencies. However, drift effects are more pronounced in populations of reduced size (Savolainen and Kuittnen 2000; Ridley 2004).

In this sense, size should not be referred as the count number of individual of the population, but the number of individuals that effectively contribute to maintain the genetic diversity of the population, which can be referred as effective population size (Ne). If a population is continuously exploited, it is expected a reduction in the effective size which means less genetic diversity and less fitness, affecting population survival over generations. Approaches for estimating Ne based on molecular markers such as microsatellites have been developed (Xu and Fu 2004).

Computational resources for population genetics evaluations

Currently, several computer programs are available to perform population genetics analyses to determine genetic parameters such as those described in this review. One of the most used for genetic structure analyses, especially in case of dominant markers although also applicable for co-dominant ones, is ARLEQUIN, which performs Analysis of Molecular Variance (AMOVA - Excoffier et al. 1992; Excoffier et al. 2005). Recently, however, STRUCTURE has been more used for such population structure inference (Pritchard et al. 2000). The latter assumes that a set of individuals is divided in K populations and this is determined probabilistically. This software can be used for all traditional molecular markers including AFLP, SSR and SNP.

Another software resources can also be cited for particular analyses: POPGENE (Yeh and Boyle 1999) for genetic diversity and genetic structure; GDA - Genetic Data Analysis (Lewis and Zaikin 2002); FSTAT: which calculates estimators of F-statistics (Goudet 1995); TFPGA (Tools for Population Genetic Analysis) (Miller 1997). Also, packages have been developed for the R software and can be easily downloaded and executed on R or R Studio.

Genomic approaches for conservation purposes

A series of new techniques have been developed for high-throughput genotyping and several model species, non-model species or agricultural crops are being widely explored through such methods. Advances in SNP genotyping are remarkable, since scientists are capable to associate single bases to specific phenotypes or traits, as for wood characteristics (Porth et al. 2013) or even phenotypes to specific *loci* polymorphisms (Ingvarsson et al. 2008). This is valuable because it opens the possibility to study adaptive traits for forest tree species, their evolutionary patterns and how they can be affected by current forest disturbance or from the global warming phenomenon (González-Martinez et al. 2006; Pauls et al. 2013).

Moreover, impressive developments have been achieved in studying population genetic diversity at the RNA level. Current technologies allow the sequencing of a whole transcriptome (RNA-seq) for individuals in a few steps and very compact equipment. Comparisons among transcriptional profiles among individuals in a population may provide useful information about genes and their regulatory pathways (Qiu et al. 2011) and this information

may be used to identify important genes for the adaptation of species (Villar et al. 2011). So far, most studies with such purposes were performed with species of *Eucalyptus* and *Populus*.

How can these statistics be applied for a conservation and sustainable management program?

So far, all genetic statistics described reveal important features of tree species and populations. Therefore, all this information can be directed to an effective program aiming to conserve a species or to establish a sustainable management strategy of its genetic resources and depending on the economic importance, propose methods for its genetic breeding.

Therefore, some hypothetical situations are listed and possible alternatives are proposed. In all cases it is assumed that forest tree species are allogamous and preferably from tropical environments.

There is no genetic information about an extremely endangered species

Sampling natural populations in a wide range of its distribution is very important to start the genetic characterization. AFLP markers with a considerable number of *loci* can give an initial basis of the genetic diversity. However, if possible, a SSR library should be performed, tracing *loci* which are considerably or highly polymorphic. This information will help to establish germplasm collections with individuals that represent the genetic diversity of the species. These germplasm collections can be maintained in the original areas of these populations (*in situ*) or in specific locations/institutions (*ex situ*) which combine every genetic resources available.

The species is highly endangered but of primary economic importance for a location

Germplasm collections (*ex situ* or *in situ*) are very important in all cases. The study of genetic diversity of populations by comparing fragmented and more conserved populations should be performed. Of course, this is only possible if there are still some protected areas where the species grows. If it would not be the case, the genetic diversity should be measured and continuously monitored in a selected population or area of occurrence. At the same time, seeds from the species should be collected and cultivated or introduced to forest fragments, being at least representative of the genetic diversity that is available yet. Heterozigoty (Ho and He) is a suitable statistics to monitor genetic diversity over time as is the mean number of alleles (A).

Low genetic diversity in a population located in a small fragment

If it is expected high genetic diversity for the species but it was detected a low level according to genetic statistics (A, Ho, He) and the small fragment is strongly perturbated, the introduction of genetic materials from other populations with different sources of allele combinations (by observing A and even at the genotype designated - AA, AB, BB, CD etc) could help to increase or restore levels of genetic diversity for next generations. But this encompasses many practical efforts and is time-consuming since plants would have to be monitored during their development so they can achieve maturity and be able to exchange pollen. Besides, if the species is pollinated by insects, wind or other sources, researchers need to know if pollination can be effective. Reproductive biology and insect populations knowledge in the area of study are needed to verify such issues and eventually design strategies to overcome it.

High and non-expected genetic differentiation between two populations

High values of genetic differentiation (F_{ST}, G_{ST}) for an allogamous species are not expected and indicate low gene flow (*Nm*) among populations. One strategy which could be used to overcome such issue is to establish a wildlife corridor, connecting populations that are close to each other. Otherwise, if populations are isolated by considerable or long distance, an exchange of genetic materials could be done, in order to minimize genetic differentiation. Researchers need to keep in mind, however, that these strategies would be practiced for genetic diversity restoration *in situ*.

High and non-expected inbreeding levels in a population

Inbreeding depression is one the major concerns that could lead to population extinction over generations. The introduction of other genetic materials, with different sources of alleles and therefore genetic diversity would also be a good strategy to overcome such issue.

Many other cases could be pointed out here, however this is not the purpose of this review. More than that, it is to show that genetic statistics are helpful in directing conservation or breeding strategies but they must not be used alone. Many practical issues need to be in mind in order to design such strategies, which means a multidisciplinary approach. Many researchers have to be engaged on such directions and we all urge in accelerating effective solutions given all fragmentation, deforestation and global warming issues.

Final considerations

Global warming is a problem that acts concomitantly with continuous exploitation and fragmentations of forests. Therefore, research interests to address their consequences and possible solutions have to be intensified. In this sense, this review highlighted one of the first steps to be taken which is the interpretation of the main population genetic parameters obtained through molecular markers fingerprinting and how these data can be directed to the development of conservation strategies for forest tree species. Forest ecosystems need to be further studied and the knowledge of the genetic status of their species has to be deciphered in order to trace public policies that promote the conservation of forest areas and specially endangered species.

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