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Full Length Research Paper

Genetic variability of sacred fir (*Abies religiosa*) in the Nevado de Toluca National Park

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Sacred fir (*Abies religiosa)* forests of the Nevado de Toluca National Park, Mexico, are threatened principally by illegal cutting and an increasing incidence of parasites as bark beetles and dwarf mistletoe. An important and sometimes ignored component of conservations plan is genetic variability hence, it is necessary to carry out studies on the genetic diversity of sacred fir to create an efficient plan of conservation and management. In the present study DNA markers were used to analyze genetic variability of seventeen populations of sacred fir. Results suggest high values of genetic variability among populations (G_{ST} > 0.5) and low levels of gene flow (Nm < 1), indicating high population differentiation. These results allow the localization of genetically unique populations and propose them as Conservation and Management of Gene Resources Zones.

Key words: Nevado de Toluca national park, *Abies religiosa*, molecular markers, conservation.

INTRODUCTION

The Nevado de Toluca National Park (NTNP) is a natural protected area in Mexico, formed principally by conifer forests that surround the Xinantecatl volcano that is part of the Trans-Mexican Neovolcanic Belt mountain system. In NTNP fir forests comprise sacred fir (*Abies religiosa* (HBK) Schld. and Cham.), the most widely distributed fir in Mexico (Rzedowski, 2006). The NTNP *A. religiosa* forests are sanctuaries for the Monarch butterfly (*Danaus plexippus* L.), which passes its hibernation period there. Also, in these woods are found a major number of species of animals and fungi, as well as the outstanding presence of moss (*Thuidium* spp.), which prevents water runoff and soil erosion. Furthermore, sacred fir is highly valued as commercial wood used in products such as beams, Christmas trees and fine papers.

The NTNP is surrounded by urban areas, implicating environmental and anthropogenic pressures for the natural resources. Illegal logging, for example, leads to habitat fragmentation, and excessive cutting of healthy trees leaving only the weak and ill ones, resulting in an increase in the incidence of parasites such as mistletoes and bark beetles (Franco-Maass and Candeau-Dufat, 2007). There are limited reports of genetic variability studies of *A. religiosa* (Eguiarte-Fruns et al., 1997; Aguirre-Planter, 2000), additionally there are no reports of genetic variability in the NTNP woods using DNA markers, despite its ecological and economical importance, and this is why this study is relevant.

It is known that populations distributed naturally across an altitude gradient, for example those which are distributed in mountain systems; tend towards differentiation

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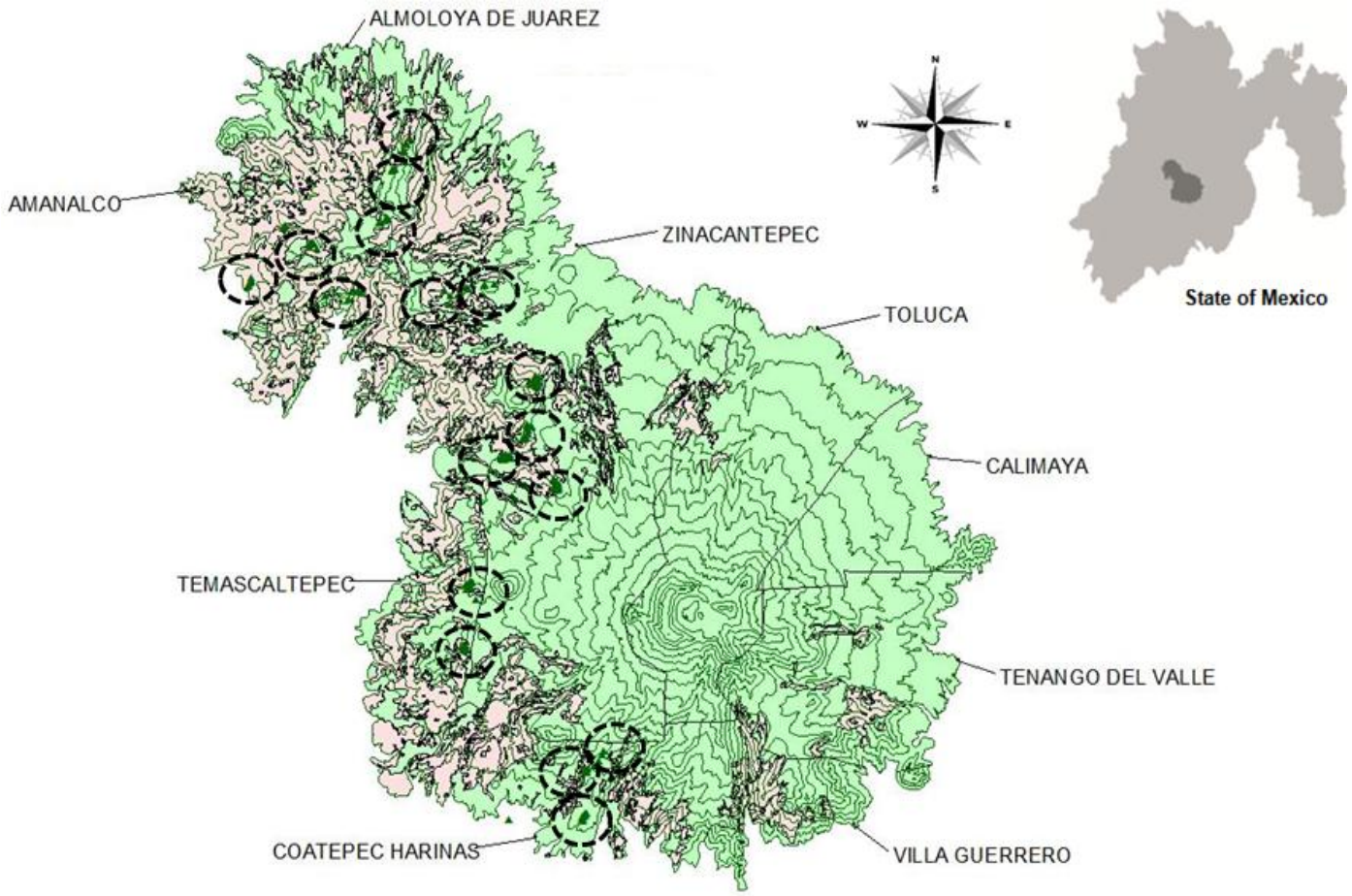

Figure 1. Distribution of the seventeen populations of *Abies religiosa* (dashed circles) sampled in the Nevado de Toluca National Park; municipalities surrounding the natural protected area are indicated.

because of the varied environmental conditions and selection pressures (Viveros-Viveros, 2005). At high altitudes, environmental conditions are much more drastic and successful plants depend on microclimates and topographical conditions, which create marked physiology differences among trees.

An imminent climate change requires preserving healthy forests, and this involves the creation of conservation plans which must consider genetic variability because, as a component of the biodiversity, it provides essential information for successful conservation and management and permits the inclusion of biological events such as evolution and adaptation.

To analyze genetic variability, it is common to use DNA markers, including the DNA of cytoplasmic genomes (mitochondria and chloroplast), which also provides information on gene flow. It has been reported that particular plastid DNA heredity in pines and firs, are inherited paternally (chloroplast DNA) and maternally (mitochondrial DNA) (Clark et al., 2000; Neale and Sederoff 1989; Vendramin et al., 1997, Xiao-Quan et al., 2000; Xu 2005, Birky, 1995), whereas nuclear DNA is inherited biparentally. In order to analyze the genetic variability among and within populations of sacred fir chloroplast DNA (cpDNA), mitochondrial DNA (mtDNA), and nuclear ASSR (Anchored Simple Sequence Repeat; ncDNA) markers were used. The genetic variability was then related with geographic distribution of sacred fir in the NTNP. Results obtained, allow us to propose *in situ* and *ex situ* conservation strategies.

MATERIALS AND METHODS

Sacred fir (*A. religiosa*) needles were obtained from 17 georeferenced sample points of the population of the NTNP, randomly selected by systematic sampling (Figure 1). Each sampled point was considered a population, defined as an aggregate of individuals of the same species within the same space, which in this study was vegetation type (Ricklefs and Miller, 2000), and contained at least 10 trees. We used ArcView GIS Version 3.1 to generate a map with the location of each sampled point (population). A total of 128 individuals were sampled. Approximately 100 g of needles were collected per sample, and diameter at breast height, height, and geographic location (latitude, longitude and altitude scored with a GPS) were recorded. In the laboratory, tissue was stored for DNA analysis: 50 g of needles

Table 1. Genetic variability of A*bies religiosa* for cpDNA, mtDNA, and ncDNA markers; observed number of alleles(na), effective number of alleles(ne), Nei's genetic diversity (h), H_T (total genetic diversity per locus), H_S (within populations genetic diversity), G_{ST} (among populations genetic diversity), and Nm (gene flow).

Genetic marker	*na*	*ne*	*h*	H_T	H_S	G_{ST}	*Nm*
cpDNA	2.000	1.7127	0.4021	0.3955	0.1870	0.5273	0.4483
mtDNA	2.000	1.4931	0.2982	0.3190	0.1571	0.5077	0.4849
ncDNA	2.000	1.4870	0.2964	0.2973	0.1589	0.4589	0.5895

were placed in polyethylene bags with silicate and stored in an incubator (REVCO) at 4°C. DNA was extracted with the CTAB method (Zhou et al., 1999) with few modifications: washing the tissue three times in order to ensure elimination of proteins, polysaccharides, polyphenols, tannins, and pigments. Two cpDNA primers were used for PCR amplification (cp-trnK and cptrnG-trnQ Parducci and Szmidt, 1998), two mtDNA primers (nad-5 Wang et al., 2000 and cox3in, Wen-Qing et al., 1999), and two anchored microsatellites for ncDNA analysis (ASSR15 and ASSR20, Yamagishi et al., 2002). Amplification products were then subjected to electrophoresis analysis 1.5% in agarose gel and PCR products were visualized with ethidium bromide. We used the Pop Gene 32 (Yeh et al., 1999) software for data analyses. The standard measures of genetic variability were recorded: mean effective number of alleles per locus (ne); Nei's genetic diversity, which represents probabilities of finding two different alleles in one locus (Berg and Hamrick, 1997); heterozygosis parameters expressed by H_T (total heterozygosis per locus), H_S (within population heterozygosis), or G_{ST} (among population heterozygosis) (Berg and Hamrick, 1997; Dobzhansky, 1993; Yeh, 1979); and finally gene flow (Nm) which is an indicator of genetic information exchange between populations; it determines to what extent the populations can be independent evolutive units: values of $Nm \leq 1$ indicate strong differentiation between populations and $Nm \geq 1$ indicates a great panmitic population (Eguiarte-Fruns et al., 1997; Solomon, 1999). We used Statgraphics 4.1 software for two statistical tests: a linear correlation to test whether a relationship exists between altitude and genetic variability and an analysis of variance (ANOVA), this last, was used to detect differences in the levels of genetic variability between the sampled zones. For this last purpose, the NTNP map was divided into three sampling zones: north (populations: 1 to 6, 9 and 10), center (populations: 11 to 14), and south (populations: 7, 8 and 15 to 17), the values considered to carry out the ANOVA were Nei's genetic diversity (h) value by population (data not shown) and sampling zone (1 North, 2 center, and 3 South); finally this information was used to analyze geographic distribution of genetic variability.

RESULTS

Genetic diversity among sacred fir populations in the NTNP was high (≥0.5) compared to the average reported for Mexican conifers (G_{ST} = 0.024-0.337, Ledig, 1997). This can be explained by the low levels of gene flow (Nm = 0.4483-0.5895), which in turn mean high levels of population differentiation (G_{ST} = 0.4589-0.5273) as we observed in the present study. The effective number of alleles per locus (ne) was high for the tree genomes (Table 1) as compared with the only mean ne reported for Mexican *Abies* on isozyme studies (ne = 1.46-1.8, Eguiarte-Fruns et al., 1997; Aguirre-Planter et al., 2000).

Heterozygosis

Nei's genetic diversity (h) was low for mtDNA, and ncDNA (h = 0.2982, h = 0.2964 respectively), while for cpDNA (h = 0.4021) it was high (Table 1). Organelle genomes show more polymorphism (Birky et al., 1983), in this case cpDNA. Variation within populations (H_S) (mtDNA = 0.1571, ncDNA = 0.1589 and cpDNA = 0.1870). Total genetic diversity per locus (H_T) was low for ncDNA (0.2973); again we observed that the organelle genomes presented more variability (cpDNA = 0.3955, mtDNA = 0.3190).

Genetic variability among populations (G_{ST}) was high for all markers (cpDNA = 0.5273, mtDNA = 0.5077, ncDNA = 0.4589) indicating that 4.5-5.2% of the total genetic variability was due to differences among populations,. This has ecological implications because it means that all the populations studied must be preserved to prevent losing genetic diversity.

Gene flow

With all markers, gene flow was low, explaining the high values of population differentiation; the lowest value was obtained at cpDNA (0.4483) and the highest at ncDNA (0.5895). For conifers the mean number of migrants per generation is 10 or higher (Ledig, 1997), and so, values for sacred fir can be considered very low (Table 1).This ecological trait in NTNP is highly influenced by forest fragmentation and rugged topography; also, a low gene flow value can suggest metapopulation behavior of the sampled points (Aguirre-Planter, 2007).

Geographic distribution of genetic variability

In the linear correlation analysis, a positive correlation between genetic variability and altitude was found for chloroplast, and nuclear markers (cpDNA: r^2 = 2.87, F=3.73, p=0.0557; ncDNA: r^2 = 12.7, F=18.33, p=0.0000). For mitochondrial data, correlations were not statistically significant. The ANOVA showed that chloroplast markers had the highest genetic variability in the north and center zones ($p < 0.05$), while mitochondrial and nuclear ($p <$

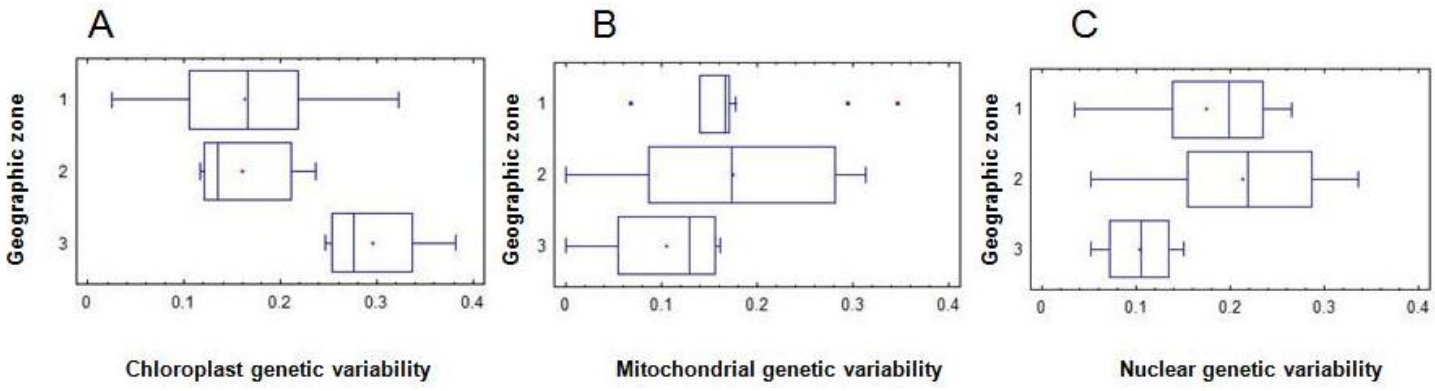

Figure 2. ANOVA analysis of genetic variability by geographic zone (1: north, 2: center, and 3: south) of 17 populations of *Abies religiosa* in the Nevado de Toluca National Park. A) cpDNA: F=57.86, p= 0.0000; B) mtDNA: F= 9.06, P = 0.0002, C) ncDNA: F= 23.26, p= 0.0000.

0.05) markers were the most variable in the south zone (Figure 2).

Despite the results obtained are the normally expected in natural fragmented populations, it allow us to propose *ex situ* and *in situ* conservation strategies which in turn will permit improve the existent conservation programs of the NTNP.

DISCUSSION

Genetic diversity

Compared with the mean G_{ST} = 0.188 reported for conifers cpDNA (Yoshihiko *et al*. 2000), our result (G_{ST} = 0.5273) constitutes a high value of genetic diversity, probably because the cpDNA has high rates of inter specific variation; it is also the most polymorphic among conifers since it has multiple variable regions (Parducci and Szmidt, 1999). Clark et al. (2000) found an average of h= 0.76 for three conifers and Parducci et al. (2001) reported an h= 0.964 for three fir species. We found an h= 0.4021 (Table 1), these value are expected to be high for sacred fir populations in the NTNP, since the genome type is transmitted paternally, there is no recombination, and it passes linearly from generation to generation. Besides the conditions of sacred fir in NTNP, such as the presence of parasites and excessive cutting, cause the elimination of individuals and consequently of alleles. On the other hand, our low h value may be explained by the value of gene flow which was also low (Nm = 0.4483), most likely because of the presence of biological and geographical barriers and population fragmentation caused by illegal cutting, which restricts gene flow between populations. It has been reported that for sacred fir gene flow is limited; the distance of dispersing pollen in this case is not far enough to prevent population isolation (Aguirre-Planter et al., 2000), and it is possible that the number of paternal individuals, is not sufficient because of forest fragmentation. A low value of gene flow causes population differentiation; a high level of gene diversity and, consequently, a larger number of populations, must be preserved because each one possesses unique genetic value.

The mtDNA genome is maternally inherited in some conifers such as pines and firs (Liepelt et al., 2002); in our study the values of mtDNA genetic variability was lower (h = 0.2982) than cpDNA (h = 0.4021). It is known that the mtDNA genome has the lowest evolutionary rates in plants (Zhang and Hewitt, 2003); seeds (which carry maternal DNA) travel shorter distances than pollen; hence, the loss of individuals as a consequence of felling trees and of parasites decreases mitochondrial variability even more in NTNP. These results were lower than those reported by Jaramillo-Correa and Bousquet (2005). Comparing two conifers (h = 0.3242), they explained that high values were obtained because of the mitochondrial hybridization between two species. *A. religiosa* is the only fir species, so possibilities of hybridization are low. Our results suggest high levels of mtDNA variability for sacred fir due in part to low values of gene flow (0.4849), indicating that populations are going through a process of isolation. For this reason, each population has a unique evolutive value, and the conservation of all of these populations is necessary to prevent loss of genetic information which would permit these organisms to adapt.

In the present study, the genetic diversity of the nuclear genome (biparental inheritance) was high (G_{ST} = 0.4589, Table 1), concordant with low levels of gene flow (Nm = 0.5895, Table 1). In other studies, populations of *Abies guatemalensis* and *A. nordmanniana* were found with a G_{ST} = 0.137, and for *A. nordmanniana,* G_{ST} = 0.007 (Petit et al., 2005). For the genus *Abies*, our results can be considered high.

Pollen has a major capacity for dispersion; this probably enhances the genetic variability of cpDNA and ncDNA. Thus, apparently gene flow by pollen is more important for sacred fir; in fact, almost all plants depend

on pollen dispersion for gene exchange (Petit et al., 2005), but values of gene flow with the biparental marker (ncDNA Nm= 0.5895) were higher. This means that gene flow of sacred fir depends mainly on seed (Latta and Mitton 1997), which in turn represents a problem for sacred fir forests of NTNP because of the low levels of mitochondrial gene flow means that more maternal individuals are needed to avoid inbreeding.

Generally, a nuclear genome is expected to have the highest levels of genetic variability because of the recombination processes that it undergoes. In the present study, however, it was found that the chloroplast genome had the highest value. Effective population size could be reduced for ncDNA; if a genome is paternally inherited, it does not only imply transmission by pollen; paternally inherited genes are transmitted only in reproductive cycles and only 50% of nuclear genes pass to the next generation. High levels of genetic variability and low levels of gene flow reflect a defined genetic spatial structure and suggest that evolutive forces such as natural selection or genetic drift may be shaping population structure (Aguirre-Planter, 2000). For sacred fir, these forces would tend to decrease genetic variability; hence, it cannot be assumed that high levels of genetic variability mean that the forest is found in a good evolutive stage (Rocha and Gasca, 2007) in the NTNP. The presence of parasites (dwarf mistletoe and bark beetles) and illegal logging causing habitat fragmentation could be creating metapopulations, and if so, populations would confront bottlenecks, which invariably lead to genetic drift (Rocha and Gasca, 2007). This could mean that the high genetic variability of *A. religiosa* found in the present study was the result of transitory genetic variability, which occurs especially when populations experience a loss of individuals (Jump and Peñuelas, 2006). This suggests that it is therefore, necessary to create a conservation plan for this species in NTNP.

Geographic distribution of genetic variability

CpDNA and ncDNA variabilities were higher at high elevations, meaning altitudinal differentiation. It is possible that effective population size was high at higher elevations due to less illegal cutting and so the forest was less disturbed, and more individuals participate in gene flow. Also, the more extreme conditions at high elevations produce better phenotypes (Bidwell, 1979).

The North and center zones were the most variable at the mitochondrial and nuclear level. This implies an interesting issue for conservation and management planning since there are more maternal progenitors in this zone, considering that seeds are not capable of traveling long distances and they contain both uniparental (mitochondrial) and biparental (nuclear) genetic information. Seeds from the north zone can promote genetic variability in the south zone of the NTNP. In the south zone, chloroplast was the most variable indicating that pollen travels longer distances, promoting population differentiation.

Conservation and management

Ex situ conservation

In an *ex situ* conservation plan, the maximum range of evolutive potential must be preserved. This could be done through seed banks, tissue conservation, or storage of pollen and seeds (Palmberg-Lerche, 2001). In this study, it was found that seed is the principal agent for gene flow; so, we propose the creation of a gene bank, and collection- preservation of *A. religiosa* seeds of populations with high levels of polymorphism (Figure 3), this could be also helpful in order to have resources to looking for parasite resistance genes (Soekotjo and Thielghes, 2001)

In situ conservation

In order to preserve genetic variability, is necessary maintain populations in their natural habitat permitting forestry practices, populations with high levels of polymorphism must be considered as units of conservation. We propose the north and center zones as Genetic Resource Conservation Units (Sáenz-Romero, 2003; Rocha and Gasca, 2007) these must be considered highly significant evolutionary units with a high priority for conservation since they are populations with high levels of polymorphism (Figure 3).

In order to assess gene flow effectively, a high number of populations must be preserved to make it possible. To make it possible we propose bringing in seeds of sacred fir from other provenances (Michoacan forests, for example) to NTNP, this also could increase genetic variability in the populations with low levels; a population increases its genetic variability because of the introduction of external genes (Alfonsín et al., 2004; Rocha and Gasca, 2007), and crosses between different provenances generate high heterozygosity, which reduces expression of deleterious alleles.

Finally we would like to emphasize that additional studies are needed to complement this conservation strategies, for example the inclusion of forestry practices like harvesting, regeneration systems, tree breeding, and nursery practices, all of this impact significantly genetic variability. Certainly genetic variability of sacred fir in the NTNP is high and this must be used as advantage to conserve this conifer and ensure their adaptation to future environmental changes and the resistance to parasites.

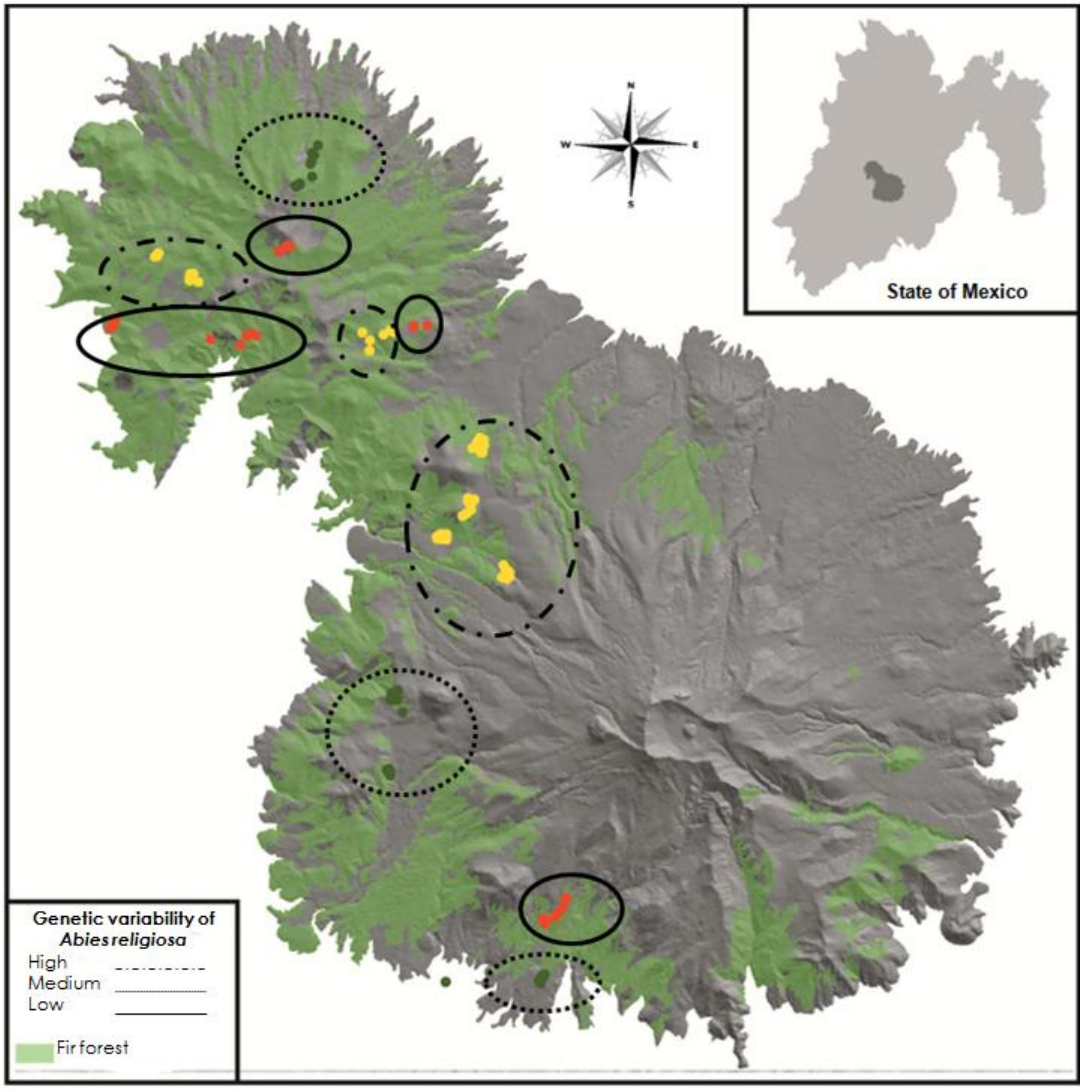

Figure 3. Ellipses indicate populations of *Abies religiosa* in the Nevado de Toluca National Park proposed as Conservation and Management of Gene Resources Units. Populations which contains high genetic variability enclosed an average of h= 0.2404-0.1913, medium: h= 0.1833-0.1573, and low: h= 0.1390-0.0597 for each marker.

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