Population genetic analysis of two Polylepis microphylla (Wedd.) Bitter (Rosaceae) forests in Ecuador

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ABSTRACT
Habitat fragmentation is a severe problem that negatively affects forests around the world. This phenomenon has consequences at a genetic level in populations such as genetic diversity and gene flow reduction. As a result, population fitness decreases, and the possibility of extinction increases. Polylepis (Rosaceae) forests are endemic to the Andes and their distribution reflects hundreds of years of human activities. Polylepis microphylla is the most endangered species of the genus in Ecuador and is represented by only two known forests in Chimborazo province, separated from each other by 1.6 km. The present study focused on i) exploring the genetic structure and connectivity, ii) analyzing the genetic diversity patterns in these two forests of P. microphylla. Parameters such as Fixation index, molecular variance (AMOVA) and genetic diversity estimators were calculated from the haplotypic sequences of one chloroplastic DNA region (trnG intron), one nuclear DNA region (ITS), and the concatenation of both. We found that DNA regions used were highly polymorphic with a total of nine, four and thirteen segregation sites, respectively, and nine, six and fifteen haplotypes, respectively. Also, we detected that both forest work as a single population unit due to low fixation index values (Fst = 0.03822, −0.03883 and −0.00469, respectively), which indicate high gene flow between these two forests. In addition, we detected high genetic diversity reflected by haplotype diversity in both forest (hd = 0.54204, 0.63664 and 0.81682, respectively). Likewise, haplotype relationships were visualized in networks, which demonstrated limited spatial structuring between haplotypes, corroborating the low genetic differentiation among these forests. In conclusion, our results suggest there is a single population unit of P. microphylla in Ecuador. Habitat fragmentation does not seem to have negative effects on the genetic diversity of this species. Nevertheless, the species may be vulnerable to rapid environmental fluctuations despite having relatively high genetic diversity. Its long generational time causes increased susceptibility of P. microphylla to environmental changes and may eventually lead to the extinction of the species in Ecuador.

Introduction
Habitat fragmentation caused by human activities is a major problem that negatively affects forests around the world, leading to isolated and small forest patches [1–3]. On a large scale, habitat fragmentation can be evidenced by decline of biodiversity, changes in community composition and alteration of ecological processes [3]. As a consequence of spatial isolation due to habitat fragmentation, genetic structure and connectivity can be altered, leading to genetic diversity and gene flow reduction, inbreeding depression, and genetic erosion [4–7]. Thus, population fitness might decrease, and populations may become more vulnerable to strong environmental changes, increasing their probability of extinction [4,5].

Genetic diversity, structure and connectivity are closely related with occupied geographical space and can be affected by habitat fragmentation [7,8]. According to Young et al. [9], genetic consequences of habitat fragmentation such as reduced variability, increased inbreeding and reduced gene flow have serious implications for species conservation. The reduction and isolation of forests can diminish the genetic pool of populations and reduce their fitness and capacity to quickly and effectively adapt to environmental changes [4,5,10–12].

The tropical Andes are considered as one of the most important biodiversity hotspots in the world [13,14], which offers a wide variety of ecosystem services to the region [15 p. 1–7, 13–19]. Due to high species richness and endemism, it has been categorized as one of the priority regions for conservation around the world [13]. However, human activities such as agriculture, which has been carried out in the region for hundreds of years [16], and fires [17], have fragmented Andean ecosystems [18–20].

Trees and shrubs of Polylepis Ruiz & Pav. (Rosaceae), endemic to the Andean region, exemplify habitat fragmentation related to anthropogenic impact. Polylepis forests have a patchy distribution that is the result of
hundreds of years of constant human activities [21]. Logging, livestock, agricultural activities and, especially, fire are determining factors that cause an accelerated reduction in the size of these forests and are a possible explanation of their currently fragmented distribution [22–25].

Fjeldså [26] considered *Polylepis* forests as small oases of life compared with the characteristic and dominant páramo grasslands in which they occur. They are also considered as biodiversity islands, providing shelter to many endemic animal and plants species [27–29]. In addition, they are valuable habitats that offer several ecosystem services [27]. Despite these characteristics, *Polylepis* forests are considered as one of the most endangered ecosystems around the world [30].

The genus *Polylepis* comprises about 45 species [31–34] that are distributed from northern Venezuela to northern Argentina and Chile [35,36] and can be found between 2700 and 5200 m a.s.l [35–37]. In Ecuador, there are ten native species ranging from 2700 to 4300 m a.s.l [36,38]. All species are wind pollinated.

*Polylepis microphylla* (Wedd.) Bitter is the most endangered species of the genus in Ecuador, being known in the country from only one location [39, p. 748–750]. In agreement with the forest definition of the United Nations Framework Convention on Climate Change (UNFCCC) [40], the species is represented by two forest remnants near Achupallas village in Chimborazo province, in an open grassland area dominated by agricultural activities between 3500 and 4000 m a.s.l. In 2004 IUCN Red List of Threatened Species, *P. microphylla* is recorded as vulnerable (VU) [41], but the 2011 Red Book of the Endemic Plants of Ecuador [42] categorizes it as critically endangered (CR) [39, p. 748–750]. Based on our field observations, we consider that the biggest threats for this species are accelerated expansion of rural settlements, logging and, especially, fires (Figure 1).

Available information about *P. microphylla* is limited and is mainly taxonomic. Simpson [35] considered the species a synonym of *P. weberbaueri* Pilg. but Bitter [43], Romoleroux [36] and Kessler & Schmidt-Lebuhn [31] recognized *P. microphylla* as a separate species. Schmidt-Lebuhn et al. [44] estimated the genome size of *P. microphylla* by flow cytometry and presumed that it is a diploid species.

It is generally assumed that long-distance pollen dispersion is operative in wind-pollinated species [11,45], implying high levels of genetic diversity and low genetic differentiation among populations [11,46]. However, Hensen et al. [20] found high genetic differentiation among populations in wind pollinated *Polylepis incana* Kunth, indicating that habitat fragmentation has a negative effect on the genetic diversity in this species. Given the limited distance (1,6 km) between the two forest stands of *P. microphylla*, we hypothesize that these two stands share a common genetic pool and work as a single population. Thus, the present study will focus on: i) exploring the genetic structure in the two known forests of *P. microphylla* in Ecuador, and ii) analyzing the genetic diversity patterns of *P. microphylla*.

Figure 1. Photograph of burnt *Polylepis microphylla* shrubs in the study area.
Materials and methods

Study species and study area. Since the first review of the genus [43], various species concepts have been applied to *Polylepis*. Here we applied the general lineage concept by which species are considered a segment of an evolutionary lineage [33, 47], because this definition is applied at the population level and takes ecological aspects into account.

*Polylepis microphylla* has been recorded from one location in Ecuador and three in Peru, ranging from 3200 to

![Figure 2. Tree habit of *Polylepis microphylla* of approximately 4 m high located in forest 1.](image1)

![Figure 3. Detail of mature flower and leaves of *Polylepis microphylla*.](image2)
4000 m a.s.l [48, 49]. Following the taxonomical description of Romoleroux [36], the species is a shrub or tree to 7 m high (Figure 2 and 3); leaves are alternate, compound and imparipinnate formed by 3–6 pairs of leaflets, always accompanied by a pair of villous to glabrescent stipules that are connate and adnate to the petiole; leaflets are oblong or slightly ovate, 0,3–0,7 × 0,2–0,6 cm, the lower surface is densely villous with sparse small red and black hairs, while the upper surface is sparsely villous; inflorescences are simple racemes; flowers are apetalous with oval and green sepals; stamens 15–20, with elongate filaments; hypanthium with irregular crests densely covered by lanose pubescence; and fruits have small, flattened spines.

The study was carried out in the surroundings of Totoras indigenous community along the road Ozogoche-Achupallas, about 18 km SE of Alausí (Chimborazo province). The two forest of P. microphylla occur here on relatively dry slopes, in open areas dominated by grassland and crops, separated by a valley of 1.6 km (Figure 4) (Figure 4 near here). According to UNFCCC [40], forests are defined as having a minimum area of 0,05–1,0 hectares with vegetation coverage of more than 10–30%, made up of trees with a capacity to reach 2–5 m height at maturity. Forest 1 was located at S 02° 16’ 16,79” W 78° 42’ 16,88”, between 3556 and 3800 m a.s.l., with a total area of ≈19.06 ha and an area of P. microphylla coverage of ≈10.56 ha (Figures 4 and 5). Forest 2 was located at S 02° 15’ 04,53” W 78° 41’ 30,77”, between 3964 and 3673 m a.s.l., and had a total area of ≈49.05 ha and an area of P. microphylla coverage of ≈30.14 ha (Figures 4 and 6).

**Sampling.** In each forest, we randomly sampled 50 adult individuals of P. microphylla at minimally 20 m distance from each other to limit genetic closeness [20]. Leaf samples were collected from each individual and stored in paper envelopes with silica gel. A GPS waypoint was noted for each individual, and vouchers were deposited in Herbarium QCA.

**DNA extraction, PCR amplification and sequencing.** Total DNA extraction was carried out with 50 mg of grinded leaf tissue using Doyle & Doyle [50] protocol with some modifications. β-mercaptoethanol was placed independently of the CTAB solution, chloroform and isooamyl alcohol were added separately too. Also, DNA was precipitated for 24 hours. Finally, the extracted DNA was quantified in NanoDrop® 1000 Spectrophotometer and stored at concentrations of 25 ng/µL in 0.1 X TE solution on the freezer at –20 °C.

After testing a total of 3 chloroplast DNA (cpDNA) regions (trnL-trnF, rpoB-trnC, and trnS-trnG), we

![Figure 4](image-url). Map of two Polylepis microphylla forests in the study area in Chimborazo province, Ecuador.
decided to select the intron trnG, which is part of the trnS-trnG intergenic spacers for the present study. We selected this cpDNA region based on the informational potential reflected as number of segregation sites. In the case of nuclear DNA, in order to make the best use of resources, we selected the ITS region, which has proven to be highly informative at the intraspecific level [51-56]. The ITS region amplified included the spacer I and II and the 5.8 subunit of nuclear ribosomal DNA (nrDNA) [51].

The final reaction volume was 25 µL consisting of 12.5 µL of GoTaq® Green Master Mix (Promega©), 4 µL of DNA (100 ng), 1 µL of each primer (10 pmol) and 6.5 µL of UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific©).

Polymerase chain reaction (PCR) protocol for both regions was carried out under the following conditions of time and temperature: 95 °C x 3 min; 35 cycles (94 °C x 1 min; 54 °C x 1 min; 72 °C x 1 min); 72 °C x 7 min. The primers used of each region are presented in Table 1. (Table 1 near here)

The PCR products were verified by electrophoresis in 1% agarose gel in a solution of Tris-borate-EDTA (TBE) 1X with 4 µL of SYBR safe 10 000X in DMCO (Thermo Fisher Scientific©). Finally, we ran it at 100 v in the electrophoresis chamber for one hour. PCR positive samples were sequenced in Macrogen Korea commercial laboratory.

Data analysis.- In each region analyzed, the forward and reverse sequences received from Macrogen Korea were reviewed and aligned using the Geneious Alignment function [57] in the Geneious Prime® 2019.2.1 program. Each consensus sequence obtained was reviewed and edited manually. For the alignment of consensus sequences between individuals of the same population we used the MUSCLE Alignment function [58,59] of the same program.

The regions were analyzed independently and concatenated. To concatenate them we used the SuperMatrix function from evobiR package in RStudio v.1.2.1335 program [60].

The estimators of genetic diversity used at the intrapopulation and interpopulation levels were: Number of segregation sites (S), number of haplotypes (h), haplotypic diversity, (hd) and nucleotide diversity (n). Haplotypes are the genetic constitution of a single chromosome [61], while haplotype diversity is defined as the probability that two randomly sampled alleles are different. On the other hand nucleotide diversity represents the average number of nucleotide differences per site in the comparison between different sequences of DNA [62]. These genetic estimators
were obtained using the DNA Sequence Polymorphism v.5 program.

To determine the percentage of variation between and within the forests, the analysis of the molecular variance (AMOVA) was performed. Excoffier et al. [63] proposed AMOVA as a method to test the hierarchical population structure. They suggested that under the null hypothesis, random samples taken from the complete pool belong to a global population. This means that the analyzed populations are structured as one. While, to determine the genetic differentiation between populations, the value corresponding to fixation index (Fst) [64] was used. This parameter is widely used to determine population structure type, and is defined as the probability of sampling randomly alleles from within a subpopulation in relation to the whole population [65]. These values were calculated using the Arlequin v.3 program.

To visualize the genealogical relationships of DNA sequences at intraspecific level and population structure, haplotypic networks were generated by means of the Median Joining method [66–68] in Population Analysis with Reticulate Trees (PopART) v.5 program. Finally, node names of haplotype networks were added using Inkscape v.0.92.4 program.

Results

DNA extraction, amplification and sequencing.- DNA extraction was successfully completed in 19 samples of forest 1 (F1) and 18 samples from forest 2 (F2), which represent the 37% of all the individuals sampled. The amplification of the trnG intron and ITS region was positive for all samples.

Reviewing, editing and concatenation of regions.- Alignments of the trnG intron and ITS region resulted in sequences of 737 and 618 bp, respectively. The trnG intron was the most polymorphic region with 9 segregation sites (S), while the ITS region showed 4 (Tables 2 and 3). The concatenated region resulted in 37 sequences, 19 from F1 and 18 from F2 with a final length of 1355 bp and a total of 13 segregating sites (Table 4). AMOVA- trnG intron. The analysis of the molecular variance showed that highest percent variation occurred within forests (96.18%), compared to among forests (3.82%) (P > 0.05). The Fst value revealed low population differentiation (Fst = 0.03822) (Table 5).

ITS. AMOVA showed the same pattern for ITS: percent variation within forests (103.88%) was higher than among forests (−3.88%) (P > 0.05). The Fst value revealed low population differentiation (Fst = −0.03883) (Table 5).

Figure 6. Photographs of forest 2. A) Polylepis microphylla represented by shrubs of maximum 1,5 m high. Area dominated by grassland. B) A slope of P. microphylla coverage.
Table 1. DNA regions used. The type of region, primers used, their sources and temperatures of annealing (TA) are indicated.

| Region       | DNA Type  | Primers | Sequence (S′-3′) | TA    |
|--------------|-----------|---------|------------------|-------|
| tmG-G2G intron | Chloroplast | 3 tmGUSC | GTA GCG GGA ATC GAA CCC GCA TC | 54 °C |
|              |           | 5′ tmG2G | GCC GGT ATA GGT TAG TGG TAA AA |      |
| ITS          | Nuclear   | IT51b   | TCC GTA GGT GAA CTT GGG |      |
|              |           | IT54    | TCC TCC GTAT TGA TAT GC |      |

Concatenated regions. Main percent of regions. Most percent within forests (100.47%) compared to among forests (−0.47%) (P > 0.05). The Fst value revealed a low population differentiation (Fst = −0.00469) (Table 5).

Intrapopulation genetic diversity estimators.-tmG intron. Within F1, 6 segregation sites were identified, generating 6 haplotypes: H3, H5, H6, H7, H8 and H9. Haplotypes H3, H6, H7 and H8 were unique for this forest, while H9 was the most frequent. Haplotypic diversity was relatively high (>0.5) (hd = 0.53,801), nucleotide diversity was low (<0.5) (π = 0.00099) (Table 6). In the case of F2, five segregating sites were identified, generating 5 haplotypes: H1, H2, H4, H5 and H9. Haplotypes H1, H2 and H4 were unique for this forest, and H9 was the most frequent. Haplotypic diversity was relatively high (hd = 0.55556) while nucleotide diversity was low (π = 0.00139) (Table 6).

ITS. Within F1, 3 segregation sites were identified, generating a total of 5 haplotypes: H1, H2, H3, H5 and H6. Haplotypes H1, H3 and H5 turned out to be unique for this forest and H6 proved to be the most frequent. Haplotypic diversity was high (hd = 0.69591) while the nucleotide diversity was low (π = 0.00194) (Table 6). In the case of F2, 3 segregation sites were identified, generating a total of 3 haplotypes: H2, H4 and H6. Haplotypic diversity was unique and H2 was the most frequent. Likewise, haplotypic diversity was high (hd = 0.58170) while the nucleotide diversity was low (π = 0.00191) (Table 6).

Concatenated regions. 9 segregation sites were identified in F1, generating a total of 9 haplotypes: H4, H7, H9, H10, H11, H12, H13, H14 and H15. Haplotypes H4, H7, H9, H10, H11 and H14 were unique for this forest, while H13 was the most frequent. Haplotypic diversity turned out to be high (hd = 0.84795) while the nucleotide diversity was low (π = 0.00142) (Table 6). In the case of F2, we found 8 segregation sites, and 8 haplotypes: H1, H2, H3, H5, H6, H8, H12 and H13. Haplotypes H1, H2, H3, H5, H6 and H8 were unique for this forest, while H12 and H13 were the most frequent ones. Haplotypic diversity was high (hd = 0.80392) and nucleotide diversity low (π = 0.00163) (Table 6).

Table 2. Segregation sites and haplotype frequencies of tmG intron with their position in the final alignment and their frequencies in the two forests. (1) represents the same nucleotide in relation to the first sequence.

|    | 1  | 2  | 3  | 4  | 17 | 27 | 32 | 70  | 728 | 738 | 807 | 1177 | 1300 | F1  | F2  |
|----|----|----|----|----|----|----|----|-----|-----|-----|-----|------|------|-----|-----|
| H1 | T  | A  | T  | A  | T  | A  | T  | A   | T   | C   | C   | 0    | 1    | 0   |
| H2 | -  | T  | -  | -  | -  | -  | -  | -   | C   | -   | -   | A    | 0    | 1   |
| H3 | -  | T  | -  | -  | -  | -  | -  | -   | -   | -   | -   | -    | 0    | 1   |
| H4 | -  | T  | A  | G  | -  | -  | -  | A   | C   | -   | -   | -    | 1    | 0   |
| H5 | -  | T  | A  | G  | -  | -  | -  | C   | C   | -   | -   | A    | 1    | 0   |
| H6 | -  | T  | A  | G  | -  | -  | -  | C   | C   | -   | -   | A    | 1    | 0   |
| H7 | -  | T  | A  | G  | -  | -  | -  | C   | C   | -   | -   | A    | 1    | 0   |
| H8 | -  | T  | A  | G  | -  | -  | -  | C   | C   | -   | -   | A    | 1    | 0   |
| H9 | -  | T  | A  | G  | -  | -  | -  | A   | -   | -   | -   | C    | -    | A   |
| H10| -  | T  | A  | G  | -  | T  | -  | -   | -   | -   | -   | -    | A    | 2   |
| H11| -  | T  | A  | G  | -  | -  | -  | -   | -   | -   | -   | -    | A    | 1   |
| H12| -  | T  | A  | G  | -  | -  | -  | -   | -   | -   | -   | -    | A    | 1   |
| H13| -  | T  | A  | G  | -  | -  | -  | -   | -   | -   | -   | -    | A    | 1   |

Table 3. Segregation sites and haplotype frequencies of ITS region with their position in the final alignment and their frequencies in the two forests. (1) represents the same nucleotide in relation to the first sequence.

|    | 1  | 70 | 440 | 563 | F1  | F2  |
|----|----|----|-----|-----|-----|-----|
| H1 | A  | T  | A   | C   | 1   | 0   |
| H2 | -  | -  | C   | -   | 6   | 9   |
| H3 | -  | -  | C   | T   | 1   | 0   |
| H4 | C  | C  | C   | A   | 0   | 1   |
| H5 | -  | C  | A   | 2   | 0   | 7   |
| H6 | C  | C  | A   | 9   | 8   | 7   |

Table 4. Segregation sites and haplotype frequencies of the concatenated regions with their position in the final alignment and their frequencies in the two forests. (1) represents the same nucleotide in relation to the first sequence.

|    | 1  | 2  | 3  | 4  | 17 | 27 | 32 | 70  | 728 | 738 | 807 | 1177 | 1300 | F1  | F2  |
|----|----|----|----|----|----|----|----|-----|-----|-----|-----|------|------|-----|-----|
| H1 | T  | A  | T  | A  | T  | A  | A  | T   | C   | C   | C   | 0    | 1    | 0   |
| H2 | -  | T  | -  | -  | -  | -  | -  | -   | C   | -   | -   | A    | 0    | 1   |
| H3 | -  | T  | -  | -  | -  | -  | -  | -   | -   | -   | -   | -    | 0    | 1   |
| H4 | -  | T  | A  | G  | -  | -  | -  | A   | C   | -   | -   | -    | 1    | 0   |
| H5 | -  | T  | A  | G  | -  | -  | -  | C   | C   | -   | -   | A    | 1    | 0   |
| H6 | -  | T  | A  | G  | -  | -  | -  | C   | C   | -   | -   | A    | 1    | 0   |
| H7 | -  | T  | A  | G  | -  | -  | -  | C   | C   | -   | -   | A    | 1    | 0   |
| H8 | -  | T  | A  | G  | -  | -  | -  | C   | C   | -   | -   | A    | 1    | 0   |
| H9 | -  | T  | A  | G  | -  | T  | -  | -   | -   | -   | -   | -    | 1    | 0   |
| H10| -  | T  | A  | G  | -  | T  | -  | -   | -   | -   | -   | -    | A    | 2   |
| H11| -  | T  | A  | G  | -  | -  | -  | -   | -   | -   | -   | -    | A    | 1   |
| H12| -  | T  | A  | G  | -  | -  | -  | -   | -   | -   | -   | -    | A    | 1   |
| H13| -  | T  | A  | G  | -  | -  | -  | -   | -   | -   | -   | -    | A    | 1   |
| H14| -  | T  | A  | G  | -  | -  | -  | -   | -   | -   | -   | A    | 1    | 0   |
| H15| -  | T  | A  | G  | -  | -  | -  | -   | -   | -   | -   | A    | 1    | 0   |
Table 5. Analysis of molecular variance (AMOVA) in trnG intron, ITS and concatenated regions in the two forests (P > 0.05). (d.f) degrees of freedom, (Va) Variance of components among forests, (Vb) Variance of components within forests, (Fst) Weir & Cockerham (1984) fixation index.

| Source of variation | d.f | Sum. of squares | Variance components | % of variation | Fst index |
|---------------------|-----|-----------------|---------------------|---------------|----------|
| trnG intron         |     |                 |                     |               |          |
| Among forests       | 1   | 0.753           | 0.07225 Va          | 3.82          | 0.03822  |
| Within forests      | 35  | 15,193          | 0.43,409 Vb         | 96.18         |          |
| Total               | 36  | 15,946          | 0.45,133            |               |          |
| ITS                 |     |                 |                     |               |          |
| Among forests       | 1   | 0.182           | −0.02203 Va         | −3.88         | −0.03883 |
| Within forests      | 35  | 20,629          | 0.58,939 Vb         | 103.83        |          |
| Total               | 36  | 20,811          | 0.56,736            |               |          |
| Concatenated regions|     |                 |                     |               |          |
| Among forests       | 1   | 0.935           | −0.00478 Va         | −0.47         | −0.00469 |
| Within forests      | 35  | 35,822          | 1.02,346 Vb         | 100.47        |          |
| Total               | 36  | 35,757          | 1.01,870            |               |          |

Table 6. Genetic diversity estimators, distribution and frequency of haplotypes within and both forests in trnG intron, ITS and concatenated regions. (n) sample size; (S) number of segregation sites; (h): number of haplotypes; (hd) haplotype diversity; (n) nucleotide diversity, * represents unique haplotypes.

| Source of variation | (n) | (S) | (h) | (hd) | (n) | Haplotype frequency |
|---------------------|-----|-----|-----|------|-----|---------------------|
| trnG intron         |     |     |     |      |     |                     |
| Forest 1            | 19  | 6   | 6   | 0.53,801 | 0.00,099 | H3(1) *, H5(3), H6(1) *, H7 (2) *, H8(1) *, H9(13). |
| Forest 2            | 18  | 5   | 5   | 0.55,556 | 0.00,139 | H1(1) *, H2(2) *, H4(1) *, H5 (3), H9(12) |
| Total               | 37  | 9   | 9   | 0.54,204 | 0.00,121 |                     |
| ITS                 |     |     |     |      |     |                     |
| Forest 1            | 19  | 3   | 3   | 0.69,591 | 0.00,194 | H1(1) *, H2(6), H3(1) *, H5 (2) *, H6(9) |
| Forest 2            | 18  | 3   | 3   | 0.58,170 | 0.00,191 | H2(9), H4(1) *, H6(8) |
| Total               | 37  | 4   | 6   | 0.63,664 | 0.00,189 |                     |
| Concatenated regions|     |     |     |      |     |                     |
| Forest 1            | 19  | 9   | 9   | 0.84,795 | 0.00,142 | H4(1) *, H7(1) *, H9(1) *, H10(2) *, H11(1) *, H12 (5), H13(6), H14(1) *, H15 (1) |
| Forest 2            | 18  | 8   | 8   | 0.80,392 | 0.00,163 | H1(1) *, H2(2) *, H3(1) *, H5 (1) *, H6(1) *, H8(1) *, H12(6), H13(6) |
| Total               | 37  | 13  | 15  | 0.81,682 | 0.00,152 |                     |

Interpopulation genetic diversity estimators.- trnG intron. Nine segregation sites were detected in both forests, generating nine haplotypes (Table 2). Haplotype diversity value was relatively high (hd = 0.54204) while nucleotide diversity was low (π = 0.00121) (Table 6).

ITS. A total of 4 segregation sites were detected in both forests, generating 6 haplotypes (Table 3). Haplotype diversity value was high (hd = 0.63664) while nucleotide diversity was low (π = 0.00189) (Table 6).

Concatenated regions. Thirteen segregation sites were detected in the two forests, generating 15 haplotypes (Table 4). Haplotype diversity value was high (hd = 0.80392) while nucleotide diversity was low (π = 0.00152) (Table 6).

Haplotype networks.- trnG intron. The network corroborates the low differentiation between forests. As shown in Figure 7, the most common haplotype H9 is shared by the two forests with a frequency of 25. Other haplotypes are separated from the most common by one or two nucleotide substitutions. Haplotype 5, the second most common one, is also shared by both forests (Figure 7).

ITS. Haplotypes H2 and H6, being the most frequent ones with frequencies of 15 and 17 m respectively, are present in both forests (Figure 8). Unique haplotypes have low frequencies: H1(1), H3(1) and H5(2) in forest 1 and H4(1) in forest 2. These haplotypes are different from the most frequent for only one nucleotide substitution, showing limited spatial structuring between haplotypes (Figure 8).

Concatenated regions. The patterns described above are repeated here, the most frequent haplotypes, H12 and H13, being shared by both forests with almost the same frequencies, respectively 11 and 12. All other haplotypes have frequencies of 1 with exception of H10 which has a frequency of 2 (Figure 9).

Discussion. The results obtained in this study represent the first analysis of genetic diversity and structure of P. microphylla forests in Ecuador. They should be handled with caution because of the possibility of different ploidy levels within the Polypleis species [44] and the relatively low number of samples used in the study.

According to Brown and Biggs [69], theoretically 60–70% of alleles are captured by sampling 10% of the individuals in a population. Therefore, the number of samples to be analyzed in population genetics studies is not defined and may depend on several factors,
such as the number of alleles captured by the type of molecular markers used [70]. Zhao et al. [71] found that 90% of population diversity as reflected by the expected heterozygosity or genetic diversity, can be
captured with 25–30 samples using AFLP’s markers and 10–15 samples using SSRs. On the other hand, Hale et al. [72] mentioned that minimally 25–30 individuals are needed using microsatellite markers.

The AMOVA results showed that the greatest source of variation is explained within forests, compared to among forests (Table 5). This points to a single population structure of *P. microphylla* in Ecuador. Due to the significance of the P value exceeding the tolerable range to take the alternative hypothesis [73], the null hypothesis of the AMOVA, which implies that all samples belong to a global population, was selected [63]. Our results are not in line with those reported by Hensen et al. [20], who found that habitat fragmentation has a negative effect on the connectivity and indicate genetic differentiation among populations in *P. incana* using AFLP markers. Our results are also not in complete agreement with those of Vranckx et al. [74], who indicated that severe habitat fragmentation can affect to wind-pollinated tree species and may lead to pollen limitation and limited gene flow.

As the studied *P. microphylla* forests were at a distance from each other of approximately 1.6 km, we suggest that the spatial separation was not a barrier for completely random reproductive processes. Since *Polylepis* trees are wind pollinated, their pollen grains can be dispersed over long distances [75] and have reproductive success between spatially separated patches. For instance, Seltmann (unpubl.) found *P. australis* pollen grains separated approximately by 80 km from the nearest forest. Additionally, she mentioned that these pollen grains had a viability rate of up to 5 days after being released.

Values corresponding to Fst index can be interpreted as low genetic differentiation between the two forests, indicating high levels of gene flow and random mating [76] (Table 5). Negative values in the ITS region and the concatenated analysis can occur when populations are not or are weakly differentiated [77]. The results obtained in this study agree with studies on population genetics of *Polylepis rugulosa* Bitter and *P. tarapacana* Philippi [78], *P. australis* Bitter [12,79], *P. pauta* Hieron [80] and *P. besseri*
Hieron [81], all of which revealed high levels of gene flow and low population differentiation.

Because of the low genetic differentiation between the two forests of *P. microphylla*, we suggest that the two forests act as a single population unit and have a panmictic reproduction system, implying random mating between individuals [82].

High diversity, reflected as haplotypic diversity, was detected in *P. microphylla* (>0.5) in each of the three genetic regions analyzed. This is consistent with results obtained in *P. australis* in Argentina [79] and *P. pauta* in Ecuador [80], where high genetic diversity was detected based on analysis of different molecular markers.

In both the individual and the concatenated analysis of the analyzed genetic regions, a well-defined pattern of genetic diversity estimators was found. The high haplotype diversity (>0.5) and low nucleotide diversity (<0.5) may be a consequence of relatively short sequences and few haplotypes in the population [83] (Table 6). This pattern can indicate a low genetic differentiation between the observed haplotypes, separated from each other by few nucleotide substitutions (Figures 7, 8 and 9) [84,85].

According to Hamrick [11], populations at geographical and ecological border situations are generally more susceptible to environmental changes. Due to the major, human-induced environmental changes [86], *P. microphylla* could be severely affected and even become locally extinct, despite having a high genetic variability. Its slow life cycle might not allow *P. microphylla* to adapt quickly to the changing habitat conditions [87]. Because of its highly restricted geographical distribution in Ecuador, together with our results that points to a single population in the country, *P. microphylla* is vulnerable to environmental changes and susceptible to extinction [11,88].

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No potential conflict of interest was reported by the author(s).

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