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Population genetics of the endangered black walnut *Juglans neotropica* (Juglandaceae) based on plastid data from the Amazonas region

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Abstract

The black walnut *Juglans neotropica* is a forest species characterized by being a monoecious and deciduous tree with a long life. This species has great ecological, environmental, and economic value, playing a fundamental role in the ecosystem. According to the IUCN, *J. neotropica* is threatened by anthropogenic activities that have drastically affected its distribution. In this study, the plastid intergenic spacer marker *trnS-trnfM* was amplified from 74 *J. neotropica* samples from eight locations in Amazonas region (Peru) to determine its haplotype network, genetic diversity, and genetic divergence. The results revealed that *J. neotropica* from Amazonas region showed i) a lineage composed of the eight populations embedded into the Rhysocaryon section; ii) three genetic groups within the haplotype network with the presence of an ancestral haplotype (H1) and possibly candidates for new taxa; iii) a high divergence between the populations of Molinopampa and Luya (1.62–2.64% of p-distance); iv) populations with high genetic diversity indices (Levanto = 0.32, Molinopampa = 0.41) with constant threats from anthropogenic activities; and v) high genetic structuring within populations (Fst = 0.04). Overall, these results collectively support a scenario of high variability with limited interpopulation genetic exchange. Our findings provide previously unavailable insights into the vulnerability of the black walnut *J. neotropica* by (i) quantifying the genetic consequences of human-induced habitat fragmentation and (ii) establishing baseline diversity metrics for future monitoring. These results directly inform *in situ* conservation priorities by identifying populations harbouring unique alleles that warrant immediate protection. Finally, further research should include nuclear markers (e.g., microsatellites or RAD-seq) to support our findings.

Introduction

The family Juglandaceae is one of the most iconic groups of nut trees (Zhang *et al.* 2022) and holds significant scientific and conservation value (Song *et al.* 2020). Notable genera within this family include *Carya*, *Juglans*, *Pterocarya*, and *Platycarya* (Stanford *et al.* 2000). Among them, the genus *Juglans* is the most representative and comprises the majority of fruit-bearing and timber-yielding species (Trouern-Trend *et al.* 2020). The genus *Juglans* consists of 21 species and is classified into four taxonomic sections (i.e., Cardiocaryon, Juglans, Rhysocaryon, and Trachycaryon) based on the morphological characteristics of its flowers and leaves (Manning 1960). One of these species, namely *Juglans neotropica* traditionally known as black walnut, is native to Colombia, western Venezuela, Ecuador, and Peru (Manning 1960).

Juglans neotropica is a woody angiosperm that can reach heights of 25–30 metres (Nieto and Rodríguez 2010). This species is characterized by having furrowed bark on its trunk and monoecious nature (Ramírez and Kallarackal 2021). The altitudinal distribution range of the black walnut *J. neotropica* is between 500 and 3300 m.a.s.l. (Reynel and Marcelo 2009). This species has been reported in the following regions of Peru: Amazonas, Cajamarca, Cusco, Huancavelica, Junín, La Libertad, Lambayeque, and Pasco (Manning 1960, Hurtado Manrique *et al.* 2015). The black walnut *J. neotropica* is recognized as one of the most ecologically, environmentally, and economically valuable tree species (Vanegas and Rojas 2018) due to its prominent role in the recovery of degraded soils and its contribution to improving air quality and water conditions in the ecosystems (Vanegas and Rojas 2018).

In Peru, the black walnut *J. neotropica* is locally known as nogal and is used as a medicinal and commercial tree due to its fruits with high nutritional value and pigments used in textile

products (Woll *et al.* 2023). In the Amazonas region, this nogal tree is also highly valued for their timber quality. For instance, *J. neotropica* is part of the official list of forest species used for timber purposes (SERFOR 2019), with extraction figures of 2457.21 m^3/year for sawn wood and 5416.64 m^3/year for round wood (SERFOR 2019). Currently, the black walnut *J. neotropica* is listed as endangered species (IUCN, 2023) due to rapid reductions in its distribution as a result of anthropogenic activities. These activities generate a loss of genetic variability (Anabat *et al.* 2020), preventing it from responding to natural selection and limiting its ecological recovery (Janat Gul *et al.* 2021).

Various studies have been conducted on different species of *Juglans* regarding its genetic improvement, potential commercial use, restoration capabilities for degraded ecosystems, genetic diversity for conservation purposes, and spatial monitoring (Ross-Davis *et al.* 2008, Vischi *et al.* 2017, Gaisberger *et al.* 2020, Veintimilla *et al.* 2020). For instance, the distribution patterns, diversity, and structure of different populations of *J. regia* have been evaluated *via* nuclear microsatellites (Magige *et al.* 2022). However, this study has found limitations in the dispersion and discontinuous geographical distribution of the species, which has made it difficult to interpret genetic patterns (Gaisberger *et al.* 2020). Strikingly, most of the genetic studies carried out in *Juglans* have focused on specimens distributed in the Northern Hemisphere (Ma *et al.* 2020, Zhou *et al.* 2021). In contrast, in the Southern Hemisphere, only two specimens of *Juglans*, which are distributed mainly in Argentina (*J. australis*) and Ecuador (*J. neotropica*), were sequenced (Aradhya *et al.* 2007).

Additionally, the most widely used molecular markers to infer the phylogeny of numerous *Juglans* species are the plastid coding markers (*rbc*L, large subunit of ribulose bisphosphate carboxylase; *mat*K, maturase K), plastid intergenic markers (*trn*T-*trn*F, *psb*A-*trn*H, *trn*S-*trnf*M), and nuclear non-coding marker (ITS, internal transcribed spacer) (Orel *et al.* 2003, Aradhya *et al.* 2007). Among these markers, the intergenic *trn*S-*trnf*M revealed a better phylogenetic resolution, greater number of sequences, and greater genetic divergences between *Juglans* species (Aradhya *et al.* 2007). For instance, genetic analysis in other Juglandaceae such as *Pterocarya* used this intergenic marker to identify evolutionary patterns (Lu *et al.* 2024). Despite the great utility of this marker, genetic studies related to the black walnut *J. neotropica* are currently lacking in Peru.

Genetic diversity studies within the *Juglans* genus have predominantly focused on *Juglans regia* (Sun *et al.* 2019, Ren *et al.* 2023). For instance, low genetic diversity was confirmed in populations from Pakistan and Kyrgyzstan (Gaisberger *et al.* 2020, Magige *et al.* 2022). Conversely, higher genetic diversity was observed in populations from Uzbekistan (Gaisberger *et al.* 2020). These studies suggested that human activities significantly influence the distribution of *Juglans regia*. Population genetics of *Juglans cinerea* revealed a high level of genetic diversity, supporting the development of conservation strategies to protect its local populations (Ross-Davis *et al.* 2008). Although populations of *Juglans hopeiensis* exhibited robust genetic structure and were different from other sympatric species within the genus, they face significant threats due to their small size and ongoing hybridization (Hu *et al.* 2017).

Accordingly, understanding the distribution and genetic diversity of *Juglans* species is crucial (Zhao *et al.* 2018) since it will provide valuable information of its current state and ability to survive against various risk factors (Govindaraj *et al.* 2015, Stojnić *et al.* 2019). The population genetic analysis of *J. neotropica* serves multiple critical purposes for both ecological understanding and conservation practice. First, it reveals historical lineages, helping reconstruct post-glacial migration patterns and identify potential refugia in the Amazonas region, which is key for understanding how this species responded to past climatic shifts (Shahi Shavvon *et al.* 2023). Second, quantifying genetic diversity and divergence directly informs conservation priorities, where populations with low diversity may require urgent protection due to their vulnerability to habitat fragmentation (Dexter *et al.* 2018). Our study thus bridges evolutionary history and actionable conservation strategies for this critically endangered species. For this reason, the present study aimed to evaluate the genetic diversity of the black walnut *J. neotropica* from eight locations in the Amazonas region. This study addressed the degree of genetic connectivity and identified genetic groups (haplotypes), allowing for the generation of crucial information for further conservation and control strategies.

Material and methods

Collection of samples

A total of 74 specimens of the black walnut *J. neotropica* (locally known as nogal) were collected from the following eight locations in the Amazonas region: Levanto (12 specimens), Leymebamba (10 specimens), Luya (13 specimens), Molinopampa (10 specimens), Nogalcucho (8 specimens), San Isidro de Maino (9 specimens), Yerbabuena (10 specimens), and Tambolic (2 specimens) (Figure 1). The identification of these specimens as *Juglans neotropica* was based on taxonomical keys (Nieto and Rodríguez 2010, Ramírez and Kallarackal 2021) and distribution reports (Manning 1960, Hurtado Manrique *et al.* 2015). The collected specimens from each location were at least 15 metres apart (Deng *et al.* 2020). The black walnut *J. neotropica* trees chosen for collection were young with new leaves and flowers; the leaves were tender with no epiphytes and free of pests and diseases. A wild flora scientific research permit (D000506-2020-MINAGRI-SERFOR-DGGSPFFS) was obtained from the National Forest and Wildlife Service (SERFOR). The collection points were defined by reviewing the distributions of the black walnut *J. neotropica* in the Amazonas region recorded in the databases of international herbaria, such as the JSTOR Global Plants (https://plants.jstor.org/), the New York Botanical Garden Steere Herbarium (https://sweetgum.nybg.org/science/), the Global Biodiversity Information Facility (https://www.gbif.org/) and Tropicos database of the Missouri Botanical Garden (http://www.tropicos.org). Additionally, each collection point was georeferenced, and each collected sample was deposited in the herbarium KUELAP of the Universidad Nacional Toribio Rodríguez de Mendoza (Table 1).

Molecular analysis

Sample preparation and extraction

A stereoscope (Labtech, Linitron) was used to thoroughly clean the specimens of black walnut *J. neotropica*. Then, small 3 cm^2 fragments were cut and deposited in 1.5 mL microtubes, immersed in liquid nitrogen and crushed using freezing and crushing equipment (Disruptor SK-10, Japan). Genomic DNA extraction from the specimens was performed using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The quality of the extracted DNA was determined on a 1% agarose gel and by fluorometric quantification with

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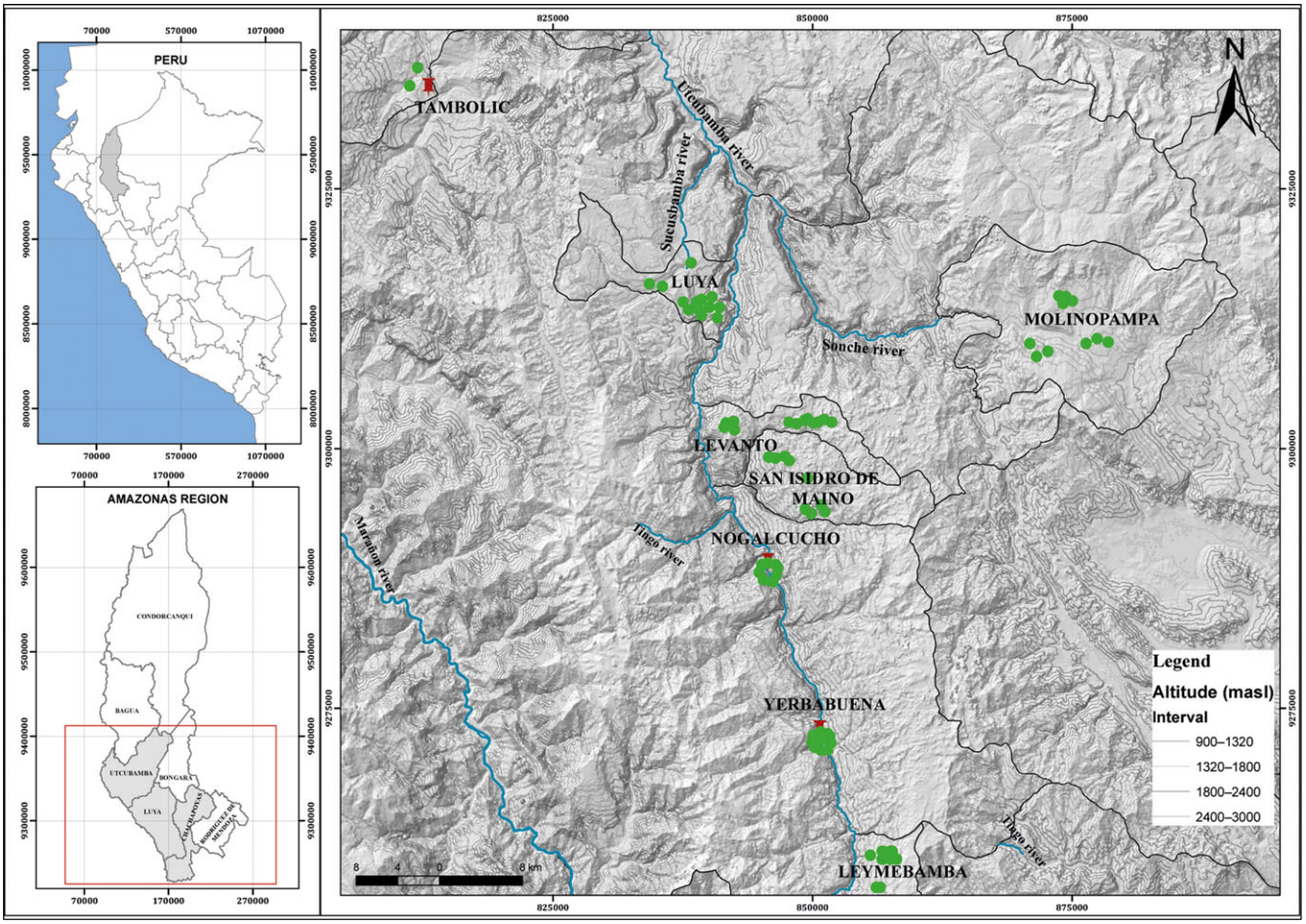

Figure 1. Collection sites for black walnut *J. neotropica* specimens in the Amazonas region, northern Peru.

Quantus™ (Promega, Madison, United States). Finally, the extracted DNA was stored in a freezer at –80 °C for preservation.

DNA amplification and purification

Preliminary analysis revealed the problematic topology observed in *Juglans* species when using the coding (*rbc*L, *mat*K) and intergenic markers (*trn*T-*trn*F, *psb*A-*trn*H) (Figures A.1–A.5). In addition to the better phylogenetic resolution of the *trn*S-*trnf*M marker (Figure A.6), it is one of the most commonly used genes for plants in genetic analysis (Aradhya *et al.* 2007; Minami *et al.* 2009). Accordingly, the intergenic marker *trn*S-*trnf*M was amplified with primers 2F (5′-CGGAGCTATCAACCACTCGG-3′) and 539R (5′-ACTCGACCAACCATCAGGAG-3′) (Aradhya *et al.* 2007). Polymerase chain reaction (PCR) was performed in a 10 µL reaction mixture containing 5.0 µL of master mix, 2.6 µL of distilled water, 0.2 µL of each primer (forwards or reverse) and 2 µL of genomic DNA (Deng *et al.* 2020). The PCR protocols for the marker were as follows: 1 cycle of predenaturation at 94 °C for 5 minutes, 40 cycles of denaturation at 94 °C for 45 seconds, alignment at 62 °C for 1 minute, extension at 72 °C for 2 minutes, and a final extension cycle at 72 °C for 7 minutes. The obtained amplicons were visualized by electrophoresis in 1% agarose gels in buffer (0.045 M Tris-borate; 0.001 M EDTA) for 15 minutes at 100 V (Porth and El-Kassaby 2014). The clear banded amplicons were purified with the Zymo Reseach DNA Clean & ConcentratorTM-5 cleaning kit (Zymo, California, United States) following the manufacturer's instructions and were sequenced commercially by Macrogen (Seoul, South Korea).

Phylogenetic analysis

A phylogenetic analysis was performed using the 74 specimens collected of the black walnut *J. neotropica* and 49 sequences downloaded from GenBank database (length of 502 nucleotides) (Figure 2). The software PartitionFinder v. 2.1.1 was used to select the evolutionary model (Coates *et al.* 2018). The reconstruction of the tree was performed using the maximum likelihood (ML) method with RaxML GUI v. 2.0.0 beta 10 considering a bootstrap of 1000 replicates (Rosenfeld *et al.* 2019, Tineo *et al.* 2020). For this analysis, the species *Juglans neotropica* (AY293368, Ecuador) was not included because it was grouped outside the taxonomic genus.

Genetic diversity and haplotype network construction

Genetic diversity was obtained by calculating the frequencies of distribution via DnaSP v6 software (Librado and Rozas 2009), which calculated the number of haplotypes (h), the diversity of haplotypes (Hd), and the diversity of nucleotides (π). In addition, a heatmap was constructed based on pairwise distances (Magige *et al.* 2022). The values of the genetic distances were obtained using the MEGAX program and then exported using the pheatmap extension to R software (R Development Core Team 3.4.2).

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Table 1. List of black walnut *J. neotropica* samples collected in the Amazonas region, northern Peru

						Coordinates		
GenBank code	Herbarium Code	Collection code	District	Province	Altitude (masl)	East	North	UTM
PP349511	KUELAP-4461	Lev1	Levanto	Chachapoyas	2319	175712	9302901	18
PP349510	KUELAP-4462	Lev2	Levanto	Chachapoyas	1725	175258	9302132	18
PP349521	KUELAP-4463	Lev3	Levanto	Chachapoyas	1731	174956	9301832	18
PP349509	KUELAP-4464	Lev4	Levanto	Chachapoyas	1735	175655	9300476	18
PP349512	KUELAP-4465	Lev5	Levanto	Chachapoyas	1735	175699	9300410	18
PP349513	KUELAP-4466	Lev6	Levanto	Chachapoyas	1750	175775	9300407	18
PP349508	KUELAP-4467	Lev7	Levanto	Chachapoyas	1739	175756	9300245	18
PP349507	KUELAP-4468	Lev8	Levanto	Chachapoyas	1740	175749	9300120	18
PP349523	KUELAP-4469	Lev9	Levanto	Chachapoyas	1738	175748	9300335	18
PP349491	KUELAP-4470	Lev10	Levanto	Chachapoyas	1736	175894	9299905	18
PP349490	KUELAP-4471	Lev11	Levanto	Chachapoyas	1740	175661	9303565	18
PP349522	KUELAP-4472	Lev12	Levanto	Chachapoyas	1742	175662	9303565	18
PP349489	KUELAP-4473	Ley1	Leymebamba	Chachapoyas	2413	190628	9255868	18
PP349535	KUELAP-4474	Ley2	Leymebamba	Chachapoyas	2413	190630	9255866	18
PP349488	KUELAP-4475	Ley3	Leymebamba	Chachapoyas	2420	190662	9255818	18
PP349487	KUELAP-4476	Ley4	Leymebamba	Chachapoyas	2422	190662	9255783	18
PP349516	KUELAP-4477	Ley5	Leymebamba	Chachapoyas	2413	190659	9255948	18
PP349480	KUELAP-4478	Ley6	Leymebamba	Chachapoyas	2413	190631	9255996	18
PP349479	KUELAP-4479	Ley7	Leymebamba	Chachapoyas	2410	190695	9256074	18
PP349475	KUELAP-4480	Ley8	Leymebamba	Chachapoyas	2406	190740	9256135	18
PP349474	KUELAP-4481	Ley9	Leymebamba	Chachapoyas	2314	190167	9256494	18
PP349505	KUELAP-4482	Ley10	Leymebamba	Chachapoyas	2303	190443	9256621	18
PP349530	KUELAP-4438	Luy1	Luya	Luya	2367	176259	9317320	18
PP349519	KUELAP-4439	Luy2	Luya	Luya	2400	175341	9317719	18
PP349520	KUELAP-4440	Luy3	Luya	Luya	2385	176247	9316589	18
PP349531	KUELAP-4441	Luy4	Luya	Luya	2311	174588	9318924	18
PP349499	KUELAP-4442	Luy5	Luya	Luya	2303	173919	9319322	18
PP349532	KUELAP-4443	Luy6	Luya	Luya	2308	175304	9316719	18
PP378486	KUELAP-4444	Luy7	Luya	Luya	2301	173754	9318669	18
PP349498	KUELAP-4445	Luy8	Luya	Luya	2307	173914	9317893	18
PP349518	KUELAP-4446	Luy9	Luya	Luya	2342	173726	9317615	18
PP349497	KUELAP-4447	Luy10	Luya	Luya	2339	174084	9317048	18
PP349517	KUELAP-4448	Luy11	Luya	Luya	2336	173741	9318701	18
PP349496	KUELAP-4449	Luy12	Luya	Luya	2340	173728	9318725	18
PP349515	KUELAP-4450	Luy13	Luya	Luya	2336	173760	9318920	18
PP349533	KUELAP-4451	Mol1	Molinopampa	Chachapoyas	1790	202275	9308646	18
PP349495	KUELAP-4452	Mol2	Molinopampa	Chachapoyas	2406	203558	9311089	18
PP349494	KUELAP-4453	Mol3	Molinopampa	Chachapoyas	2372	206566	9311688	18
PP349534	KUELAP-4454	Mol4	Molinopampa	Chachapoyas	2342	206566	9311688	18
PP349493	KUELAP-4455	Mol5	Molinopampa	Chachapoyas	2410	203736	9312776	18
PP349492	KUELAP-4456	Mol6	Molinopampa	Chachapoyas	2047	200765	9310140	18
PP349486	KUELAP-4457	Mol7	Molinopampa	Chachapoyas	2015	203859	9309997	18

(*Continued*)

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Table 1. (*Continued*)

GenBank code	Herbarium Code	Collection code	District	Province	Altitude (masl)	Coordinates		
						East	North	UTM
PP349476	KUELAP-4458	Mol8	Molinopampa	Chachapoyas	1991	204884	9312264	18
PP349473	KUELAP-4459	Mol9	Molinopampa	Chachapoyas	2004	202303	9313973	18
PP349502	KUELAP-4460	Mol10	Molinopampa	Chachapoyas	1936	203006	9313074	18
PP349524	KUELAP-4483	Nog1	Nogalcucho	Chachapoyas	1848	181863	9289332	18
PP349527	KUELAP-4484	Nog2	Nogalcucho	Chachapoyas	1843	181865	9289291	18
PP349528	KUELAP-4485	Nog3	Nogalcucho	Chachapoyas	1837	181852	9289140	18
PP349529	KUELAP-4486	Nog4	Nogalcucho	Chachapoyas	1837	181886	9289113	18
PP349525	KUELAP-4487	Nog5	Nogalcucho	Chachapoyas	1827	181851	9289139	18
PP349526	KUELAP-4488	Nog6	Nogalcucho	Chachapoyas	1829	182014	9289290	18
PP349500	KUELAP-4489	Nog7	Nogalcucho	Chachapoyas	1822	182058	9289303	18
PP349481	KUELAP-4490	Nog8	Nogalcucho	Chachapoyas	1821	182090	9289380	18
PP349506	KUELAP-4503	May1	San Isidro de Maino	Chachapoyas	1802	182658	9294197	18
PP349514	KUELAP-4504	May2	San Isidro de Maino	Chachapoyas	1806	183450	9294433	18
PP349485	KUELAP-4505	May3	San Isidro de Maino	Chachapoyas	1800	183494	9293866	18
PP349484	KUELAP-4506	May4	San Isidro de Maino	Chachapoyas	1801	183858	9294022	18
PP349483	KUELAP-4507	May5	San Isidro de Maino	Chachapoyas	1807	184904	9293531	18
PP349482	KUELAP-4508	May6	San Isidro de Maino	Chachapoyas	1812	184378	9293459	18
PP349501	KUELAP-4509	May7	San Isidro de Maino	Chachapoyas	1821	184354	9293658	18
PP349504	KUELAP-4510	May8	San Isidro de Maino	Chachapoyas	1860	184105	9294258	18
PP349503	KUELAP-4511	May9	San Isidro de Maino	Chachapoyas	1828	182145	9294481	18
PP349472	KUELAP-4491	Yer1	Yerbabuena	Chachapoyas	1846	187971	9272493	18
PP349471	KUELAP-4492	Yer2	Yerbabuena	Chachapoyas	1835	187486	9273612	18
PP349470	KUELAP-4493	Yer3	Yerbabuena	Chachapoyas	1837	188327	9273657	18
PP349469	KUELAP-4494	Yer4	Yerbabuena	Chachapoyas	1831	188267	9271701	18
PP349468	KUELAP-4495	Yer5	Yerbabuena	Chachapoyas	1829	187787	9273973	18
PP349467	KUELAP-4496	Yer6	Yerbabuena	Chachapoyas	1830	188019	9273174	18
PP349466	KUELAP-4497	Yer7	Yerbabuena	Chachapoyas	1827	188796	9271123	18
PP349465	KUELAP-4498	Yer8	Yerbabuena	Chachapoyas	1831	188794	9271123	18
PP349478	KUELAP-4499	Yer9	Yerbabuena	Chachapoyas	1830	188774	9272381	18
PP349477	KUELAP-4500	Yer10	Yerbabuena	Chachapoyas	1830	188774	9272381	18
PP349464	KUELAP-4501	Tam1	Tambolic	Utcubamba	2258	148011	9336263	17
PP349463	KUELAP-4502	Tam2	Tambolic	Utcubamba	2313	145917	9333615	17

Connectivity analyses between the haplotypes of the populations of the Amazonas region and of the other countries were carried out by means of the median junction network (MJ) method and PopArt v1.7 software (Xu *et al.* 2021) (Table A.1).

Molecular variance analysis

The Analysis of Molecular Variance (AMOVA) was performed using Arlequin 3.11 software (Excoffier *et al.* 2005) to determine the distribution of variance and the level of significance among walnut populations and to determine the source of genetic variation.

Results

Phylogenetic analysis

Phylogenetic analysis of the Juglandaceae family, which includes *Cyclocarya paliurus* as external group, revealed the grouping of three taxonomic sections (Cardicaryon, Juglans, and Rhysocaryon). The Rhysocaryon section encompassed eight species including the specimens from Amazonas region (*J. cinerea, J. guatemaiensis, J. hindsii, J. major, J. microcarpa, J. neotropica, J. nigra,* and *J. olanchana*). Additionally, the Cardiocaryon (*J. ailanthifolia, J. californica, J. cathayensis, J. hopeiensis* and *J. mandshurica*) and Juglans (*J. olanchana, J. regia* and *J. sigillata*) sections were composed of five and three species, respectively.

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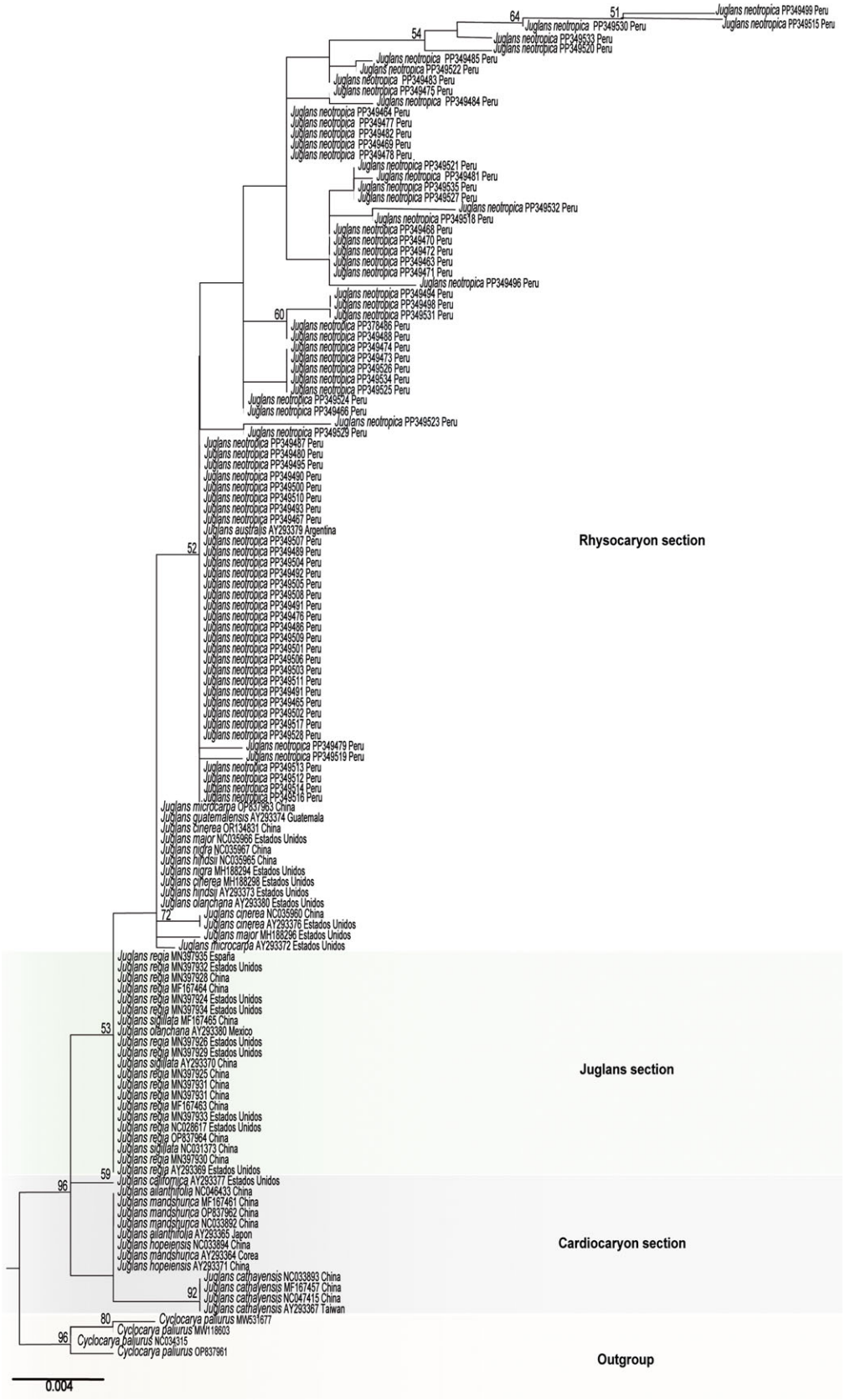

Figure 2. Phylogenetic tree based on the maximum likelihood analysis of the *trn*S-*trn*fM marker for sections of the genus *Juglans*. Maximum likelihood bootstrap values are indicated in the branches. The scale indicates the number of nucleotide substitutions per site. The specimens generated in this study are in bold.

Genetic divergence

Interspecific genetic divergences based on pairwise distances of *Juglans* individuals collected in the Amazonas region and in Argentina (AY293379), China (AY293370, MF167461, KX671977, NC047415), Ecuador (AY293368), Spain (MN397935), Guatemala (AY293374), Japan (AY293365), Mexico (AY293380), the United

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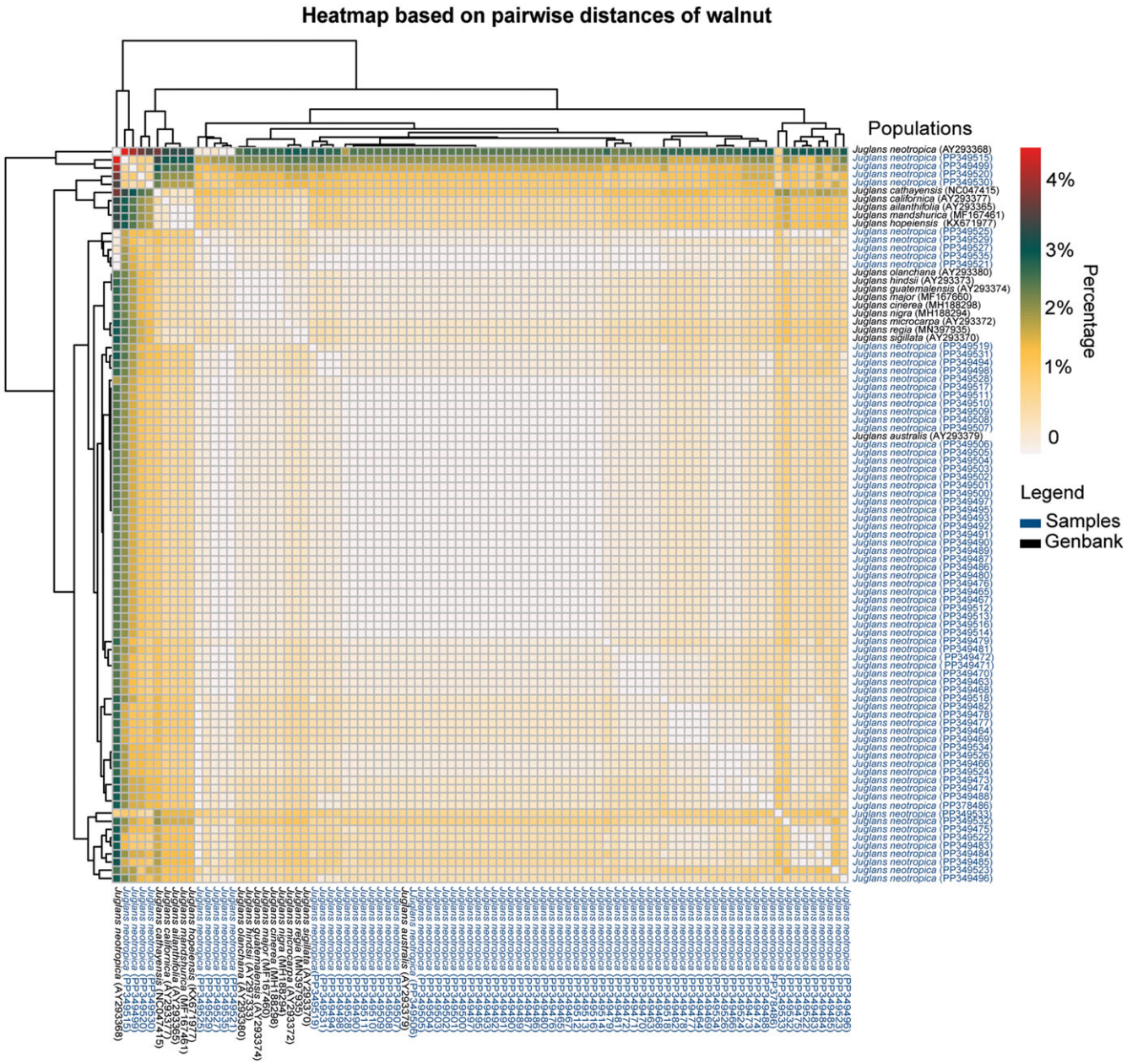

Figure 3. Heatmap based on pairwise distances (p-distance) of black walnut *J. neotropica* populations from the Amazonas region and foreign populations.

States (AY293377, AY293373, MF167460, MH188298, MH188294, AY293372) ranged from 0 to 5%.

The greatest genetic distance was detected between the Luya population and the Ecuador population (AY293368) (4.75%). On the other hand, the lowest genetic divergence (0.19%) was obtained between the populations of Molinopampa, Tambolic, and Yerbabuena and the populations of Argentina (AY293379), the United States (MH188298, MF167460), and Guatemala (AY293374), respectively. Considering only the samples from the Amazonas region (intraspecific genetic divergence), the most divergent populations were Luya and Molinopampa (1.62–2.64% of p-distance), while the most similar populations were Levanto, Molinopampa, San Isidro de Maino and Yerbabuena (0.19%) (Figure 3).

Genetic diversity

The nucleotide compositions of the specimens from the Amazonas region were A = 30.88%, G = 17.33%, C = 14.54%, and T = 37.25%. The data from the Amazonas region showed a mean haplotype diversity (Hd) of 0.57 and a relatively low nucleotide diversity (Pi) of 0.0035 (Table 2). Luya stood out among all populations since it was i) the population with the greatest number of haplotypes ($h = 7$), ii) the population with the greatest number of polymorphic sites ($s = 11$), iii) the population with the greatest number of diverse nucleotides (Pi = 0.01), and iv) the population with the greatest average number of nucleotide differences per pair ($k = 3.97$). On the other hand, the population with the greatest diversity of haplotypes was Tambolic (H = 1.00) (Table 2).

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Table 2. Genetic diversity of black walnut *J. neotropica* trees in the Amazonas region. n: number of samples; h: number of haplotypes; s: number of polymorphic sites; Hd: diversity of haplotypes; Pi: nucleotide diversity; K: average number of nucleotide differences per pair

Populations	N	h	s	Hd	Pi	k	Tajima´s D
Levanto	12	3	5	0.31818	0.00200	0.83333	−1.89423
Leymebamba	10	3	2	0.60000	0.00160	0.66667	−0.65748
Luya	13	7	11	0.73077	0.00955	3.97436	0.09127
Molinopampa	10	3	5	0.41111	0.00278	1.15556	−1.38818
Nogalcucho	8	3	2	0.67857	0.00189	0.78571	0.06935
San Isidro de Maino	9	3	2	0.66667	0.00227	0.94444	0.97505
Tambolic	2	2	1	1.00000	0.00240	1.00000	0.00000
Yerbabuena	10	2	1	0.53333	0.00128	0.53333	0.98627
Total	74	12	12	0.57127	0.00350	1.45428	−0.22724

Tajima's D test revealed negative values ($P < 0.05$) for the Levanto, Molinopampa, and Leymebamba populations. This would indicate that these populations recently expanded after a selective sweep (Ebrahimi *et al.* 2017). For the other populations of Luya, Nogalcucho, San Isidro de Maino, Tambolic, and Yerbabuena, the Tajima's D test values were positive, indicating balanced selection (Magige *et al.* 2022).

Haplotype network analysis

The haplotype network based on the median-joining network analysis of the eight populations of *J. neotropica* from the Amazonas region ($n = 74$) allowed the identification of three groups. Group I was the most diverse and representative, comprising 7 haplotypes: the first haplotype (H1) included 46 individuals from the 8 populations; haplotype 2 (H2) included 16 individuals from the Leymebamba populations (3), Luya (1), Molinopampa (2), Nogalcucho (3), San Isidro de Maino (2), Tambolic (1) and Yerbabuena (4); and haplotype 3 (H3) included only 2 individuals from the populations of Levanto (1) and San Isidro de Maino (1). Additionally, group I was composed of 4 unique haplotypes from Levanto (1), Leymebamba (1), Luya (1), and Nogalcucho (1). Group II consisted of 2 unique haplotypes from Luya (1) and Molinopampa (1). Group III was composed of three unique haplotypes belonging to Luya individuals (Figure 4).

The second network was composed of a total of 124 individuals. The formation of two additional groups (Group IV and Group V) was observed. Group IV consisted of two haplotypes (H14, H18), and its individuals came from only Asian populations (China, Korea, Japan, Taiwan) (Figure 5, Table A.2). Group V included 5 haplotypes (H13, H15, H16, H17, H19), of which haplotype 19 was the most predominant and consisted of 35 individuals from populations from China (NC035960, NC035967, NC035965, OR134831, OP837963, MN397931, MN397930, MN397928, MN397925, MF167465, MF167464, MF167463, NC031373, OP837964, AY293370), Guatemala (AY293374), Mexico (AY293380), Spain (MN397935) and the United States (MH188296, MH188298, MH188294, NC035966, AY293373, MN397934, MN397933, MN397932, MN397929, MN397927, MN397926, NC028617, AY293369, MN397924, AY293372, AY293376, AY293377).

Genetic structure of populations

The global fixation index (Fst), based on the calculation of correlation values between genetic distances, allowed the estimation of connectivity values, and its values ranged from 0–0.05 (low connectivity), to 0.05–0.15 (moderate connectivity), and to 0.15–0.25 (high connectivity) (Aguirre-Pabon *et al.* 2022). In the Amazonas region, a value of 0.04 was obtained, which represents a low level of genetic connectivity between populations. The populations with the highest level of genetic connectivity were Leymebamba – Levanto (Fst = 0.10052), Leymebamba – Molinopampa (Fst = 0.10077), Nogalcucho – Levanto (Fst = 0.07791) and San Isidro de Maino – Leymebamba (Fst = 0.08939). The populations with the lowest level of genetic connectivity were Yerbabuena with the following populations: Nogalcucho (Fst = −0.33397), Luya (Fst = −0.37748), Molinopampa (Fst = −0.34328), Leymebamba (Fst = 0.15559), San Isidro de Maino (Fst = −0.30178) and Tambolic (Fst = −0.22845) (Figure 6, Table A.3).

The AMOVA at the population level in the Amazonas region revealed that most of the genetic structuring and/or differentiation occurred within the populations (96.59%, $P < 0.05$, Fst = 0.03940), while the lowest percentage of genetic variation occurred between populations (3.41%, $P < 0.05$, Fst = 0.02843) (Table 3).

Discussion

Phylogenetic analysis

In Peru, *Juglans neotropica* is distributed mainly in the Amazonas, Cajamarca, Cusco, Huancavelica, Junín, La Libertad, Lambayeque, and Pasco regions (Hurtado Manrique *et al.* 2015). The phylogenetic analysis of this study confirmed the presence of the black walnut *J. neotropica* in the Amazonas region and resolved the diversity of the family Juglandaceae in lineages that correspond to three taxonomic sections (Cardyocarion, Juglans, and Rhysocaryon). The lineage of the Rhysocaryon section, which is mainly endemic to North and Central America, included eight taxa (*J. cinerea*, *J. guatemaiensis, J. hindsii, J. major, J. microcarpa, J. neotropica, J. nigra,* and *J. olanchana*) (Fitz-Gibbon *et al.* 2023). The specimens of *J. neotropica* from the Amazonas region formed numerous small lineages that could be grouped in a sibling section to Rhysocaryon and probably different from the other two sections. However, the use of only one marker does not fully support this hypothesis. Therefore, further analysis including multiple markers and additional genomic information is strongly recommended. Additionally, the sequence of *J. australis* (AY293379 from Argentina) was grouped with samples of *J. neotropica* from the Amazonas region. This would suggest that *J. australis* would

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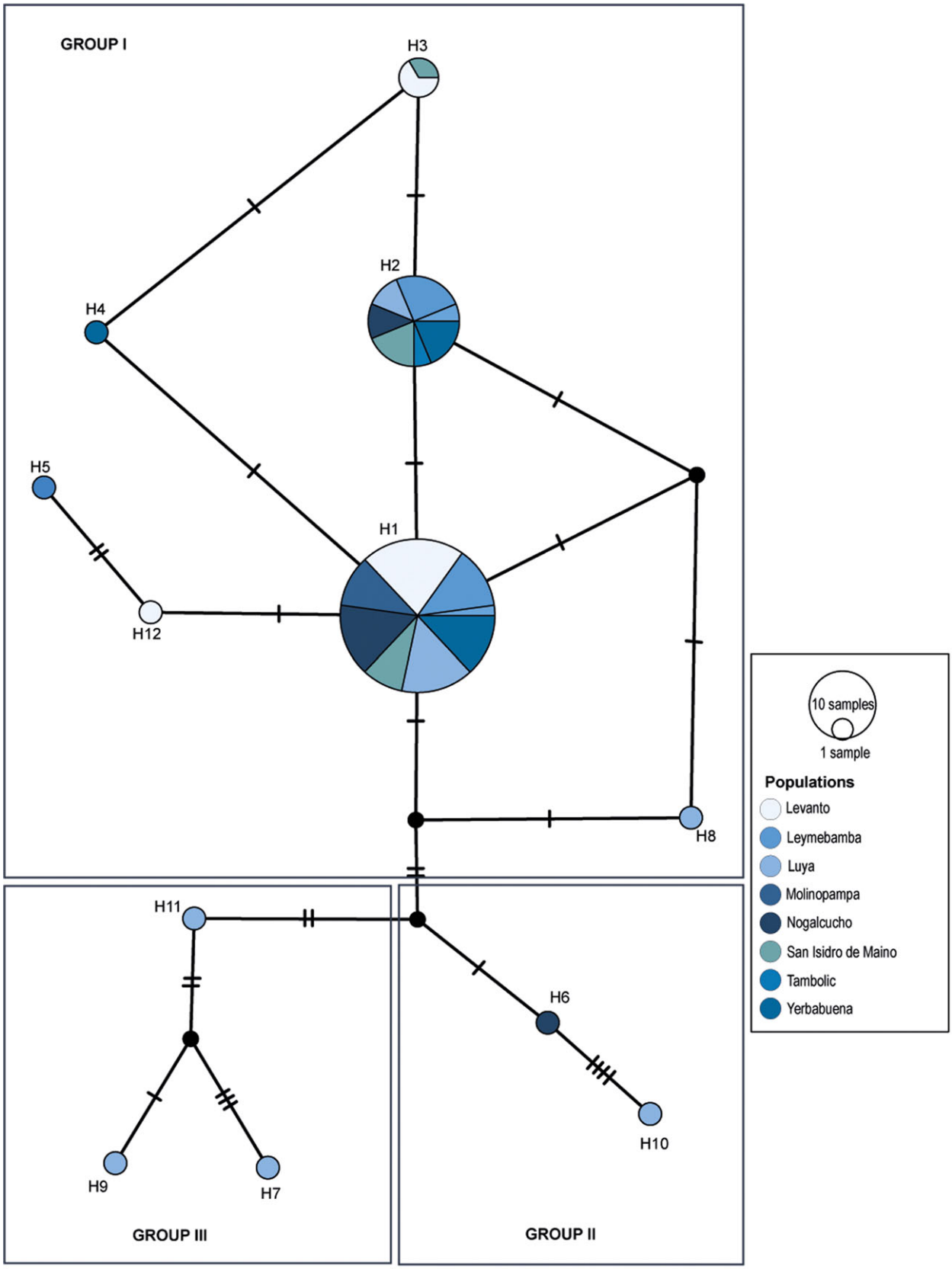

Figure 4. Haplotype network of black walnut *J. neotropica* populations in the Amazonas region. The populations are represented by colours. The lines perpendicular to the branches represent the mutational points. H: haplotype.

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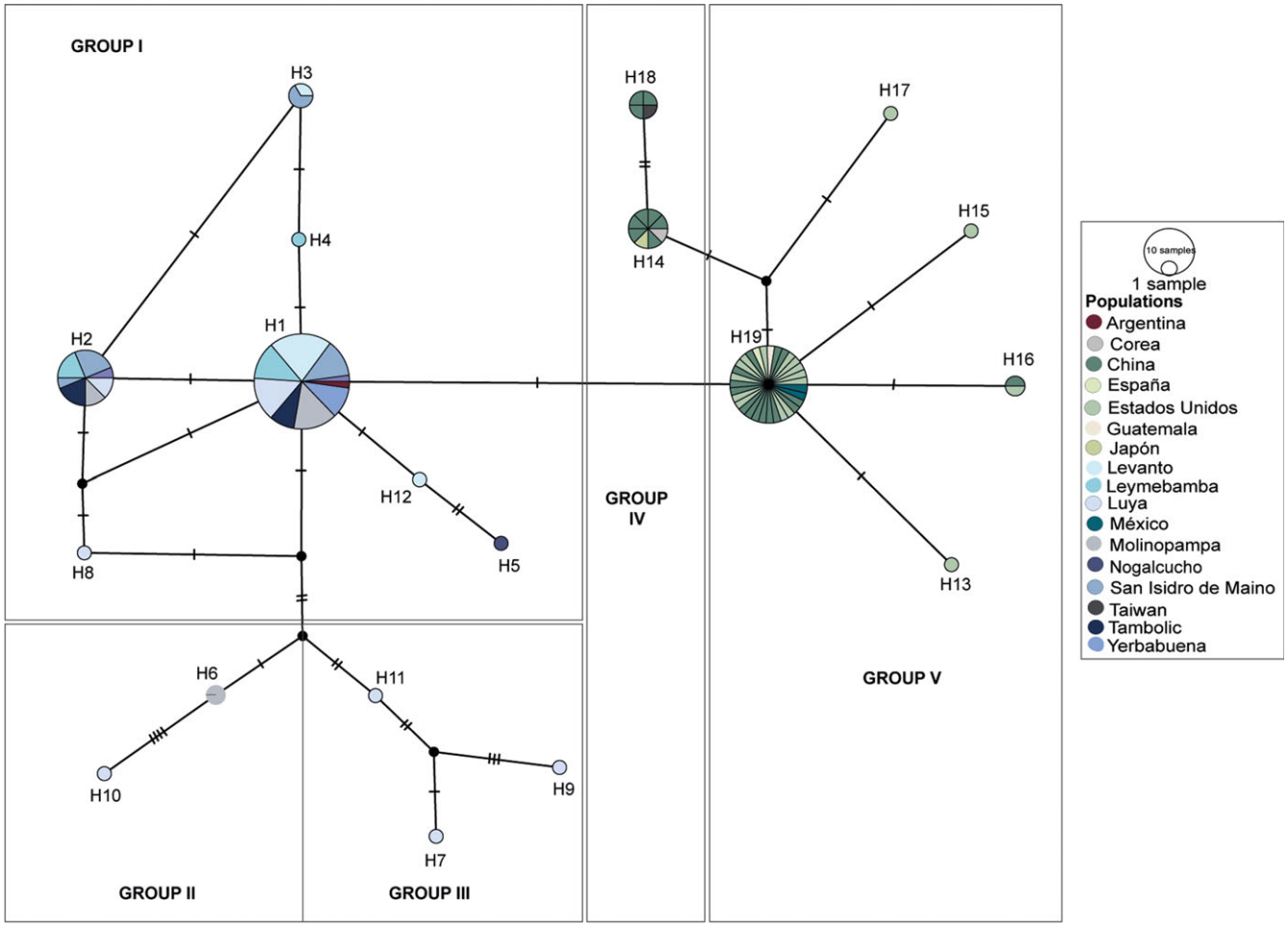

Figure 5. Haplotype network of black walnut *J. neotropica* populations from the Amazonas region and other countries (74 from the Amazonas region and 51 additional individuals). The lines perpendicular to the branches represent the mutational points. H: haplotype.

belong to the Rhysocaryon section. However, type materials from *J. australis* must be sequenced to confirm their correct phylogenetic position (Manning 1960, GBIF 2023). The lineage in the Cardyocarion section included four species native to East Asia (*J. ailanthifolia, J. cathayensis, J. hopeiensis* and *J. mandshurica*) and one from Europe (*J. californica*) (Aradhya *et al.* 2007, Dong *et al.* 2017). The lineage of the *Juglans* section, known as the Persian or cultivated English walnut, was composed of three species (*J. olanchana*, *J. regia,* and *J. sigillata*), which are distributed from southeastern Europe to China and the Himalayas (Ren *et al.* 2023).

Genetic divergence

In the Amazonas region, the genetic divergence based on the p-distance of the black walnut *J. neotropica* was less than 3%, unlike that of the populations of *Juglans* spp. from Europe, Asia and North America, which varied by 2%–5.2% (Ebrahimi *et al.* 2017, Magige *et al.* 2022). The most divergent populations in the Amazonas region were those of Luya and Molinopampa (1.62% – 2.64% of p-distance). This could be because the flow of genes begins to change in response to genetic and geographical barriers (Vischi *et al.* 2017). For example, Molinopampa is located 63 km from the Luya and contains populations of *Juglans neotropica* with a low level of genetic diversity and a high level of historic threat due mainly to livestock, excessive agriculture, grazing and over-exploitation (Oliva *et al.* 2016). Additionally, Molinopampa and Luya are populations historically separated by two rivers (i.e., Utcubamba River and Sonche River) and a mountain range (i.e., Huancas). Consequently, we recommend integrated *in situ* conservation strategies for Molinopampa and Luya, including seed banking and habitat corridor establishment, to safeguard the genetic integrity of *J. neotropica*. On the other hand, the least divergent populations were Levanto, Molinopampa, San Isidro de Maino, and Yerbabuena (0.19%). The genetic similarity between Levanto and San Isidro de Maino is explained by their geographical proximity (8 km) (Alarcón-Méndez *et al.* 2023). However, the localities of Yerbabuena and Molinopampa have a wider geographical distance (104 km), so genetic similarity could be explained by other factors, such as i) a common gene pool, ii) previous genetic connections of areas of distribution that were lost, or iii) possible anthropogenic or natural transport (Gaisberger *et al.* 2020).

Genetic diversity

Despite the reduced geographic space analysed in this study, the mean haplotype diversity in the Amazonas region (Hd = 0.57) was slightly lower than those in other studies conducted in *Juglans*. For example, Ebrahimi *et al.* (2017) obtained a value of Hd = 0.61 for

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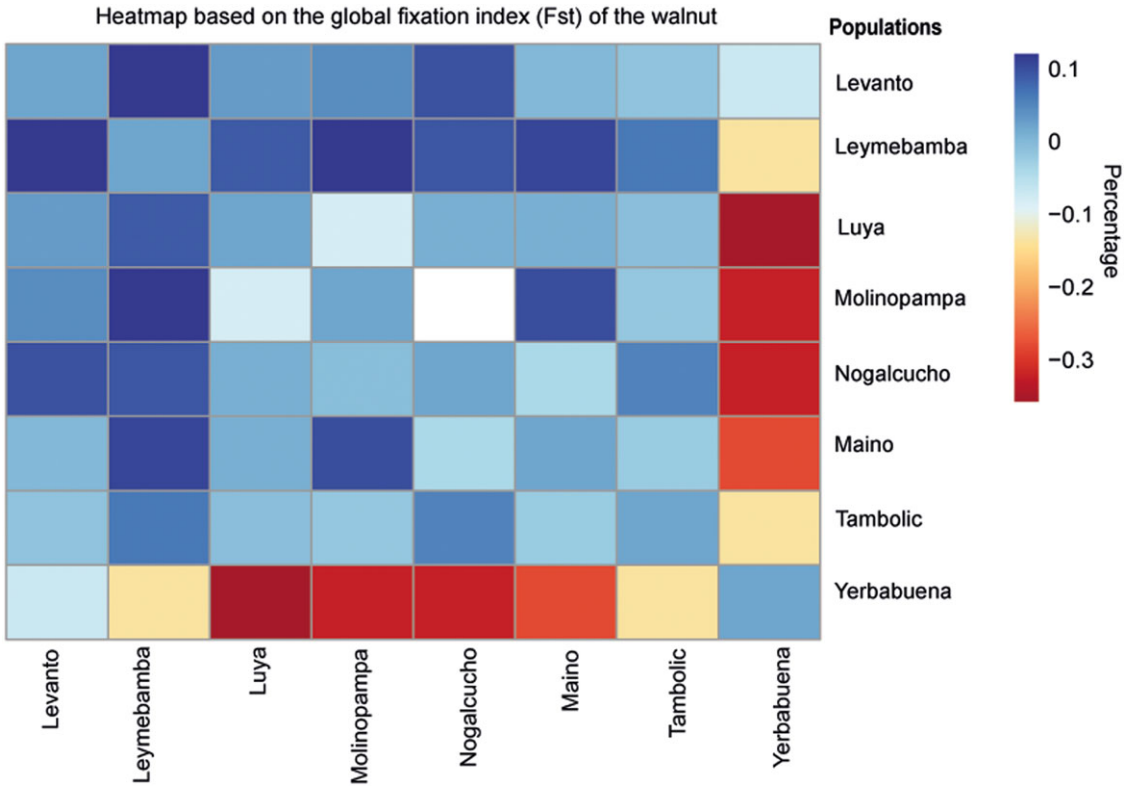

Figure 6. Heatmap based on the global fixation index (Fst) of the black walnut *J. neotropica* populations of the Amazonas region.

specimens from Europe, while Dangl *et al.* (2005) reported Hd = 0.60 for specimens from Asia, Europe, and North America. This suggests a relatively high genetic diversity within *Juglans neotropica* in the Amazonas region. This diversity is greater than in the genetically diverse common walnut from the Iranian Plateau (Shahi Shavvon *et al.* 2023). It is also confirmed by the high nucleotide diversity (Pi = 0.004) of *Juglans* in the Amazonas region compared to those found in specimens from Asia (Pi = 0.002) (Hu *et al.* 2017). For instance, in the population of Luya, nucleotide diversity (Pi = 0.009) and haplotype diversity ($h = 7$) were the highest in the Amazonas region. The observed high Hd and high Pi suggest either secondary contact of differentiated populations or long evolutionary history in a large stable population (Grant and Bowen 1998). The second scenario is supported by the haplotype network which shows star-like arrangement for H1 and H2, being a signature of prolonged demographic stability (Grant and Bowen 1998). In Amazonian trees, such signatures often reflect Pleistocene refugia populations (Dexter *et al.* 2018).

The negative values of Tajima's D for the Levanto, Leymebamba, and Molinopampa populations are caused by a sudden reduction in population size, which could be related to a possible bottleneck or a selective sweep (Ebrahimi *et al.* 2017). These populations are constantly facing threats such as livestock, excessive agriculture, grazing and overexploitation (Casiano 2015, Oliva *et al.* 2016, Cachay 2021), which lead to the constant loss of habitat, causing fragmentation and, therefore, loss of genetic material (Vahdati *et al.* 2015). On the other hand, the populations of Luya, Nogalcucho, San Isidro de Maino, and Yerbabuena presented positive values, indicating balanced selection (Muriira *et al.* 2018). This is because these populations do not present many threats due to their isolated locations and access difficulties and thus generate an increase in genetic diversity. This pattern may reflect ecological mechanisms such as (i) frequency-dependent pollinator foraging, where Andean bees and hummingbirds preferentially visit rare floral morphs (Eckhart *et al.* 2006), and (2) rodent-mediated seed dispersal, a process previously documented in the closely related *J. mandshurica* (Wang *et al.* 2017). These results coincide with what was found by Sun *et al.* (2019), who mentioned that there could be a greater connection of diversity in places where there is a lower incidence of human activities; therefore, these areas with fewer ecosystem disturbances lead to better adaptability with balanced selection and greater gene flow (Magige *et al.* 2022).

Haplotype network analysis

The haplotype network of the individuals of the populations of *J. neotropica* from the Amazonas region was divided into 3 groups, comprising predominantly individuals from the population of Luya, with the presence of 7 haplotypes distributed in the three groups (H1, H2, H7, H8, H9, H10, H11). This high frequency of haplotypes could be caused by adaptation to changes in habitats or by probable fragmentation or isolation over time (Shigaeva and Darr 2020, Gaisberger *et al.* 2020). However, the hypothesis of a recent separation in small, fragmented populations is discarded because the results of genetic diversity yielded a balanced selection of populations of *Juglans neotropica* in the population of Luya (0.09). Additionally, the prevalence of haplotypes H1 and H2, displaying structural patterns characteristic of expanding populations in Amazonas, indicates their likely ancestral origin. The negative Tajima's D values observed in our analysis provide statistical support for this conclusion.

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Table 3. Analysis of molecular variance (AMOVA) of the black walnut *J. neotropica* populations in the Amazonas region

Variation source	df	Sum of squares	Variance components	Percentage of variation	Fixation index
Between populations	3	3.739	0.02669	3.41	0.02843
Within populations	69	52.644	0.76269	96.59	0.03940
Total	72	56.384	0.78993		

The inclusion of new individuals from other populations outside the Amazonas revealed the differentiation of populations present in the Amazonas region with respect to the groups formed by specimens from other countries (e.g., groups IV, V). This difference could be related to genetic groups not previously reported, in addition to the scarce molecular information on genus *Juglans* in the Southern Hemisphere. For instance, group IV is exclusively distributed in Asia (Hu *et al.* 2017), while group V is more dispersed throughout the Northern Hemisphere. The individuals in group IV are impacted by geographic barriers, cultivated material or local autochthonous plants that restrict their distribution (Magige *et al.* 2022), while the wide distribution of group V is a consequence of possible recent human or natural transport of propagules at long distances (Gaisberger *et al.* 2020).

Additionally, the three groups identified in *Juglans neotropica* from the Amazonas region could probably represent three distinct taxa based on the high genetic divergence and haplotype grouping. Nevertheless, the use of a unique maker in this study hampers the confirmation of this finding. As previously stated, a multilocus approach and plastome data are crucial for the establishment of this potentially new taxa (Mao *et al.* 2014).

Genetic structure of populations

AMOVA revealed greater genetic variation in partitioning within populations (Fst = 0.04), a common scenario, particularly for species with a cross-breeding system such as walnut (Magige *et al.* 2022). The AMOVA results were consistent with the differences between the populations (Fst = 0.02), which represents a low level of connectivity (Aguirre-Pabon *et al.* 2022). This could be due to fragmentation of the habitat caused by anthropogenic activities in the region (Zhou *et al.* 2021). Habitat fragmentation limits or prevents long-distance pollination events, resulting in pollination only within groups of close relatives (Shigaeva and Darr 2020).

Conclusion

The use of the intergenic marker *trn*S-*trnf*M in specimens of the black walnut *J. neotropica* from the Amazonas region resolved them in the Rhysocaryon section. These specimens were found in a genetic group not previously reported and further genetic information (i.e., plastid genomes, multilocus phylogeny) could probably delimit candidates for new taxa in the genus *Juglans*. The genetic divergences of black walnut *J. neotropica* in the Amazonas region were slightly lower than those reported in Europe, Asia, and North America. Within the populations of the Amazonas region, the greatest genetic diversity was found in the Luya population and high divergence was detected in the populations of Molinopampa and Luya, probably due to the presence of genetic and geographical barriers, while the populations of Levanto and San Isidro de Mayno were less divergent due to their geographic proximity and greater genetic interaction. The populations of Levanto, Leymebamba and Molinopampa showed a reduction in population size, which could be linked to a selective sweep or a possible bottleneck, while the populations of Luya, Nogalcucho, San Isidro de Maino, Yerbabuena, and Tambolic showed balanced selection. Collectively, our findings indicate pronounced genetic variability alongside restricted gene flow between populations, a pattern consistent with anthropogenic habitat fragmentation. Consequently, we propose a multi-faceted *in situ* conservation strategy for Molinopampa and Luya to preserve the evolutionary potential of *Juglans neotropica*. This should integrate (i) targeted seed banking of genetically distinct populations to capture extant diversity, (ii) establishment of habitat corridors to facilitate gene flow between fragmented subpopulations, and (iii) community-based protection initiatives to mitigate anthropogenic pressures. Such measures are critical for maintaining both the species' adaptive capacity and the ecological functionality of these Andean forest ecosystems. Our study yields novel insights into *Juglans neotropica* conservation by empirically linking deforestation to genetic erosion and generating critical baseline genetic parameters for monitoring.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0266467425100102

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Competing interests. The authors declare none.

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