International Journal of Agriculture and Environmental Research

ISSN: 2455-6939

Volume:02, Issue:05

GENETIC DIVERSITY WITHIN AND BETWEEN ACCESSIONS OF *Elaeis oleifera* FROM THE ECUADORIAN AMAZON

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ABSTRACT

The species *E. oleifera* is a promising genetic resource for oil palm breeding programs. The main purpose of this study was to quantify the genetic diversity within and between accessions collected in the Ecuadorian Amazon. Nine microsatellite markers were genotyped in 40 plants from the *E. oleifera* germplasm bank of INIAP in Ecuador. The number of alleles varied from two to five, with a total of 26 alleles and a mean number of 2.89. The polymorphism information content was 0.35, indicating that all markers were informative and enough to access the variability within and between *E. oleifera* plants. The average inbreeding coefficient was -0.03, the mean expected heterozygosity 0.41, the average observed heterozygosity 0.42 and seven of the nine markers were in Hardy–Weinberg equilibrium (HWE). This result shows that the analyzed population was close to the assumed HWE, showing high variability between plants and no inbreeding or sampling effect. The 40 plants were clustered in seven groups differentiated by the Tocher method. Seedlings derived from a same accession were grouped separately, indicating variability within the sampled accessions. This variability was illustrated by grouping the 40 plants by the UPGMA method and confirmed by the molecular variance analysis (AMOVA). Of the total variation, 72% was detected among plants within the sampled accessions. These results have implications for breeding purposes and the collection of new *E. oleifera* germplasm.

Keywords: Caiaué, oil palm, microsatellite markers, Ecuador

ISSN: 2455-6939

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INTRODUCTION

The species *E. oleifera* is seen as a promising genetic resource for oil palm breeding programs, since it has some important traits, e.g., a low annual trunk growth rate, resulting in shorter trees and reduced operating costs. The species is also resistant to diseases that currently threaten the crop, as for example bud rot, known as fatal yellowing disease in Brazil and as "Pudrición del Cogollo" in the other countries of South America, which is the greatest problem for oil palm cultivation, killing palms on plantations of hundreds of hectares in Colombia, Brazil, Ecuador, Suriname, Costa Rica, Nicaragua, and Panama (Bergamin Filho et al., 1998, Renard et al., 1980; Hardon et al., 1985; Franqueville, 2001). Moreover, the content of unsaturated fatty acids of *E. oleifera* is high, liquefying the oil under natural conditions, allowing its use for the manufacturing of biodiesel (Hartley, 1988; Meunier, 1975). Although the two species are geographically isolated, compatible and fertile interspecific hybrids can be bred, with gains in oil content of up to 90%, aside from other desirable characteristics (Amblard et al. 1995).

Molecular markers are an important tool to quantify genetic variation and its distribution among and within populations (Robinson, 1998). Once they are not influenced by the environment, they can be used to determine genetic polymorphisms at any stage of the plant, cell or tissue development and provide more genetic information per locus in co-dominant markers (Faleiro, 2007).

Moretzsohn et al. (2002) estimated the genetic diversity of Brazilian germplasm collections and evaluated the genetic relationship between *E. guineensis* and *E. oleifera* with RAPD (Random Amplified Polymorphic DNA) markers. Barcelos et al. (2002) also studied the genetic diversity of *E. oleifera* and identified four distinct populations from the respective geographic origins Brazil, Peru, northern Central America, and Suriname/French Guiana. Additional studies showed the development of microsatellite markers (SSRs) and their application in the characterization of *E. oleifera* germplasm (Singh et al. 2008; Zaki et al., 2010). The results of Araya et al. (2009), who used eight microsatellites of the *E.oleifera* germplasm bank of the company ASD in Costa Rica for characterization, corroborated the findings of Barcelos et al. (2002) with regard to the four geographically distinct populations.

In the Amazon Rainforest, *E. oleifera* germplasm is usually found in small populations near rivers, in fertile and well-drained areas (Andrade, 1983; Barcelos, 1986). In Ecuador, because of the great concern about the bud rot disease that affects hundreds of hectares of commercial hybrids of *E. guineensis*, a source of resistance and other traits of interest are being sought by the INIAP (Instituto Nacional de Investigaciones Agropecuarias, Ecuador). At the experimental

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station Santo Domingo of INIAP, seeds from native *E. oleifera* plants were collected in three surveys in the Ecuadorian Amazon to establish a genebank of the species.

The purpose of this study was to quantify the genetic variability within and between *E. oleifera* accessions from the Ecuadorian Amazon by microsatellite markers.

MATERIAL AND METHODS

1. Plant material

We evaluated 16 *E. oleifera* accessions represented by 40 plants, of the germplasm bank of the experimental station Santo Domingo of INIAP, at km 38 of the connecting road Quinindé (latitude 79º20' West, 00º 06' North; 300 m asl). The germplasm was collected in the province of Morona Santiago, in the Amazon region Taisha, in 2004 and 2006.

2. DNA extraction

Genomic DNA was extracted from 20 g of young apical tissue of the central leaves of each plant and accession using a modified CTAB protocol (Grattapaglia and Sederoff, 1994) and sorbitol (Morillo, 2002). The quality and concentration (ng/uL) of DNA samples were analyzed by fluorescence with ethidium bromide (EtBr) by horizontal electrophoresis on 1% agarose gel, at the Experimental Station Santa Catalina of the Department of Biotechnology.

3. SSRs genotyping

The amplification was performed using a reaction cocktail with a final volume of 10 L at a concentration of 1X PCR buffer (500 mM Tris pH=8.5, 10mM KCl, 2mM MgCl2, 500 mg/ml BSA), 1µM dNTPS, 0.1µM primer Fw-M13, 0.3 µM primer Rv, 1µM M13-IrDye 700 u 800, 0.6 U Taq, and 30 ng DNA. Amplification was performed in a Biometra Tprofessional basic thermocycler (gradient model) with an initial denaturation cycle of 5 min at 95 ºC, followed by 25 amplification cycles at 94 ºC for 20 sec, 50 - 53.5 ºC for 30 sec and 72 ºC for 30 sec with a final extension cycle at 72 ºC for 3 min. Nine SSRs primers were used, described by Singh et al. (2008) and Norziha et al. (2008) in studies on oil palm.

4. Data logging

The data were recorded by SAGA-GT 4200-507, Version 3.3 (LI-COR BIOSCIENCES), which allows automatic genotyping by "allele fingerprinting" of a self-learning, background error-free algorithm, using the molecular weight ladder 350 bp size standard (LI-COR IRDye). By reading and debugging of information, a genotypic data matrix was constructed for each plant or sample

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with each SSR primer. When the marker was not amplified or generated difficult-to-interpret products, the missing data were considered, recorded as "?". Further amplifications were performed to reduce the amount of missing data to a maximum of three (genotypes/loci) per sample.

5. Statistical analyses

For the number of alleles of each marker, the frequencies, inbreeding coefficient (F) and the average polymorphic information content (PIC) were estimated.

For inbreeding, we used the estimator $F = 1 - \frac{H_{obs}}{H_{esp}}$, where:

H_{obs} = observed frequency of heterozygotes in a population subjected to mating/crossing of relatives given by 2pq-2ε, when considering only two alleles per locus and ε = pqF, where F is the inbreeding coefficient

H_{esp} = expected frequency of heterozygotes in a population supposedly in Hardy-Weinberg equilibrium (HWE) given by 2pq, when considering only two alleles per locus.

To estimate H_{esp}, the following expressions were used:

$$f(Homozygote_{SobHWE}) = \sum_{i=1}^{a} pi^2$$

$$f(Heterozygote_{SobHWE}) = H_{esp} = 1 - \sum_{i=1}^{a} pi^2$$

where:

a = total number of alleles;

p_i = frequency of the i^{th} allele of each SSR marker, with a alleles.

The average polymorphic information content was estimated as proposed by Botstein et al. (1980):

$$PIC = 1 - \sum_{i=1}^{a} pi^2 - \sum_{i,j=1}^{a} \sum_{i \neq j}^{a} p_i^2 p_j^2$$

The Hardy-Weinberg equilibrium of the estimated genotype frequencies for the nine markers was tested by Fisher's exact, the z and chi-square tests.

For the analysis of genetic diversity among accessions, the genotypic distance matrix was initially estimated by the weighted index (Cruz et al., 2011), where each element was expressed

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by the complement of the similarity index, considering the number of alleles common to each pair of genotypes, weighted by the total number of alleles per marker.

Subsequently, the accessions were grouped by the Tocher method. The hierarchical grouping method was also used, by the unweighted pair-group method based on arithmetic averages (UPGMA), to construct the dendrogram.

To quantify the variability between and within accessions, the analysis of molecular variance (AMOVA) was used by the model proposed by Excoffier et al. (1992), as shown below:

SV	DF	SS	MS	E(MS)	Φ_{ST}
Between accessions	a-1	SSb	MSb	σ_i^2+Ñσ_p^2	$\frac{\sigma_p^2}{\sigma_T^2}$
Within accessions	N-a	SSw	MSw	σ_i^2	
Total	N-1	SST	-	σ_T^2	

a: number of accessions, N: total number of plants; Ñ: harmonic mean of the number of plants per accessions $\tilde{N} = \frac{N-\sum_i \frac{N_i^2}{N}}{a-1}$; σ_i^2 : Variance in accession; σ_p^2: variance among accessions and σ_T^2: total variance.

All analyses were performed using the software package Genes (Cruz, 2006).

RESULTS AND DISCUSSION

The number of alleles and their frequencies for nine microsatellite markers used to characterize 40 plants of 16 *E. oleifera* accessions are shown in Table 1; the number of alleles per marker ranged from two to five, with a total of 26 alleles and an average of 2.89. Similar results were obtained by Singh et al. (2008) with EST-microsatellites for the genus *Elaeis* (mean of 2.56), by Zaki et al. (2012) with 14 genomic microsatellite markers in four *E. oleifera* populations from different countries (mean of 2.66), and by Araya et al. (2009) with eight microsatellite markers, also in *E. oleifera* from different countries (mean of 2.75 and total of 22 alleles). Zaki et al. (2010) however obtained 2 to 11 alleles per marker, with a mean of 5.1, also in *E. oleifera*. A similar mean was reported by Billotte et al. (2001), with microsatellite markers in *E. guineensis*.

The frequencies of the 26 alleles of 9 markers (Table 1) ranged from 0.02 (markers PAM5 and PAM14) to 0.96 (PAM2). For the most common allele of each marker, a variation of 0.40 (PAM4 marker) to 0.96 (marker PAM2) was observed.

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Table 1. Number of allelles and allelle frequency of 9 microsatellite markers applied to 40 plants derived from 16 *E. oleifera* accessions sampled in the Ecuadorian Amazon

Marker	Number of allelles	f(A1)	f(A2)	f(A3)	f(A4)	f(A5)
PAM1	2	0.94	0.06			
PAM2	2	0.96	0.04			
PAM4	4	0.40	0.26	0.24	0.10	
PAM5	5	0.42	0.36	0.14	0.06	0.02
PAM6	2	0.58	0.42			
PAM7	2	0.60	0.40			
PAM8	3	0.87	0.09	0.04		
PAM14	4	0.73	0.18	0.07	0.02	
PAM3	2	0.66	0.34			

The structure of the *E. oleifera* population in terms of expected observed and expected maximum heterozygosity is shown in Table 2. The values of expected heterozygosity (0.12 and 0.08) were lowest for markers PAM1 and PAM2, and highest (0.71 and 0.67) for the markers PAM4 and PAM5. The observed heterozygosity ranged from 0.0 for PAM3 to 0.85 for PAM4. According to Frankham (2008), the expected heterozygosity is less sensitive to sample size than the observed heterozygosity. However, only for three of the nine markers (PAM4, PAM7 and PAM6) the observed was higher than the maximum expected heterozygosity.

The average expected was similar to the average observed heterozygosity (0.41 and 0.42, respectively). In populations with random mating, the observed and expected heterozygosity values are similar (Frankham, 2008). In other words, these results indicate that the accessions in this study are in Hardy-Weinberg equilibrium for the tested markers. Araya et al. (2009) estimated an average observed heterozygosity (0.33) similar to the average expected heterozygosity (0.32) for the open-pollinated Taisha germplasm. Frankham (2008) also mentioned that the average heterozygosity of multiple loci can be used to characterize the genetic diversity of a species.

According to Ott (1992), a marker is considered polymorphic when $H_o \geq 0.1$ and highly polymorphic when $H_o \geq 0.7$. Of the nine markers, seven were polymorphic (Table 2), two of which (PAM4 and PAM7) highly polymorphic; it is worth mentioning that these markers had four and five alleles, respectively. According to Frankham (2008), a locus is considered

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polymorphic when the frequency of the most frequent allele is lower than 0.95. Thus, only marker PAM2 showed low polymorphism.

Table 2. Estimates of allele frequency, expected heterozygosity (H_e), observed heterozygosity (H_o), maximum heterozygosity, PIC values, percentage of maximum PIC and inbreeding (F) values for 9 microsatellite markers used in 40 plants derived from 16 *E. oleifera* accessions sampled in the Ecuadorian Amazon

Markers	Allelle frequency	Expected heterozygosity (H_e)	Maximum heterozygosity	Observed heterozygosity (H_o)	PIC	% PIC	F
PAM1	0.94	0.12	0.50	0.13	0.11	29	-0.07
PAM2	0.96	0.08	0.50	0.09	0.08	21	-0.05
PAM4	0.40	0.71	0.75	0.85	0.65	93	-0.21
PAM5	0.42	0.67	0.80	0.53	0.61	79	0.21
PAM6	0.58	0.49	0.50	0.60	0.37	97	-0.23
PAM7	0.60	0.48	0.50	0.81	0.37	97	-0.68
PAM8	0.87	0.24	0.67	0.26	0.22	37	-0.12
PAM14	0.73	0.42	0.75	0.50	0.38	54	-0.18
PAM3	0.66	0.45	0.50	0.00	0.35	92	1.0
MEAN	0.68	0.41		0.42	0.35		-0.03

The average polymorphic information content (PIC) of the nine markers (Table 2) ranged from 0.08 (PAM2) to 0.65 (PAM4). Considering the percentage of polymorphic information related to the maximum PIC, the markers PAM6, PAM7, PAM4, PAM5, and PAM14 were the most informative. The PIC value can estimate the discriminatory power of a marker, since not only the number of alleles per locus, but also the relative frequency of these alleles is taken into consideration (Cruz et al., 2011). Singh (2008) used 10 microsatellite markers in oil palm and reported PIC values of 0.19 - 0.84. In other perennial palms (*Cocos nucifera* L.), the PIC ranged from 0.07 to 0.88 (Kumar et al., 2011). According to the classification of Botstein et al. (1980), markers with PIC values higher than 0.50 are considered very informative, values between 0.25 and 0.50 moderately informative, and values below 0.25 uninformative. This classified the markers PAM4 and PAM5 as highly informative, PAM3, PAM6, PAM7, and PAM14 as moderately informative and PAM1, PAM2 and PAM8 as uninformative.

The estimates of the inbreeding coefficient (F) of seven of the nine markers were negative (from -0.05 to -0.68) (Table 2). For the markers PAM3 and PAM5, positive inbreeding values (0.21

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and 1.0, respectively) were observed. These results associated with similar values of average expected and average observed heterozygosity indicated that the accessions were derived from randomly crossed plants.

The Hardy-Weinberg equilibrium (HWE) test for the nine markers was performed considering only two alleles, ***A*** (most frequent allele) and ***a*** (sum of the frequencies of other alleles) (Table 3). In the population consisting of the 40 plants, HWE was not only observed for the markers PAM3 and PAM7 in the three tests. According to Cruz et al. (2011), an absolute z value exceeding 1.96 indicates that the observed proportion of homozygotes is not according to the expected ratio when assuming equilibrium. Additionally, an imbalance rate greater than zero ($D_A > 0$) indicates excess of homozygotes and $D_A < 0$ lack of homozygotes. Thus, the imbalance rate only indicates lack and excess of homozygotes for the markers PAM7 and PAM3, respectively.

Table 3. Summary of the tests exact Fisher [$P(N_{Aa}\backslash n_A)$ and P(Acum)], Chi-square [(X^2 and $P(X^2)$] and the deviation from the homozygote frequency (D_A and z) for the EHW assumption of 40 plants derived from 16 *E. oleifera* accessions sampled in the Ecuadorian Amazon

				Exact Fisher test		D_A Test		Chi-square Test	
Marker	AA[1/]	Aa	aa	$P(N_{Aa}\backslash n_A)$	P(Acum)	D_A	z	X^2	$P(X^2)$
PAM1	34	5	0	0.87	1.0	-0.0	-0.43	0.18	0.67
PAM2	31	3	0	0.96	1.0	-0.0	-0.27	0.07	0.79
PAM4	3	21	10	0.08	0.15	-0.07	-1.70	2.86	0.09
PAM5	6	15	11	0.27	1.0	0.01	0.22	0.05	0.83
PAM6	11	24	5	0.10	0.21	-0.06	-1.44	2.07	0.15
PAM7	6	25	0	0.00	0.00*	-0.16	-3.76*	14.15	0.00*
PAM8	28	10	0	0.51	1.0	-0.02	-0.93	0.87	0.35
PAM14	15	14	1	0.25	0.64	-0.04	-1.06	1.11	0.29
PAM3	23	0	12	0.0	0.0*	0.23	5.92*	35.0	0.0*

[1/]. Analysis based on the most frequent allele (allele "A") and the other alleles, represented by "a" as reference

From the dissimilarity matrix based on nine markers and by the weighted index, the 40 plants of the 16 accessions were grouped by the Tocher method (Table 4). Seven groups were formed with the 40 plants, of which 32 formed group I. Thus, the plants in this group were also clustered by the Tocher methodology, resulting in nine subgroups (Ia - Ii). Group II consisted of three plants: two originating from accession 7 and one from accession 8. The other groups consisted of one plant, derived from different accessions. In general, plants of a same accession were allocated to different groups, e.g., plant 6, 5, 7, 28 and 29, among others. These results indicate variability among plants within accessions.

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Table 4. Clustering by the Tocher method of 40 plants derived from 16 *E. oleifera* accessions sampled in the Ecuadorian Amazon

Groups	Plants (accessions)
I	
Ia	**8**(a_3), **33**(a_{14}), **32**(a_{14}) , **6**(a_2), **5**(a_2), **28**(a_{12}), **34**(a_{15}), **35**(a_{15}), **40**(a_{16}), **39**(a_{16})
Ib	**11**(a_5), **37**(a_{16}), **38**(a_{16}), **12**(a_5), **15**(a_7), **14**(a_6), **13**(a_6), **29**(a_{12}), **16**(a_7)
Ic	**22**(a_9), **23**(a_9), **26**(a_{11})
Id	**30**(a_{13}), **31**(a_{13})
Ie	**3** (a_1), **4**(a_1)
If	**24**(a_{10}), **25**(a_{10})
Ig	**10**(a_4), **36**(a_{15})
Ih	**19**(a_8)
Ii	**9**(a_4)
II	**17**(a_7), **18**(a_7) and **21**(a_8)
III	**1**(a_1)
IV	**20**(a_8)
V	**27**(a_{11})
VI	**7**(a_2)
VII	**2**(a_1)

a_j: accession j.

To visualize the variability between accessions within groups analyzed by the methodology Tocher, the accessions were dispersed by the unweighted pair-group method based on arithmetic averages (UPGMA) (Figure 1). Considering Mojena's criterion (Mojena, 1977), K = 1.25, a cut was established at 80% variation. By this cut, six groups were established which generally agreed with those established by the Tocher method. Only has difference in the Group I what comprising 33 plants and including group III established by the Tocher method. These results corroborate the existence of plant variability within groups.

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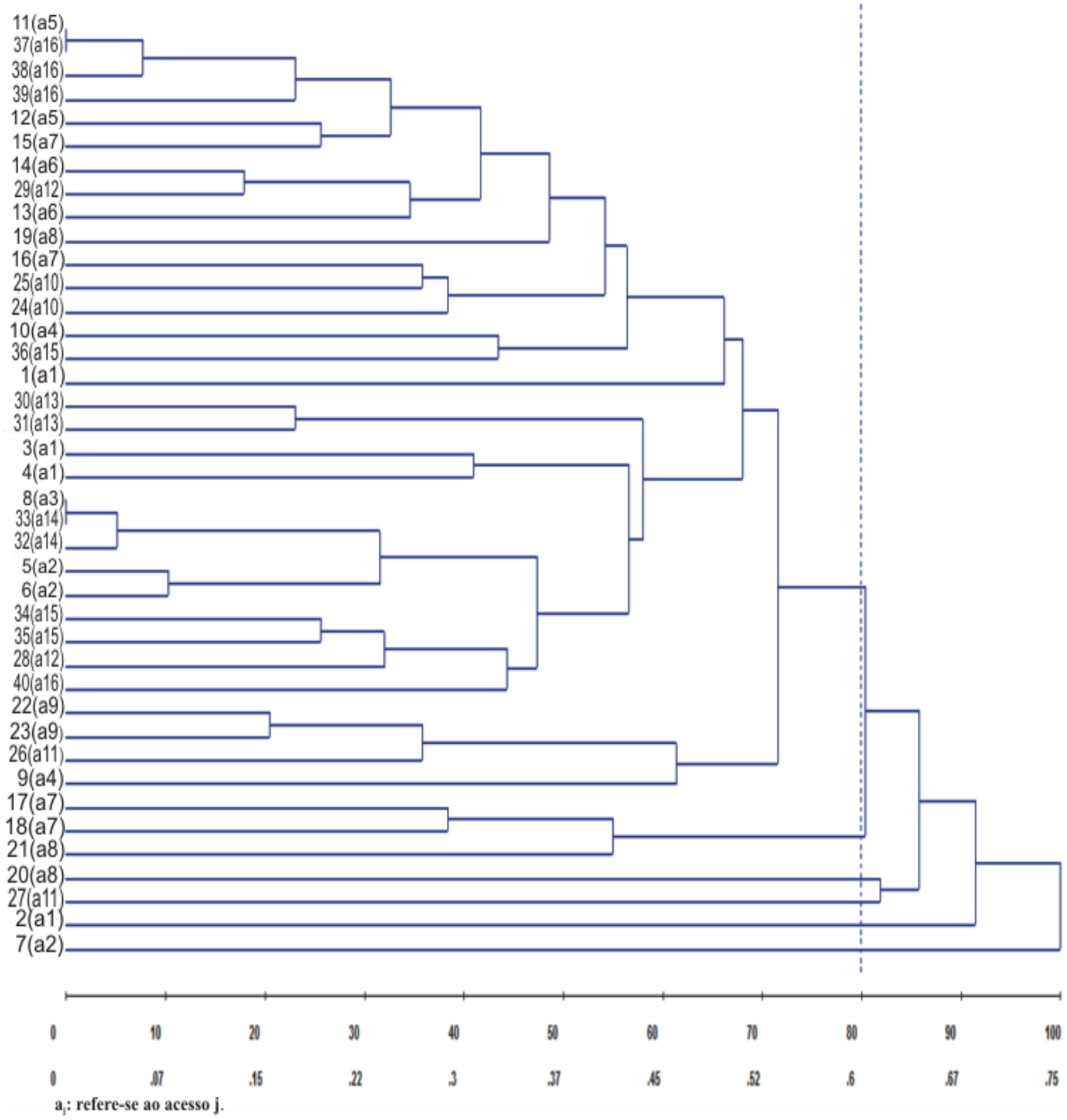

Figure 1. Dendrogram based on the clustering by the unweighted pair-group method based on arithmetic averages (UPGMA) of 40 plants derived from 16 *E. oleifera* accessions sampled in the Ecuadorian Amazon

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The variability between and within the 16 accessions was quantified by the analysis of molecular variance (Table 5). The mean square value found between accessions (0.375) was greater than that within accessions (0.189). It is worth remembering that the mean square between also includes variation within accessions. The decomposition of the total genetic variability (0.264) in variance components between and within resulted in estimates of 0.075 between accessions and 0.189 within accessions. Thus, the greatest genetic variability observed for *E. oleifera* germplasm is within the accessions, representing 72% of the total genetic variability. The statistic phi (Φ_{ST}), proposed by Excoffier et al. (1992), was 0282, which also corroborates the greatest genetic variability within accessions. Moretzohn et al. (2002) studied diversity in *E. oleifera* in the Brazilian Amazon Rainforest and also detected greater variation within than between populations, as expected in autogamous perennial and long-lived species. These results indicate that the diversity in the *E. oleifera* germplasm should be exploited, prioritizing variability between plants, i.e., considering each plant a separate accession, while the collection of *E. oleifera* germplasm should prioritize the variability within plants in their natural environment.

Table 5. Analysis of molecular variance (AMOVA) of the 40 plants derived from 16 *E. oleifera* accessions sampled in the Ecuadorian Amazon.

Source of Variation	DF	MS	Components of Variance	% de Variation	Φ_{ST}
Among accessions	15	0.3745	0.075	28.2	0.282
Within accessions	24	0.1895	0.189	71.8	
Total	39	0.2606	0.264		

ACKNOWLEDGEMENTS

The authors are indebted to the Instituto Nacional de Investigaciones Agropecuárias INIAP-Ecuador, experimental station Santo Domingo, and to the technical staff of the oil palm breeding program of the experimental station Santo Domingo.

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