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Amplification of Cherimoya (*Annona cherimola* Mill.) with Chloroplast-Specific Markers: Geographical Implications on Diversity and Dispersion Studies

Nerea Larranaga [1,2], Gustavo Fontecha [3], Federico J. Albertazzi [4], Margarita Palmieri [5] and Jose I. Hormaza [1,*]

[1] Instituto de Hortofruticultura Subtropical y Mediterránea La Mayora (IHSM La Mayora-UMA-CSIC), Algarrobo-Costa, 29750 Málaga, Spain
[2] CONACYT-Departamento de Botánica y Zoología, Universidad de Guadalajara, Zapopan 45200, Mexico
[3] Microbiology Research Institute, Universidad Nacional Autónoma de Honduras (UNAH), Tegucigalpa 11101, Honduras
[4] Centro Investigación en Biología Celular y Molecular (CIBCM), Universidad de Costa Rica, San José 11501-2060, Costa Rica
[5] Laboratorio de Protección Vegetal, Centro de Estudios Agrícolas y Alimentarios (CEAA), Instituto de Investigaciones UVG, Universidad del Valle de Guatemala (UVG), Ciudad de Guatemala 01015, Guatemala
* Correspondence: ihormaza@eelm.csic.es; Tel.: +34-952-55-26-77

Abstract: Previously developed *Annona cherimola* specific primers based on the plant barcode gene *mat*K were tested in 546 cherimoya accessions. Of those, 296 belong to an ex situ world reference germplasm collection maintained at the IHSM La Mayora-CSIC-UMA in Málaga (Spain) and 250 to cherimoya samples collected in situ in three Central American countries (Honduras, Guatemala and Costa Rica). Results showed the existence of two different haplotypes in the samples analyzed with geographical association. A new marker that amplified all the samples was designed based on the *trn*L-F locus using DNA information from seven species of the *Annona* genus. The information generated will be useful to analyze and conserve the extant genetic diversity present in situ and ex situ and to understand de dispersion of the crop from its center of origin in Central America.

Keywords: *Annona cherimola*; specific marker; *mat*K; *trn*L-F; haplotypes

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1. Introduction

Both morphological and molecular (protein and nucleotide) characters have been used for the identification and classification of taxa, relying on the hypothesis that lineages diverge in the accumulation of variants. DNA-based approaches show several advantages in comparison to morphological or protein-based molecular techniques, since DNA is a stable molecule that provides a lot of information due to the degeneration of the genetic code and the high rate of mutation of non-informative sites. Several DNA-based molecular methods, such as hybridization, restriction enzyme digestion, PCR-based techniques, real-time PCR, and analysis of nucleotide sequences including barcoding, have been used for species identification [1]. DNA barcoding is a taxonomic method whose ultimate goals are to identify and characterize species by comparing the sequences of each species with robust databases using few selected short standardized genome portions. Several loci and combinations of them have been proposed for the barcode of plants; the most important, among others, are *mat*K, *rbc*L, *trn*H-*psb*A, *trn*L-*F*, ITS1, and ITS2 [2–7]. More recently, next-generation sequencing technologies are expanding the possibilities of characterizing specimens by DNA barcoding, as target sequences can be acquired in parallel from hundreds of samples simultaneously [8].

In this work, we apply the concept of plant barcode in cherimoya (*Annona cherimola* Mill.), a member of the Annonaceae, a pantropical family in the order Magnoliales, that, together with the Canellales, Laurales, and Piperales, form the magnoliid clade, sister to

the eudicot and monocot clades of angiosperms. The Annonaceae includes approximately 107 genera and 2400 species of flowering trees, shrubs, and lianas [9] distributed all over the world mainly in tropical regions. A limited number of species, belonging to two genera, *Annona* and *Asimina*, produce edible fruits. *Asimina*, the only genus of the family adapted to cold climates, includes 12 species [10], among which *Asimina triloba*, a species native to temperate zones of North America, is the most interesting agronomically. The genus *Annona* includes around 175 species [11] and is distributed mainly in the tropical areas of America with a few native species of Africa. Several species of this genus produce edible fruits with diverse importance, but all can still be considered as underutilized crops restricted mainly to local markets: *A. cherimola*, *A. reticulata*, *A. squamosa*, *A. muricata*, *A. macroprophyllata*, *A. glabra*, and *A. purpurea*. According to the *Annona* dichotomous key of Fries [12], the section Atta, which includes *A. cherimola*, would have its center of origin in Central America and the Caribbean and is characterized by ripe, smooth-skinned fruits with raised areoles delimited by lowered furrows. *A. cherimola* is a semi-deciduous tree, which can reach 7–8 m at maturity. The leaves are simple, whole, alternately arranged, and oval to elliptical in shape, with a variable size, generally 7–18 cm long and 4 to 10 cm wide, and with a short petiole [13]. The flowers are hermaphrodite and, as most species in the family, show dichogamous (temporal separation of female and male functions) protogyny (maturation of the female part before the male one) which limits self-pollination within the same flower and between flowers of the same tree, since the sexual state is synchronized [14,15]. Pollination is mostly cantharophylous depending on the presence and visit of small Coleoptera species mainly from the family Nitidulidae; manual pollination for fruit production is common in Spain and other countries where the population of pollinating insects is low [16]. The fruit is compound conical or heart-shaped. The thin or thick skin may be smooth with fingerprint-like markings or covered with conical or rounded protuberances. The white flesh is sweet and juicy. Each segment of the flesh surrounds a single hard black bean-like seed [17]. Cherimoya grows best in subtropical climates where the average annual rainfall ranges from about 600 to 1700 mm with low seasonal and interannual temperature fluctuations and mean temperatures ranging from 17 °C to 22 °C. It is distributed from Mexico and Central America to South America at altitudes above 900–1000 m above sea level.

The cherimoya is probably the most important species of agronomic interest in the Annonaceae family, and it is considered as a neglected and underutilized species (NUS) with a high agronomical potential in subtropical areas in different regions of the world [18]. Its area of origin has recently been set in Central America [19,20], from where it was dispersed naturally to Mexico, and by humans to South America in pre-Columbian times [21,22]. In a previous work [23], one of the combinations of two chloroplast loci (*rbc*L and *mat*K) proposed for the barcode of plants [2] was tested in different *Annona* species with significant agronomic interest. In addition, specific primers based on *mat*K were designed to discriminate among them through a single PCR and observation of presence/absence of expected bands in agarose electrophoresis gels.

In order to test whether the primers designed for *A. cherimola* included all the intraspecific variation, in this work, of 546 samples, 410 were mapped and some of them sequenced. A second set of primers was designed based on the *trn*L-F locus, used in several phylogenetic studies in the Annonaceae [24–27].

2. Materials and Methods

2.1. Sample Collections

A total of 546 cherimoya accessions were analyzed in this work. Of those, 296 are conserved in a field germplasm collection maintained at the IHSM La Mayora, Spain. The number of samples per country of origin of those accessions is indicated in Table 1. The remaining 250 samples were collected in Guatemala (40), Honduras (137) and Costa Rica (73). The design of new specific *trn*L-F-based primers was carried out according to Larranaga and Hormaza [23]. Two individuals belonging to each of the most agronomically interesting species of in the genus *Annona* (*A. cherimola* Mill., *A. reticulata* L., *A. squamosa* L.,

A. muricata L., *A. macroprophyllata* Donn. Sm., *A. glabra* L., and *A. purpurea* Moc. & Sessé ex Dunal) were selected. To validate these new primers, one individual of six additional *Annona* species (*A. liebmanniana* Baill., *A. longiflora* S. Watson, *A. montana* Macfad., *A. senegalensis* Pers., and *A. emarginata* (Schltdl.) H. Rainer, and *A. salicifolia* Ekman & R.E.Fr.), as well as an *Asimina trilobal* (L.) Dun. individual were also analyzed. The codes and geographic origin of the individuals are indicated in Table 1.

Table 1. Country of origin, codes, and number of accessions, and plant genotypes used in this study.

	Country of Origin	Number of Accesions		Code	Species	Country of Origin
Ex situ cherimoya accessions from the IHSM La Mayora collection	Australia	13	Genotypes used for primer design	Che1	*A. cherimola*	Spain
	Bolivia	16		Che2	*A. cherimola*	Ecuador
	Chile	9		Che3	*A. cherimola*	Peru
	Colombia	5		Ret1	*A. reticulata*	Honduras
	Costa Rica	1		Ret2	*A. reticulata*	Honduras
	Ecuador	47		Squ2	*A. squamosa*	Unknown
	Italy	3		Squ3	*A. squamosa*	Honduras
	Japan	1		Mur2	*A. muricata*	Honduras
	Mexico	6		Mur3	*A. muricata*	Guatemala
	Peru	126		Mac2	*A. macroprophyllata*	Honduras
	Portugal	26		Mac3	*A. macroprophyllata*	Guatemala
	Spain	7		Gla1	*A. glabra*	Unknown
	USA	36		Gla3	*A. glabra*	Unknown
In situ cherimoya samples from Central America	Guatemala	40		Pur1	*A. purpurea*	Honduras
	Honduras	137		Pur3	*A. purpurea*	Costa Rica
	Costa Rica	73		Lie1	*A. liebmanniana*	Honduras
				Lon1	*A. longiflora*	Mexico
				Mon1	*A. montana*	Unknown
				Sen1	*A. senegalensis*	Unknown
				Ema1	*A. emarginata*	Paraguay
				Neo1	*A. neosalicifolia*	Paraguay
				Tri1	*Asimina triloba*	Unknown

2.2. DNA Extraction, Amplification, Visualization, and Sequencing

Plant DNA extraction was performed from 50 mg of young leaf tissue by a modified CTAB method [28]. Each PCR reaction contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween 20, 3 mM $MgCl_2$, 0.1 mM of each dNTP, 0.3 µM of each primer (Table 2), 20 ng of genomic DNA and 1 unit of BioTaq™ DNA polymerase (Bioline, London, UK) in a final volume of 15 or 35 µL. The analyzed universal and specific *mat*K and new *trn*L-F loci are indicated in Table 2. PCR reactions were carried out in an I-cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following program: (i) 94 °C for 1 min, (ii) 35 times: denaturation at 94 °C, 30 s; hybridization at 52–66 °C (Table 2), 30 s; extension at 72 °C, 1 min and (iii) 72 °C for 5 min. Amplicons were visualized in 1% agarose gels stained with Gel Red (1X) and/or with a Beckman Coulter Genome Lab GeXP™ capillary DNA analysis system after labeling forward primers with a fluorescent dye at the 5′end. In the latter case, samples were denatured at 90 °C for 120 s, injected at 2.0 kV, 30 s, and separated at 6.0 kV for 35 min. Sequencing was done by the fluorescent dye terminator sequencing method in the forward direction, and, prior to sequencing, PCR solutions were purified with NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany). The same primers were used for amplification and sequencing.

Table 2. Details of the primers used and designed including sequence, PCR annealing temperatures, expected PCR product sizes, target species, and references.

Name	Direction	Sequence 5′—3′	Annealing Temp. (°C)	Expected Size (bp)	Specificity	Reference
AChF1	F	GTATATGAATGTGAATCGGTATTC	65	396	*Annona cherimola*	[23]
AChR1	R	TTGACTCCTTACTGCGGAAT				
AChF2	F	CCATTTTCCCCCCCTAATT	66	197	*Annona cherimola*	
AChR2	R	CAATAGTGGAGATTCCTTGCCT				
1RKim	F	ACCCAGTCCATCTGGAAATCTTGGTTC	52		*Universal-matK*	[29]
3FKim	R	CGTACAGTACTTTTGTGTTTACGAG				
C	F	CGAAATCGGTAGACGCTACG	60		*Universal-trnL-F*	[30]
F	R	ATTTGAACTGGTGACACGAG				

2.3. Design of Specific Primers

In order to design a second pair of PCR cherimoya specific primers (AChF2 and AChR2; Table 2), *trn*L-F universal markers were selected to amplify and sequence 15 previously used DNA samples [23] (Table 1). Newly raw sequenced *trn*L-F fragments were aligned by MUSCLE (MEGA5 version 5.05) [31] using the default options. Inter-species polymorphisms among *A. cherimola* and other species were manually identified while intra-specific variations were not considered. Two specific primers were designed locating the 3′-end of each primer, targeting cherimoya specific variant sites. The expected size of the amplicon was 197 bp. An optimal annealing temperature was established by empirical means. A homology search was conducted with the BLAST tool to indicate which species could be amplified by these new primers.

2.4. Amplification and Sequencing of Specific Loci

Specific *A. cherimola mat* K-based primers (AChF1 and AChR1) [23] were tested in 546 cherimoya accessions, 296 from the ex situ cherimoya Spanish collection, that holds accessions from all over the world, and 250 collected in situ in Central America. Accession samples from the germplasm collection with an American origin and known geolocation (160) and all accessions from Central America (250) were mapped geographically using ArcMap 10.1 with presence/absence of amplification. WGS84 geographical projection was settled, and a layer with country boundaries downloaded from the DIVA GIS web page and a 1:10 m Cross-blended Hypsometric Tints layer from Natural Earth were used. Specific *A. cherimola trn*L-F-based primers (AChF2 and AChR2) were tested for the same 546 cherimoya accessions. Randomly selected thirteen DNA samples out of the 110 that did not amplify and 7 that showed amplification with AChF1 and AChR1 were amplified and sequenced with universal *mat*K primers to analyze the reason for the absence of amplification. Since these specific primers were based on a single nucleotide variant positioned on each 3′-end, these locations were primarily observed. Both forward and reverse primers were designed to hybridize with a cytosine and an adenine, while the rest of the sequences belonging to other species showed a thymine and a cytosine respectively. Obtained sequences were deposited in GenBank (Table 3) and a neighbor joining (NJ) phylogenic tree, using the *p*-distance, was constructed in MEGA 7 [32] taken also into account the three sequences previously used to design *mat*K cherimoya primers.

Table 3. Sequenced samples with *mat*K universal primers. The table shows the code utilized in this article, the germplasm bank code, whether they amplified with Achf1/AChR1 and AChF1/AChF2 primers, and GenBank codes.

Article Code	GB Code	Country of Origin	AChF1/AChR1	AChF2/AChF2	GenBank Code
Che1	FDJ	Spain	Yes	Yes	KM068846 [23]
Che2	SP74	Peru	Yes	Yes	KM068847 [23]
Che3	Hach10	Honduras	Yes	Yes	KM068848 [23]
Che52	HAch246	Honduras	Yes	Yes	OP286917
Che53	Bonita	U.S.A	No	Yes	OP286918
Che55	Hach28	Honduras	No	Yes	OP286919
Che56	Hach38	Honduras	No	Yes	OP286920
Che57	Hach3	Honduras	No	Yes	OP286921
Che58	Hach46	Honduras	Yes	Yes	OP286922
Che59	Hach80	Honduras	No	Yes	OP286923
Che60	Hach97	Honduras	No	Yes	OP286924
Che61	Hach111	Honduras	Yes	Yes	OP286925
Che63	Hach213	Honduras	Yes	Yes	OP286926
Che64	Hach234	Honduras	No	Yes	OP286927
Che65	Hach243	Honduras	No	Yes	OP286928
Che67	Gach26	Guatemala	No	Yes	OP286929
Che68	Gach28	Guatemala	Yes	Yes	OP286930
Che69	Gach62	Guatemala	Yes	Yes	OP286931
Che70	Gach84	Guatemala	Yes	Yes	OP286932
Che71	BS2	Bolivia	No	Yes	OP286933
Che72	BS3	Bolivía	No	Yes	OP286934
Che73	Booth	U.S.A	No	Yes	OP286935
Che74	Lucida	U.S.A	No	Yes	OP286936

3. Results

3.1. Specific Markers

Fifteen DNA samples belonging to seven *Annona* species (Table 1) were amplified with *trn*L-F universal primers to design the primers AChF2 and AChR2 based on their sequences (Table 2). Raw sequences were aligned, and two SNPs were shared by all the *A. cherimola* samples, but revealed differences in the sequences in the other species analyzed. These SNPs were used to locate the 3′-end of each new primer. Primer specificity was verified using 15 samples of different *Annona* species and one sample of *Asimina triloba* (Figure 1). Just one single band of the expected size (197 bp) appeared when the *A. cherimola* DNA was used.

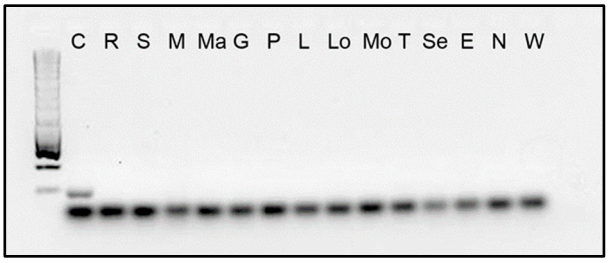

Figure 1. Electrophoresis in 1% agarose gels of cherimoya specific PCR reactions using the AChF2 and AChR2 primers (expected amplicon: 197 bp) and 14 DNA samples from different species in the Annonaceae family used as templates. (C) *A. cherimola*; Che1, (R) *A. reticulata*; Ret1, (S) *A. squamosa*; Squ2, (M) *A. muricata*; Mur2, (Ma) *A. macroprophyllata*; Mac2 (G) *A. glabra*; Gla1, (P) *A. purpurea*; Pur1, (L) *A. liebmanniana*; Lie1, (Lo) *A. longiflora*; Lon1, (Mo) *A. montana*; Mon1, (T) *A. triloba*; Tri1, (Se) *A. senegalensis*; Sen1, (E) *A. emarginata*; Ema1, (N) *A. neosalicifolia*; Neo1, (W) Water. Taxon codes are indicated in Table 1. Hiperladder 1 Kb of Bioline was used as size marker.

Interestingly, some of the raw sequences showed an electropherogram with a mixed pattern of peaks after a certain point, which could indicate a compression in the sequencing process since the mixed pattern starts after several thymines or cytosines. In particular, both sequences of *A. reticulata*, *A. squamosa* and *A. muricata* showed multiple peaks after the position 285–315 (depending on each raw sequence) and 12–15 thymines. *A. macrophyllata* sequences showed multiple peaks after position 582 or 583 and 11 cytosines. These results could also be due to the presence of more than one PCR product of the same size, although just a single band was observed in the agarose gels.

The homology search conducted with BLAST (03-2014) showed a 100% query cover for the forward primer and identity with *trn*L-F sequences of *A. cherimola*, *A. pruinosa*, *A. dumetorum* and *A. bicolor*, while the reverse primer showed identity in the 3′-end for *trn*L-F entries of *A. cherimola* and *A. pruinosa* only.

3.2. Amplification and Sequencing of Specific Loci

The primers AChF1 and AChR1 were successful to amplify 291 (98.3%) DNA samples from the cherimoya germplasm bank, while five (1.7%) failed to amplify. These five genotypes (coded as Bolivia Seedling2, Bolivia Seedling3, Bonita, Booth, and Lucida) originated from an ex situ collection in California, USA, with Mexican, Bolivian, or unknown origin genotypes. In addition, 66% of the *A. cherimola* samples from Guatemala (35/40), Honduras (60/137), and Costa Rica (70/73), were successfully amplified with the primers AChF1 and AChR1. American accessions from the Spanish collection with well-known geographic coordinates and all the Central American samples were mapped geographically in Figure 2, indicating whether successful amplification was obtained for each sample.

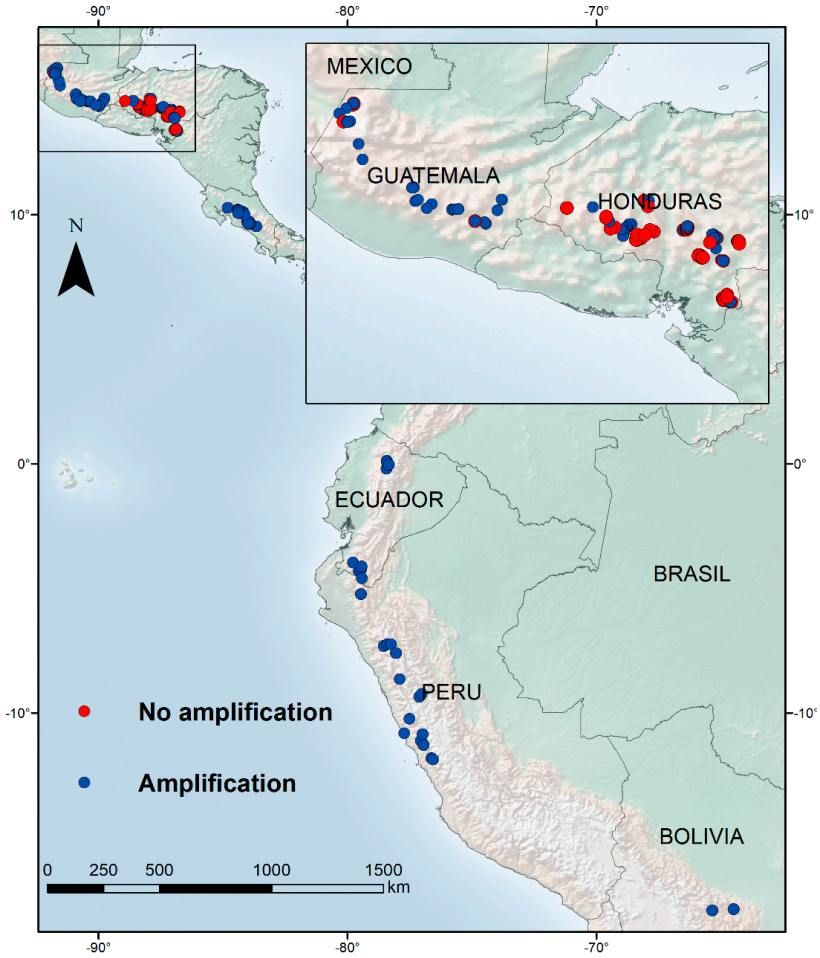

Figure 2. Geolocation of 411 *A. cherimola* samples amplified with AChF1 and AChR1. The blue color indicates the presence of an amplicon while the red color indicates the absence of amplification.

Primers AChF2 and AChR2 amplified most of the accessions analyzed (546) except four (0.73%). Two of those belong to the germplasm field collection and their origin is the island of Madeira (Portugal) and the other two were collected in Guatemala. These last two accessions also failed to amplify with the first set of primers AChF1/AChR1.

3.3. Sequence Analysis

Thirteen samples that showed no amplification using the primes AChR1/AChF1 were amplified with *mat*K universal primers, and the amplification fragments sequenced. Sequencing results were aligned, and the positions used to design AChF1 and AChR1 were analyzed manually. None of them showed any of the two cherimoya-specific *mat*K variants (cytosine and adenine) used to design the primers but shared instead two nucleotides, thymine and cytosine, with the rest of the species used in the previous alignment [23]. In addition, seven samples from Honduras and Guatemala that showed an amplicon with the set of primers AChF1/AChR1 were also amplified and sequenced with *mat*K universal primers and analyzed in the same positions; all of them showed a cytosine and an adenine, as expected. The NJ tree (showing only topology) constructed with the obtained sequences is shown in Figure 3 which clearly separates the samples according to the two identified haplotypes.

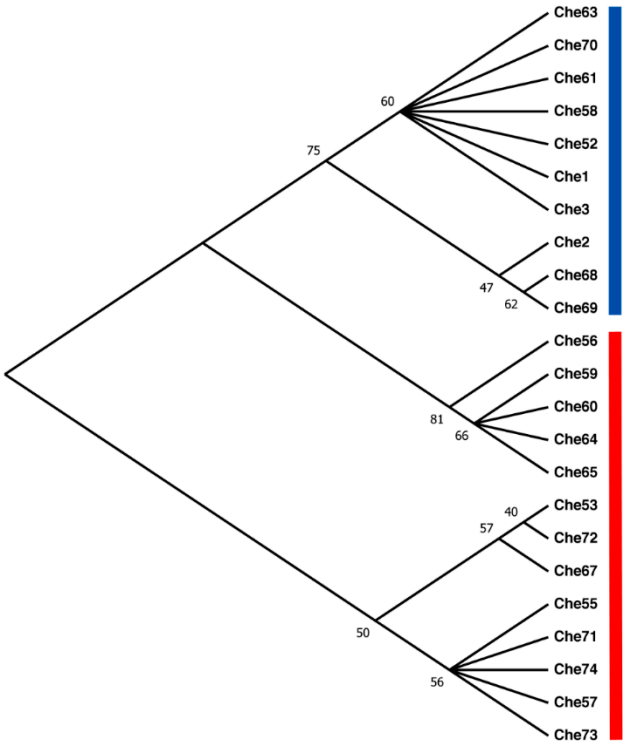

Figure 3. Evolutionary relationships of taxa based on *matK* sequences inferred with a Neighbor-Joining method using the *p*-distance. Bootstrap values are shown, and branches with values below 20 are collapsed. A total of 773 positions were present in the final dataset.

4. Discussion

Both morphological and molecular characters can be used to identify and classify species. However, the development of new techniques to produce large amounts of molecular data is revolutionizing many areas of the biological sciences including taxonomy. DNA barcoding would allow the identification and discovery of new species simply by amplifying query DNA with established barcode genes, sequencing the amplicon, and comparing the result against highly robust databases. This method allows a more effective taxa identification, especially in cases where morphological identification is difficult.

In 2015, a DNA barcoding study was conducted among species of the genus *Annona* with significant agronomic interest and some other species included in public databases using the proposed *mat*K and *rbc*L genes for the barcode of life in plants [23]. Only *mat*k could discriminate among all the species analyzed. In addition, specific primers based on this locus were designed to have an even easier tool to recognize these species by a simple PCR and visualization of a band in an agarose electrophoresis gel. The presence of a band would indicate that the DNA template belongs to the species it was designed for or to another species not included in the alignment and that could share the same primer targets.

Primers AChF1 and AChR1 were designed using three cherimoya individuals from three distant geographic regions (to recover as much intraspecific variability as possible): Spain, Honduras, and Peru. The analysis of 296 accessions of the *A. cherimola* worldwide collection in Spain showed that just a few samples (n = 5) did not produce an amplification band. These samples were obtained from a USA collection with genotypes from Central and South America. In addition, amplification was not obtained for 85 of 250 DNA samples collected in Central America. A total of 411 samples were mapped geographically and the results revealed that the samples that did not amplify were mainly obtained in Honduras (56.2%), followed by Guatemala (12.5%) and Costa Rica (4.1%). This could indicate a spatial association between the presence and absence of the PCR product. The two Californian accessions with a Bolivian origin that did not amplify could be an indicator of a direct exchange between Central America and this South American country although the possibility of an error in the passport data cannot be excluded.

In order to assess if the non-amplifying samples missed one or both SNPs, several samples were amplified with the *mat*K universal primers, and their sequences showed just two haplotypes: genotypes with the two variants (showing amplification) or genotypes without them (showing absence of amplification). The NJ tree differentiated samples according to this two polymorphic sites. If such a pattern is true, haplotype 1 is present all over the cherimoya distribution area, while haplotype 2 is characteristic of Central America, mainly Honduras. These results would be in line with the recently proposed hypothesis of a Mesoamerican origin of the species and dispersion by humans to South America in pre-Columbian times [20,22]. In addition, the presence of two genotypes (and no intermediates) would also be consistent with a hybrid origin of the species in which each chloroplast haplotype could derive from different species. Previous work has shown a close relation between *A. cherimola* and *A. pruinosa* [21]. Further molecular research is needed to provide additional information on this hypothesis.

With the aim of finding a global specific marker for *A. cherimola*, the *trn*L-F locus from several species was sequenced, and two shared SNPs among *A. cherimola* accessions were used to design a species-specific set of primers. Five hundred and forty-seven DNA samples were analyzed with a successful global amplification. Remarkably, several genotypes showed several peaks after a certain point (after a group of approximately 10 thymines or cytosines) and, although this could be due to sequencing problems, we cannot discard a case of heteroplasmy or DNA polymorphisms, that is, having more than one PCR product with similar electrophoresis mobility. Chloroplast heteroplasmy could be due to DNA damage, but biparental plastid inheritance has been reported as another source of variation [33], which could be a quite widespread trait (20%) in angiosperms [34]. In fact, Pierie et al. [26] reported ancient paralogy copies in different species in the cpDNA *trn*L-F region in the Annonaceae family.

The homology search conducted with the BLAST tool indicates that AChF2 and AchR2 could also amplify *A. pruinosa*. Again, since not all species of *Annona* have been sampled in this work and their *trn*L-F sequences have not been uploaded to GenBank, these new primers could also amplify other taxonomically close species not included in this work, in addition to *A. pruinosa*, using the combination of the forward and reverse sequences. In any case, the results obtained provide one marker (AchF1/AchR1) that seems not to be present in some cherimoya genotypes from Central America, and another marker (AchF2/AchR2)

present in all the cherimoya samples analyzed. Both markers could be useful to discriminate this species from other taxonomically related *Annona* species.

In summary, the results of this work are consistent with the origin of cherimoya in Central America and provide useful tools to easily distinguish species of Annona using conserved chloroplast sequences, avoiding the problems derived from relying on morphological characterization. Further work is needed to increase the number of species characterized as well as to determine the putative hybrid origin of *A. cherimola*.

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