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The Genetic Characterization of the Canarian Endemic Palm (*Phoenix canariensis*) by Simple Sequence Repeats and Chloroplast Markers: A Tool for the Molecular Traceability of *Phoenix* Hybridization

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Abstract: The endemic palm from the Canary Islands, *Phoenix canariensis*, is one of the most distinctive elements of the Canarian vegetation landscape, contributing to cultural, economic and environmental aspects. One of the main conservation problems facing this iconic palm is anthropogenic hybridization with other *Phoenix* species, particularly *Phoenix dactylifera*, which has been introduced extensively throughout its geographical range. Therefore, it is important to obtain a genetic tool that addresses different issues that may have an impact on the protection of *P. canariensis*, including ornamental applications and wild population conservation purposes. Our main goals were to detect a molecular tracer that could reliably distinguish between *Phoenix canariensis* and *P. dactylifera* in the Canary archipelago and to characterize the presence and extent of genetic hybridization events between the two species. We used 19 nuclear microsatellites and 1 chloroplast minisatellite set and analysed a large sample size (N = 433) of plants using both Bayesian methods and ordination techniques. Our data showed that a set of 13 nuclear markers revealed diagnostic alleles for *P. canariensis*, which were defined as the Canarian nuclear genotype (CNG). Moreover, *P. canariensis* exhibited an exclusive chlorotype of 266 bp that together with the GNC serve as an indicator of genetic purity in the Canarian palm. These markers are sufficient to detect any hybrid, even if it is not related to morphological differences. The occurrence of a considerable number of specimens with different degrees of hybridization is discussed in terms of the existence of different generations of hybrids and different types of crosses. Thus, the genetic tracers represent an invaluable tool to address any proposal for the genetic conservation of *Phoenix canariensis*.

Keywords: Canary Islands; chloroplast minisatellite; conservation genetics; genetic introgression; nuclear microsatellites; *Phoenix* hybrids

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1. Introduction

The molecular characterization of the genetic patterns of the *Phoenix* species has been subjected to numerous studies in the last decades, mainly focused on varieties of *Phoenix dactylifera* due to their great economic relevance [1]. Frequent hybridization between species and the scarcity of suitable taxonomic and morphological characters to distinguish different *Phoenix* species have motivated the search for a useful molecular marker [2–5]. Hypervariable simple sequence repeats (SSRs) and minisatellite markers have been well adapted to generate barcoding markers given the lack of sufficient sequence variation in both the nuclear and chloroplastic genomes to discriminate between *Phoenix* species [3]. Currently, a battery of highly informative markers has been developed to assess genetic processes and to identify hybrids among very closely related taxa such as *Phoenix* species [3,6–8]. In addition, these molecular markers have been proved to be effective in several *Phoenix* studies to reconstruct phylogenetic trees [3,9,10], to clarify genetic structure across biogeographic ranges of species [5,8,11–15], to determine paternal linages through sex-specific genetic markers [16] and even to identify wild relatives of *P. dactylifera* [17].

Phoenix canariensis, the Canarian date palm is one of the most representative endemic plant species from the Canarian archipelago, being designated as its plant symbol by the Canarian Government [18]. It is one of the most distinctive elements of the Canarian vegetation landscape, contributing to cultural, economic and environmental aspects. Also, the Canarian palm has an important economic relevance as ornamental palm, and it is widely distributed across all the archipelago, but it is also planted across the subtropical and tropical world for its ability to grow on a wide range of site types [19]. Different *Phoenix* species have been introduced in the Canary Islands as ornamental species. *P. dactylifera* is by far the most widespread species. It was possibly introduced across different Canarian islands since the aboriginal occupation from Northern Africa 2000 years ago [20]. Later, it was widely dispersed across the archipelago considering its agricultural and ornamental importance. This scenario has promoted anthropogenic *P. dactylifera* geneflow into *P. canariensis*, generating hybridization processes in geographic areas in which both species overlap, threatening the genetic integrity of the island endemism. There is also hybridization with other *Phoenix* species, but it is not so relevant and is of a much lesser extent than that with *P. dactylifera* [18,21,22]. In fact, in the Canarian archipelago, *Phoenix dactylifera* is included in the list of alien and invasive species following the Spanish Catalogue of Invasive Alien Species regulation (BOE No. 185, 3 August 2013). Therefore, conservation management of *P. canariensis* must preserve and expand genetically pure populations since the genetic value of hybrid populations is considerably reduced due to genetic erosion of the endemic species [23]. In fact, the Canarian Island Government published a specific decree (Decree 62/2006 of 16 May 2006) establishing measures to promote the protection and conservation of the genetic identity of the *P. canariensis* but did not specify the methods and the requirements to be met. Moreover, the morphological similarity and the great ecological and morphological plasticity between both species of the genus *Phoenix* make their classification and the morphological identification of pure and hybrid populations a very subjective task currently. In this sense, an assessment of interspecific hybridization and introgression between both species must be implemented to develop an appropriate genetic conservation strategy for *P. canariensis*, compelling researchers to generate genetic tracers that unambiguously identify the existence of genetic hybridization events in the Canarian date palm germplasm.

Previous genetic studies have demonstrated the existence of hybrids between the endemic *Phoenix canariensis* and the widespread *P. dactylifera* in the Canary Islands using RAPD markers [18,21]. Other attempts to assess genetic hybridization between both species using isozyme markers supported considerable genotype differentiation between both species [22] but with limited discriminatory power due to low levels of detectable genetic variability [24]. Nevertheless, previously detected SSR marker polymorphism in *Phoenix* species could be used to fix RAPD and isoenzyme drawbacks, being especially useful to identify genetic purity tracers of *P. canariensis* [3]. Additionally, [25] have identified a

polymorphic cpDNA minisatellite locus composed of 12 bp repeats located between the trnG(GCC)-trnfM(CAU) intergenic spacer, whose haplotypes were strongly associated with the taxonomy of *Phoenix* species [3,26–28]. Thus, *P. canariensis* seems to harbour a specific haplotype characterized by a 5X-tandem-repeat motif resulting in a fragment of 266 pb [3,26]. On the other hand, the date palm always exhibits two chlorotypes associated with an Occidental (242 pb fragment size) and an Oriental (254 pb fragment size) geographic distribution, respectively [1,3,8,17]. Overall, the combined implementation of nuclear and chloroplast markers represents a good starting point to characterize the genetic identity of the *P. canariensis* for genetic conservation of the species.

Nevertheless, although the date palm has been extensively sampled and analysed with the chloroplast minisatellite [1,8,14], only a small sample of *P. canariensis* individuals has been studied together with other *Phoenix* species [3,26,27], mostly from cultivated palms of France, Italy, United Kingdom and Spain. In this sense, this sample represents a minimal and biased fraction of the wild genetic pool of *P. canariensis* populations, which, in addition, could have originated from the wild restricted areas of La Palma and Northern Tenerife islands [13,18]. Therefore, it makes sense to consider that other haplotypes could have not been sampled across the archipelago.

In this study, we utilized nuclear SSR and chloroplast minisatellite markers to characterize genetic hybridization events between the Canarian endemic species and its Continental relative *P. dactylifera*. Specifically, we addressed the following aims: (i) to evaluate a group of nuclear and plastid markers to discriminate among *Phoenix canariensis*, *P. dactylifera* and their hybrids; (ii) to analyse the utility of this set of genetic markers to characterize the extent and the grade of the genetic introgression in the wild populations of the Canarian palm and (iii) to identify the taxonomic specificity of the *Phoenix canariensis* chlorotype employing extensive sampling from natural populations. The use of these molecular markers will serve as a powerful tool to be incorporated into genetic conservation programs of *Phoenix canariensis*.

2. Materials and Methods

2.1. Plant Material

We used a total of 433 samples from *Phoenix*: 271 of *P. canariensis*, 88 of wild putative hybrids distributed in natural and seminatural palm groves of Tenerife and Gran Canaria islands (Canary Islands), 26 of *P. dactylifera* specimens from Gran Canaria and 48 non-Canary Islands *P. dactylifera* from Tunisia and the Iberian Peninsula. The taxonomic classification of samples was based on carefully scored morphological traits such as form and colour of the leaf, trunk shape, spines sizes, presence of offshoots and shape of the crown and trunk [2,4,18] (Table 1 and Figure S1).

Table 1. List of traits considered for the morphological assignation of the *Phoenix* species that samples belonged to. Hybrids were assigned when the samples exhibited intermediate characteristics between both species of *Phoenix*. See Figure S1.

Attributes	*Phoenix canariensis*	*Phoenix dactylifera*
Stem	Completely straight. Columnar appearance	Usually, crooked. Not excessively thick
Presence of offshoots	Absence	Occasional presence
Size of leaves	Long, broad leaves with elongated, dense leaflets	Slightly shorter leaves, leaflets not very dense
Colour of the leaves	Dark green	Glaucus
Spines	Long and thick, sometimes yellowish	Short and thin
Shape of the crown	Spherical	Open leaf crown

Samples were collected from two different islands to provide a wider range of morphological and genetic variation in *P. canariensis* and putative hybrids. Specimens with

intermediate morphological features between *P. canariensis* and *P. dactylifera* were considered as putative hybrids. Moreover, we expected that *P. dactylifera* specimens would exhibit the Oriental (254 bp fragment size) or Occidental chlorotype (242 bp), as these had been previously detected in this species across its distribution [1,8,9,17]. Overall, we assumed six morpho-geographic types according to a combination of morphological features and geographic sampling origin: *Phoenix canariensis* morphotype from Gran Canaria (PCGC, N = 169) and Tenerife (PCTF, N = 102); putative hybrids from Gran Canaria (HBGC, N = 51) and Tenerife (HBTF, N = 37); and *Phoenix dactylifera* morphotype with eastern chlorotype (PD254, N = 31) and western chlorotype (PD242, N = 43).

2.2. DNA Isolation and Molecular Analysis

Total genomic DNA was extracted from approximately 50 mg of silica-dried leaf using the Invisorb® DNA Plant HTS 96 Kit, following the manufacturer's protocol. Subsequently, the DNA templates were amplified with 19 nuclear microsatellite loci previously developed for *P. dactylifera*, including dinucleotide repeats from an SSR genomic library [6], introns coding for transcription factors [29], the cup-shaped cotyledon 3-related protein (CUC3) gene and the Pd-AP3-SSR1 sequence [8]. We also included tri-/hexanucleotide repeats from coding sequences identified by in silico mining [7] in the whole-date-palm genome sequence [30]. The chlorotypes of all the palms were characterized according to the number of scored repeats of the dodecanucleotide minisatellite located in the intergenic spacer psbZ-trnfM [25]. Amplification reactions and alleles size scoring were performed as in [8], using a combination of all the loci to define an exclusive multilocus genotype for each sample.

2.3. The Genetic Characterization of the Hybridization Process between Phoenix canariensis and Phoenix dactylifera

The nuclear allele and chlorotype frequencies and sizes were calculated with GENALEX v6.5 [31]. To explore the molecular relationships among the samples and infer pure and putative hybrid specimens according to their group or intermediate membership in the main components axis, a principal component analysis (PCA) was carried out on the allele's frequency according to the dudi.pca function implemented in the ade4 package for R software v2.15.3 (R Development Core Team, Vienna, Austria, 2011).

To infer the genetic ancestry of multilocus genotypic data, we applied two Bayesian clustering methods. First, the STRUCTURE 2.3.4 software [32,33] was used to detect admixture between *P. canariensis* and *P. dactylifera*, considering that both species were well differentiated into two clusters under an admixture and uncorrelated alleles frequency ancestral model. Then, we assumed that pure samples showed a priori ancestry (*q*-value) greater than 95% for one of the two clusters according to the Popinfo setting of [32]. Admixed ancestry is modelled by assuming that an individual, i, has inherited some fraction (q) of its genome from any of the two ancestors. Thus, hybrids could be inferred when observing individuals with q-values less than 95% for any of the two clusters. We used Markov chain Monte Carlo (MCMC) iterations as implemented in the software's algorithm to explore a parameter space fixing individual memberships to a K = 2 cluster (pre-defined pure species class), which was replicated 10 independent times for each K, each with a burn-in and chain length of 105 and 106 replicates, respectively. Second, to further validate our STRUCTURE analysis, we characterized specimens according to the posterior distribution of individual assignment into pure parental (*P. canariensis*, pure I class; *P. dactylifera*, pure II class) and hybrid categories (F1, F2 or backcross with pure species I and II) as implemented in NEWHYBRIDS [34]. We used default genotypic classes with no prior information on allelic frequencies and included uniform and Jeffereys priors for θ and π following the default setting [34]. Five runs were conducted with 105 sweeps of burn-in and 107 sweeps of data collection. We calculated Pearson's correlation between STRUCTURE's proportion of membership (qi) and NEWHYBRIDS' posterior probabilities (pps) to visualize the relationship between these two analyses, using R software v2.15.3.

2.4. The Feasibility of Molecular Markers for the Detection of a Hybrid Signal in Phoenix canariensis

The molecular genotypes were checked for diagnostic alleles to assess the purity and hybrid status of the samples of *Phoenix* according to three strategies. First, we identified diagnostic nuclear alleles that had frequencies greater than 0.9 in *P. canariensis* PCGC and PCTF and were not detected in *P. dactylifera* PD254 and PD242, which also contributed the most to the PCA load plot specified in the previous section. Such alleles were considered as a tracer of absolute genetic purity in the Canarian endemism, defining altogether the *Canarian nuclear genotype* (hereafter, CNG). Thus, a measure of the hybridization extent was established as the expected loss of purity in natural populations of *Phoenix*, assuming different molecular types according to the proportion of CNG alleles: (i) *pure P. canariensis*: higher than 90% of CNG alleles; (ii) *hybrid*: between 10% and 90% of CNG alleles; (iii) *pure P. dactylifera*: lower than 10% of CNG alleles. Second, we analysed the frequency and distribution of different expected chlorotypes along the molecular types. Third, we used a *q*-value threshold to make a distinction between hybrid and pure individuals in STRUCTURE analysis, as it has been recommended for conservation purpose [35]. So, individuals with a *q*-value between 0 and <0.10 or >0.9 and 1 were classified as pure, and any individuals with a *q*-value between 0.1 and 0.9 were classified as hybrid. Also, we computed the proportion of times we observed a correspondence between the morpho-geographic types and the *molecular types;* that is, if the following condition is met: PCGC + PCTF = pure *P. canariensis*; HBGC + HBTF = hybrids; PD254 + PD242 = pure *P. dactylifera*.

3. Results

3.1. The Genetic Characterization of Phoenix canariensis and P. dactylifera and Their Hybrids

According to the 20 SSR loci, the PCA separated the samples of *P. canariensis* (PCGC + PCTF) and *P. dactylifera* (PD254 + PD242) morpho-geographic types into different groups and differentiated the within-island genetic structure in Canarian palm samples (Figure 1a).

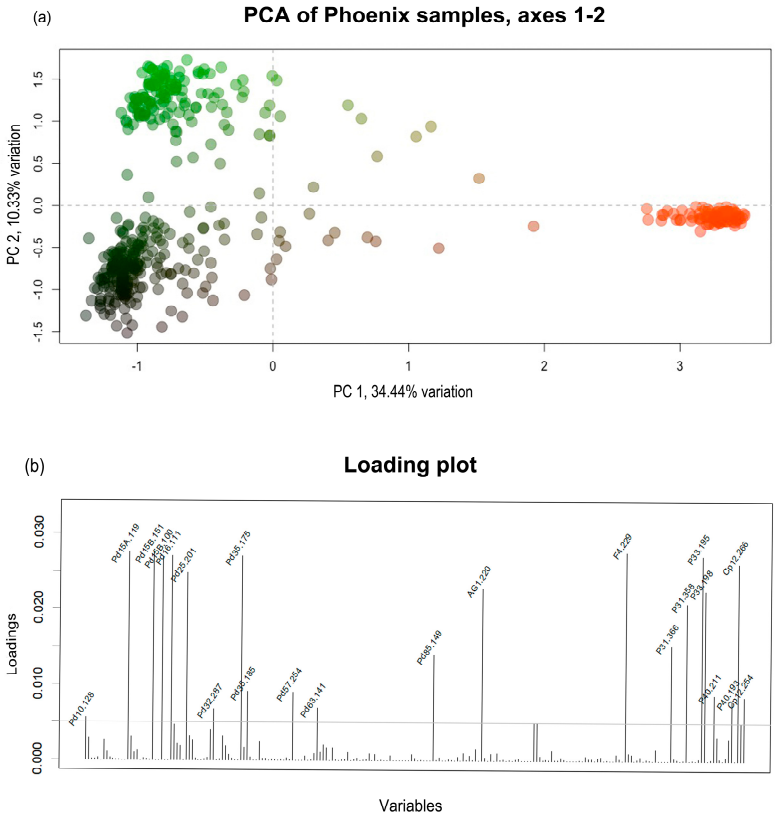

Figure 1. (**a**) PCA analyses based on allele frequency obtained from 19 nuclear SSR loci on *Phoenix* samples. The percentage of explained variance of two first components is given in parenthesis. The

genetic diversity is represented by a gradient of colour, the PC1 separated *Phoenix canariensis* (in green) from *Phoenix dactylifera* (in orange). The PC2 discriminated *Phoenix canariensis* samples according to island origins, from Gran Canaria (in green) and Tenerife (in grey). (**b**) Loading plot of PCA representing the alleles that exceeds a threshold established by 75% of the values plotted, indicating the weight of these alleles on the multidimensional ordination of *Phoenix* simples. Only the alleles above a 0.05 threshold in the loadings axis are labelled.

Both species were clearly separated by the combination of the first two axes of the PCA (44.4% of total variance), indicating a strong genetic differentiation between them. Samples located at intermediate positions of the PCA, suggesting a hybrid genetic composition, were mostly related to specimens with the putative hybrid morpho-geographic types HBGC and HBTF. The PC2 discriminated *P. canariensis* samples according to island origins, separating specimens from Gran Canaria (PCGC) and Tenerife (PCTF) (Figure 1a). On the other hand, both Bayesian inference analyses showed strong agreement with each other, supported by a significant correlation between the ancestry coefficients generated by STRUCTURE and NEWHYBRIDS (r = 0.952; $p > 0.0001$). Both software revealed two distinct clusters associated with the *P. canariensis* and *P. dactylifera* morpho-geographic types, respectively, whose samples showed assignation scores of q (STRUCTURE) or pp (NEWHYBRIDS) higher than 0.99. Also, substantial genetic introgression levels were detected in some samples, which were assigned to a greater extent to F2 genotype classes according to STRUCTURE, or backcrosses to pure *P. canariensis* as indicated by NEWHYBRIDS' outputs (Figure 2).

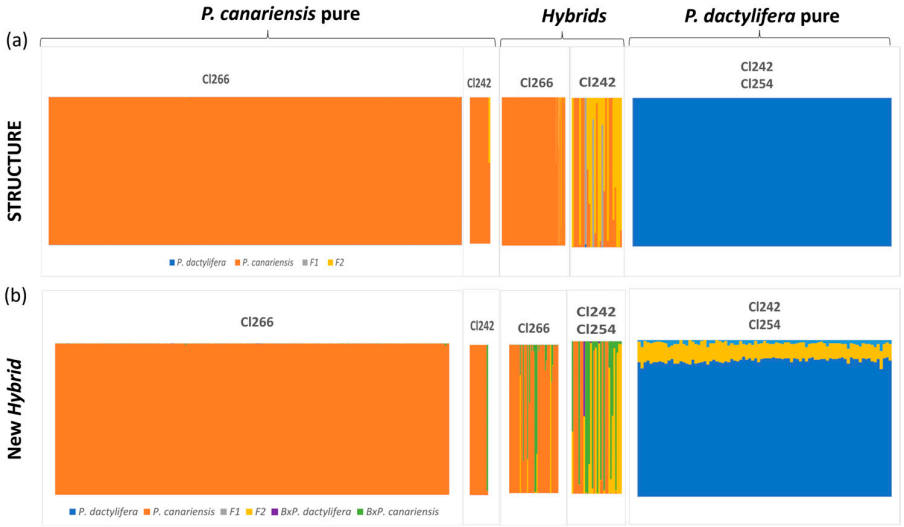

Figure 2. Identification and classification of hybrids: (**a**) STRUCTURE results showing the proportion of the genome of every individual originating from each of the two inferred clusters; orange is cluster 1 (*Phoenix canariensis*), and blue is cluster 2 (*P. dactylifera*). The bars between the two vertical black lines show the introgressed individuals with Q-values between 0.1 and 0.9. (**b**) NEWHYBRIDS classification for every individual with posterior probabilities for a given class; Cl266; Cl242 and Cl254: chlorotype of *P. canariensis*, *P. dactylifera* occidental and *P. dactylifera* oriental, respectively.

3.2. The Feasibility of SSR Markers for the Detection of a Hybrid Signal in Phoenix canariensis

Some SSR loci revealed very similar allelic frequencies between the three morpho-geographic types (e.g., PdCUC3-ssr1, mPdIRD013 or mPdIRD057). However, seven loci showed alleles with contrasting frequencies between *P. canariensis* (frequency > 0.9) and *P. dactylifera* (frequency = 0). These loci were mPdCIR015 (P15A and P15B), mPdCIR016 (P16), mPdCIR035 (P35), PdAP3-ssr-F4 (F4), mPdIRD033 (P33) and PdAG1-ssr (PAG1) (Table 2).

Table 2. Allele frequency of loci that characterize the *Canarian nuclear genotype*. PC: *Phoenix canariensis*; HB: *hybrids*; PD242: *P. dactylifera Occidental*; PD254: *P. dactylifera* Oriental. In bold, allele frequencies higher than 0.900.

Locus	Abbreviated Name	Allele	PC	HB	PD242 242	PD254 254
mPdCIR010	P10	*128*	0.478	0.455	0.000	0.000
		129	0.292	0.188	0.000	0.000
		131	0.068	0.034	0.000	0.000
mPdCIR015	P15A	*119*	**0.978**	0.795	0.000	0.000
	P15B	*151*	**0.980**	0.830	0.000	0.000
mPdCIR016	P16	*111*	**0.970**	0.801	0.000	0.000
mPdCIR035	P35	*175*	**0.994**	0.869	0.000	0.000
mPdCIR063	P63	*139*	0.179	0.182	0.000	0.000
		141	0.482	0.403	0.000	0.000
		143	0.227	0.119	0.000	0.000
mPdCIR085	P85	*147*	0.002	0.000	0.000	0.000
		149	0.694	0.608	0.000	0.000
		151	0.125	0.074	0.000	0.000
		153	0.028	0.000	0.000	0.000
		155	0.048	0.011	0.000	0.000
		161	0.033	0.000	0.000	0.000
PdAP3-ssr-F4	F4	*229*	**0.994**	0.847	0.000	0.000
mPdIRD031	P31	*343*	0.002	0.000	0.000	0.000
		355	0.009	0.006	0.000	0.000
		360	0.045	0.034	0.000	0.000
		363	0.017	0.011	0.000	0.000
		366	0.664	0.608	0.000	0.000
		368	0.141	0.142	0.000	0.000
		372	0.099	0.006	0.000	0.000
mPdIRD033	P33	*195*	**0.976**	0.858	0.000	0.000
PdAG1-ssr	PAG1	*220*	**0.911**	0.574	0.081	0.000
mPdIRD040	P40	*199*	0.124	0.142	0.000	0.000
		205	0.354	0.250	0.000	0.000
		211	0.509	0.358	0.000	0.000
		217	0.004	0.000	0.000	0.000

Some of these alleles were found in varying proportions in the putative hybrids. The locus mPdCIR015 (P15) presented four peaks in some individuals of *P. canariensis* and in some of the putative hybrids; therefore, it was considered as a putative duplication, being treated as two independent loci (P15A and P15B). Interestingly, none of the specimens considered as molecularly pure *P. dactylifera* had duplication of the mPdCIR015 (P15) locus. That is, they all had only two alleles (which were included in the P15A locus; Table 2). However, all *P. canariensis* specimens and all molecular hybrid specimens had four alleles for this P15 locus (distributed in loci P15A and P15B; Table 2). Other loci, mPdCIR010 (P10), mPdCIR063 (P63), mPdCIR085 (P85), mPdIRD031 (P31) and mPdIRD040 (P40), harboured more than one predominant allele in *P. canariensis*, but all these alleles were exclusives of Canarian species. Moreover, the sum of these allele frequencies ranged between 0.838 for mPdCIR010 (P10) and 0.991 for mPdIRD040 (P40) in *P. canariensis*, and they were not detected in *P. dactylifera* (Table 2). The loci and alleles of this group were considered as a tracer of genetic purity in the Canarian endemism, defining altogether the CNG. Therefore, we classified the samples into three different molecular types according to the proportion of CNG alleles: Pure *P. canariensis* specimen, showing a total of between 22 and 24 alleles of the CNG (i.e., 90–100% of the CNG alleles). On the contrary, a pure *P. dactylifera* specimen

harboured a range of alleles between zero and two CNG alleles (i.e., 0–10%). Between these two values (9 to 21 CNG alleles), we classified samples as hybrid specimens but with different degrees of hybridization or genetic introgression. No hybrids were found harbouring between three and eight CNG alleles (Table 3).

Table 3. Distribution of the Canarian nuclear genotype (CNG) in molecular assignation and morphologic type groups. No. CNG: number of alleles of Canarian nuclear genotype. NT: total number of individuals. Morphology group: PD: *Phoenix dactylifera* (both types, Oriental and Occidental). PC_GC: *Phoenix canariensis* from Gran Canaria. PC_TF: *Phoenix canariensis* from Tenerife Island. HB_GC: hybrids from Gran Canaria. HB_TF: hybrids from Tenerife. PC: *Phoenix canariensis* (both islands). HB: hybrids (both islands).

Molecular Assignation	No. CNG	NT	Chlorotype				Morphology Group				% Morphotype Match		
			C266	C242	C254	PD	PC GC	PC TF	HB GC	HB TF	PC	HB	PD
Phoenix canariensis pure	22–24	275	264	11	0	0	151	83	20	21	85.1	14.9	0
Molecular hybrids	9–21	77	47	27	3	0	18	19	29	11	48.1	51.9	1
Phoenix dactylifera pure	0–2	81	0	44	37	74	0	0	2	5	0	7.3	92.7
Total	-	433	311	82	40	74	169	102	51	37			

The vast majority of specimens of pure *P. canariensis* with 22–24 CNG alleles also harboured chlorotype 266. Only 11 of 275 specimens with 22–24 CNG alleles (4%) showed chlorotype 242, but they had morphological traits of *P. canariensis*. Then, among the samples with 10–22 CNG alleles that were classified as hybrids, 47 samples (61.0%) harboured chlorotype 266, 35.1% harboured chlorotype 242 and only 3 hybrid specimens (3.9%) had the eastern chlorotype (C254). In addition, the Bayesian inference largely agreed with the molecular types established on the samples. Furthermore, the samples attributed to hybrids with the date palm chlorotype (Clp242 and Clp254) were more consistently assigned to Bayesian hybrid clusters than those with the Canarian palm chlorotype (Clp266). This seems to indicate that introgression from date palms in Canarian palms with Clp266 is less detectable in appearance than that in hybrids and introgression from mother date palm linages (e.g., Clp242 and Clp254).

Lastly, when comparing the established morpho-geographic and molecular types, in general, a high proportion (85.1% to 92.7%) of pure molecular types agree with the morpho-geographic type, suggesting a scenario in which it is likely to detect the correct species by the morphological traits (Table 3). No individual morphologically classified as *P. dactylifera* was included as a pure *P. canariensis* molecular type and vice versa; only a few proportions (7.3% to 14.9%) of pure molecular type individuals were wrongly attributed to hybrid morpho-geographic type individuals. However, the matching success significantly decreases when we consider the hybrid molecular type, in which only 51.9 were correctly assigned to putative hybrids based on their morphological traits (Table 3).

4. Discussion

In this study, the molecular markers used generated substantial genetic differentiation to reliably characterize *P. canariensis* and *P. dactylifera* and infer their putative hybrids. Of the 217 alleles detected, more than half were specific to one of the two species, as suggested by the allelic loadings of the PCA (Figure 1b). Overall, our data supported previous expectations in the *Phoenix* genus about the ability of the SSR markers to discriminate *Phoenix* species [3,7,8,10]. Out of the 20 loci, 12 of them, named the Canarian nuclear genotype (CNG), exhibited 30 specific alleles for *P. canariensis* and, consequently, were considered as genetic tracers of the purity of this species, which appeared at different frequencies in the hybrid individuals (Table 2). Nevertheless, this set of diagnostic alleles

should be treated as sample-specific markers to the *P. canariensis*—*P. dactylifera* complex, since some alleles not found in *P. dactylifera* could exist in other *Phoenix* species.

We observed that the most frequent chlorotype within the *P. canariensis pure type* was the 266 bp fragment (Cl266), confirming its specificity in the Canarian palm (Figure S2), which agrees with previous studies with smaller sample sizes representing the Canarian endemism [3,26–28]. Nevertheless, some exceptions to this trend were observed in a very small sample of 11 specimens with the chlorotype 242, showing at the same time the CNG and morphological traits of *P. canariensis* (Table 3). These specimens were detected in three localities of Gran Canaria, Acusa (7), Telde (1) and Arucas (3), which coincidentally harbour several of the hybrids detected in this study and wild date palms, coinciding with the fact that specimens of uncertain origin have been planted in the surrounding area. We did not find this molecular type in any other population from Tenerife or the La Gomera islands, despite the large number of *Phoenix* specimens analysed so far by our research group. Considering that we cannot completely rule out that these specimens could be pure Canary palm individuals with the chlorotype 242, we are inclined to think that they could be in fact ancestral hybrids, whose primary parents were a female *P. dactylifera* (Clp242) and a male *P. canariensis* (Clp266). Consequently, current individuals were then generated by continuous crossbreeding by several generations of backcrossing to the more abundant *pure P. canariensis* palms, diluting the original hybrid signal in the nuclear genome but maintaining the 242 chlorotype by maternal chloroplast inheritance. The discordance among the nuclear and plastid genes is very common in hybrid lineages, a process referred to as chloroplast capture, which would perfectly explain these results [36–38].

The rest of the hybrids found fit into a very varied casuistry, although all 77 specimens detected as true molecular hybrids seem to come from the crosses between specimens of *P. canariensis* and *P. dactylifera*, since we only detected the exclusive chlorotype of these two species (Clp242, Clp254 or Clp266), but none from other *Phoenix* species [26]. The Bayesian analyses showed that the detection of backcross hybrids and evidence of historic introgression between *P. dactylifera* and *P. canariensis* are complex, and not limited to the F_1. It is very difficult to determine the exact origin of the *P. dactylifera* specimens introduced into the Canary Islands. However, it is important to mention that the aboriginal Canary Islanders were Berbers from North Africa, who had relevant knowledge of agriculture. They arrived on the islands some almost 1800 years ago, with goats, cultivated various vegetable "crop packages", especially hulled barley (*Hordeum vulgare*), durum wheat (*Triticum durum*) or lentil (*Lens culinaris*) among others [39]; therefore, we cannot rule out the possibility that they brought seeds of *Phoenix dactylifera* from their places of origin (North of Africa) and cultivated them. Under this scenario, it is likely to think that hybrid palms were generated more than 1500 years ago. More recently, we do know, however, that most of the palms imported into the Canary Islands as ornamental elements are mainly from the Mediterranean coast, where the western date palm type prevails. This is possibly part of the reason why most of the date palms in the Canary Islands are Clp242, with a scarce representation of eastern individuals. However, it is interesting to note that the western chlorotype of date palm (Clp242) was not observed in samples collected in Tenerife (Figure S2). Furthermore, the eastern specimens found in Tenerife are in the same locality (Rambla de Castro), a cultivated population that was created in a very recent period. Anyway, taking to account the great amount of F2 hybrids or backcrosses with *P. canariensis* detected in this study, it would be interesting to study the evolutionary implications of late-generation hybrids such as a possible loss of fertility in *P. canariensis*, as well as the environmental conditions that favour the anthropogenic hybridization [40].

Hybrids and specimens of *P. dactylifera* are widely distributed throughout the Canary Islands, either for historical reasons, in which the aboriginal Canary Islanders brought seeds of *P. dactylifera*, or due to their very recent introduction in tourist and urban areas as ornamentals. Most of these specimens are found in urban and peri-urban areas, where massive planting of these specimens has been very important. The effect of these urban specimens on the genetic conservation of the natural populations of *P. canariensis* is nil, due

to the remoteness and, therefore, certain isolation from the natural palm groves. However, many other hybrid specimens (and date palms) grow in areas close to pure *P. canariensis* palm groves, so the threat of hybridization could progressively increase in these areas. The lack of knowledge and the difficulty of morphologically identifying the species to which the young individuals belong has led to the plantations established in semi-natural areas becoming areas of strong hybridization (for example, Rambla de Castro in Tenerife or Acusa in Gran Canaria).

In conclusion, we detected a molecular marker set that unambiguously serves as a tool to identify and separate both species and their hybrids, especially when morphological identification is in doubt. We also recommend the use of this molecular set to check the levels of genetic introgression in the natural palm groves of the Canary Islands. The use of this group of molecular markers could be implemented permanently in the official identification of the natural palm groves of *Phoenix canariensis* as seed sources for the conservation of the genetic resources of *Phoenix canariensis* in the Canary Islands archipelago.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d16070411/s1: Figure S1: Morphological differences between *Phoenix dactylifera* and *P. canariensis* [41]; Figure S2: Number of individuals of different chlorotypes for each population; Figure S3: Number of individuals in each molecular category, according to the number of CNG alleles.

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