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Elite Genotype Characterization and Genetic Structure Analysis of the Medicinal Tree *Archidendron clypearia* (Jack) I. C. Nielsen Using Microsatellite Markers

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Abstract: *Archidendron clypearia* (Jack) I. C. Nielsen is an important tree species for traditional medicine purposes such as anti-inflammatory, detoxification, uric acid control and neuro-protection. Here, a set of 15 microsatellite markers were used to fingerprint 248 elite genotypes from six origins of *A. clypearia* and investigate the genetic structure of these genotypes. A total of 170 alleles were amplified. The elite genotypes showed moderate genetic variability, with mean observed heterozygosity (H_o) and expected heterozygosity (H_e) being 0.651 and 0.699, respectively. Each of the elite genotypes could be fingerprinted uniquely. The cumulative probability of identity (*PI*) and the cumulative probability of paternity exclusion (*PE*) were 2.25×10^{-12} and 0.999992, respectively, demonstrating the extremely high power of the markers for genotype identification. In STRUCTURE and clustering analyses, the same origin of elite genotypes tended to be grouped in a sub-population and clustered closely, but some genotypes from different geographic origins were mixed, suggesting a low level of genetic differentiation between the origins. These results can contribute to the protection and management of the elite genotype resources of *A. clypearia* and the comprehensive understanding of its genetic structure.

Keywords: *Archidendron clypearia*; medicinal tree; microsatellite marker; elite genotype; fingerprinting; genetic structure

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1. Introduction

Archidendron clypearia (Jack) I. C. Nielsen (syn. *Pithecellobium clypearia* Benth, family Leguminosae Juss. or Fabaceae Lindl.) is an important medicinal tree species that has a wide distribution across the Asian tropics [1]. It can reach 20 m in height and 50 cm in breast-high diameter [2]. Its branches, leaves and twigs serve as excellent herbal sources of such chemical compounds as flavonoids, phenolics and terpenoids for pharmaceutical purposes including anti-inflammatory, detoxification [3], uric acid control [4] and neuro-protection [5]. Also, the tree is valuable for other industrial applications, for instance, pulp and paper, furniture and veneers from its wood, as well as tannins from its bark [2]. In addition, the herbal residues can be reused for the production of several composites, e.g., fiber/polypropylene composite [6].

Raw herbal materials of *A. clypearia* have been collected mainly from natural forests over a long period of time, which, in addition to the deforestation caused by human activities, leads largely to a sharp decrease in natural resources [2]. Though artificial plantations have commenced in some regions, the planting stock is usually of unimproved seedlings raised with seeds from natural forests or earlier plantations. In order to make sustainable utilization of *A. clypearia* resources and exploit the greater economic potential, it is

imperative to carry out breeding programs and develop improved varieties with higher biomass and medicinal value. In this respect, efforts have been directed toward the collection of germplasm, field trials of the germplasm collections [2] and development of microsatellite (or simple sequence repeats, SSR) markers for germplasm diversity investigation [7]. Some preliminary progress has been achieved nowadays, for instance, more than 200 elite trees have been selected for vegetative propagation, and a breeding population has been established.

Vegetative propagation of elite genotypes allows for the creation of clonal cultivars and thus facilitates the quick realization of genetic gains from large-scale clonal plantations. As misuse of planting material may result in economic damage and intellectual-property dispute, the accurate identification of clones is essential for the registration and certification of newly released varieties and the protection of the legitimate interests of breeders, growers and the industry [8,9]. In addition, such an identification can be extraordinarily useful for the authentication of medicinal herbs and consequently for distinguishing the genuine material from adulterants, substitutes and spurious drugs [10]. A variety of methods have been used for plant cultivar identification, such as morphology traits, biochemical markers and DNA-based molecular markers [11]. As morphological traits are usually ambiguous, difficult and time-consuming to measure, and biochemical markers are of limited number, molecular markers have been widely adopted in recent decades. Among the multitude of molecular marker types, SSR has been widely recognized to meet the criteria of plant-variety DNA profiling [12] due to its characteristics of co-dominance, multiallelism, high reproducibility and abundance within the genome [13,14]. In *A. clypearia*, 456 SSR markers have been developed [7], demonstrating a rich marker resource for the molecular identification of elite genotypes.

Elite genotypes also represent candidate genetic material that can be integrated into the next generation of a breeding population. As information on genetic variation and population structure is critical for efficient breeding and the conservation of genetic resources [15], it has been a major goal to explore such information in plant breeding and genetics studies. Moreover, the presence of population structure, though generally weak for outbreeding tree species [16], may be a potential limitation to the usage of the association mapping of important traits in plants [17]. In forest trees, genetic diversity and population structure have been explored for breeding populations, e.g., in *Eucalyptus cloeziana* F. Muell. [18]. However, only a few studies have been conducted to reveal the genetic diversity and population structure of elite genotypes, such as *Quercus suber* L. [19], *Populus tremula* L. [20] and *Pinus koraiensis* Siebold & Zucc. [21].

In his study, we used a set of 15 SSR markers to genotype 248 elite genotypes of *A. clypearia*. Our objectives were to establish the molecular fingerprint of each of the elite genotypes and investigate their genetic variation and population structure.

2. Materials and Methods

2.1. Plant Materials and DNA Isolation

A total of 248 elite genotypes of *A. clypearia* were leaf sampled for DNA isolation (Table 1). Of these genotypes, 148 were cutting clones propagated from super trees selected from local plantations at Huadu District (HDp; 23°26′ N, 113°13′ E), Guangdong Province, China, and coded as HDp001–148; the remaining 100 were elite sibs of open-pollinating families (six natural origins; Table 1) selected from a 5-year-old provenance/family trial at Huadu District (HD). The selection of these elite genotypes was based on their superior growth in tree height and breast-high diameter under an approximate selection intensity of 10%. Tree growth is usually considered to be related to branchlet and leaf biomass, which represents the harvest productivity for medicinal usage in *A. clypearia*.

Table 1. Origins of the 248 *Archidendron clypearia* (Jack) I. C. Nielsen elite genotypes.

Serial No.	Origin	Latitude (N)	Longitude (E)	N_{OPfam}	N_{Egeno}
1	Jianfengling National Forest Park (JNFP)	18°45′	108°50′	16	22
2	Erlongshan Ecological Park (EEP)	23°21′	113°44′	21	51
3	Huadu District (HD)	23°26′	113°13′	2	4
	Plantations at Huadu District (HDp)	23°26′	113°13′	–	148
4	Baijianghu Forest Park (BFP)	23°30′	113°55′	5	18
5	Dalingshan Forest Farm (DFF)	23°38′	113°46′	1	2
6	Liangkou Forest Farm (LFF)	23°43′	113°44′	2	3
Total				47	248

JNFP is located in Hainan Province while the other five origins are from Guangdong Province, China. N_{OPfam}: number of open-pollinating families; N_{Egeno}: number of elite genotypes.

Genomic DNA was isolated from about 300 mg of leaf sample of each genotype using a modified cetyltrimethyl ammonium bromide method [22]. DNA quality and quantity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. SSR Markers and Elite Clone Genotyping

A set of 15 expressed sequence tag (EST)-derived SSR (EST-SSR) markers (Table 2) was used for genotyping the 248 elite genotypes. These markers had been also used for the genetic analysis of two *A. clypearia* populations [7]. Forward and reverse primers of each of the EST-SSR markers were synthesized by Generay Biotechnology (Shanghai, China), with the forward primer labeled at the 5′ end with fluorescent dye TAM, HEX, ROX or 6-FAM.

Table 2. The fifteen simple-sequence repeat (SSR) markers used and their statistical estimates based on the 248 *Archidendron clypearia* (Jack) I. C. Nielsen elite genotypes.

Serial no.	SSR Marker	*ASR* (bp)	N_a	N_e	H_o	H_e	*NG*	*NC*	*PI*	*PE*
1	ARCeSSR006	159–179	11	1.606	0.385	0.377	18	7	0.184	0.520
2	ARCeSSR075	133–155	8	3.672	0.711	0.728	21	8	0.140	0.587
3	ARCeSSR095	181–209	18	6.517	0.837	0.847	13	3	0.159	0.556
4	ARCeSSR141	117–149	9	3.974	0.765	0.748	29	12	0.199	0.477
5	ARCeSSR266	163–201	10	3.869	0.482	0.742	8	2	0.128	0.603
6	ARCeSSR277	121–145	13	3.053	0.615	0.672	10	3	0.225	0.467
7	ARCeSSR288	184–210	6	1.899	0.496	0.473	8	4	0.137	0.592
8	ARCeSSR304	178–202	16	4.568	0.837	0.781	10	1	0.115	0.622
9	ARCeSSR366	119–155	9	4.753	0.815	0.790	11	4	0.405	0.309
10	ARCeSSR425	144–162	12	2.841	0.692	0.648	9	1	0.208	0.485
11	ARCeSSR448	278–302	8	3.396	0.582	0.706	12	2	0.151	0.558
12	ARCeSSR464	102–122	14	4.159	0.525	0.760	20	7	0.086	0.676
13	ARCeSSR474	157–181	9	3.454	0.643	0.710	5	3	0.173	0.535
14	ARCeSSR649	285–306	12	3.226	0.506	0.690	14	8	0.236	0.454
15	ARCeSSR665	105–132	15	5.226	0.866	0.809	16	1	0.134	0.593
Total or Cumulative			170				248	248	2.25×10^{-12}	0. 999992
Mean (±SE)			11.3 (±0.9)	3.747 (±0.321)	0.651 (±0.039)	0.699 (±0.032)	–	–	0.179 (±0.019)	0.536 (±0.022)

The primer sequence and repeat motif for the SSR markers were as reported earlier [7]. *ASR*, allele size range; N_a, number of alleles; N_e, number of effective alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; *NG*: number of genotypes; *NC*: number of clones with a unique genotype; *PI*: probability of identity; *PE*: paternity exclusion probability; SE, standard error.

Polymerase chain reaction (PCR) was carried out in a 10 µL volume, containing 1.0 µL 10× buffer (100 mM Tris-HCl pH9.0, 80 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgCl_2$ and 0.5% NP-40), 0.2 µL 10 mM each of the four dNTPs, 0.5 µL 5.0 µM each of the forward and reverse primers, 0.1 µL 10 U/µL *Taq* DNA polymerase (Biocolors Technology Co., Shanghai, China), 0.2 µL 50 ng/µL DNA template and 6.9 µL deionized water. PCR amplification was performed on a thermal cycler DNA Engine (Bio-Rad, Hercules, CA, USA) following the program: 4 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 or 60 °C and 50 s at 72 °C;

and finally, 5 min at 72 °C. A mixture of 1 µL PCR product, 0.16 µL GeneScan 500LIZ standard and 9.34 µL deionized formamide was denatured at 95 °C for 5 min followed by immediate cooling on ice and then used for allele calling on a genetic analyzer ABI 3130xl using GeneMapper 4.1 (Applied Biosystems, Foster City, CA, USA).

2.3. Data Analysis

For each SSR marker, the number of alleles (N_a), number of effective alleles (N_e), allele size range (*ASR*), observed heterozygosity (H_o), expected heterozygosity (H_e) and fixation index (*F*) across the 248 elite genotypes were calculated using GenAlEx 6.5 [23]. The probability of identity (*PI*) was estimated using GENECAP [24]. The least number of markers for the effective identification of genotypes was determined empirically through the progressive selection of markers for distinguishing the closest genotypes.

Principal coordinate analysis (PCoA) was performed for the six origins using GenAlEx 6.5 [23]. Genetic structure was investigated using STRUCTURE v2.3.4 [25] based on an admixture model with the length of the burn-in period at 10,000 and the number of Markov chain Monte Carlo replications after burn-in at 100,000. Ten iterations were run for a number (K = 1–6) of homogeneous clusters. The most probable K value was determined using the highest ΔK method [26] in STRUCTURE HARVESTER 0.6 [27] and then used for the estimation of the membership coefficient of each individual genotype. In addition, a matrix of Nei's genetic distance values [28] between the genotypes was generated in PowerMarker 3.25 [29], which was then used for constructing a dendrogram based on the unweighted pair-group method with arithmetic mean (UPGMA) in MEGA 11 [30].

3. Results and Discussion

3.1. Characterization of the Elite Genotypes

The 15 SSR markers amplified a total of 170 alleles across the 248 *A. clypearia* elite genotypes (raw data shown in Supplementary Dataset), averaging at 11.3 alleles per marker (Table 2). The mean H_o and H_e over the 15 SSRs were 0.651 and 0.699 (Table 2), respectively, indicating the moderate genetic variability of these elite genotypes.

For each of the 15 SSR markers, *NG*, *NC*, *PI* and *PE* estimates are also given in Table 2. The two markers ARCeSSR304 and ARCeSSR288 were the most informative, as the former showed the highest *NG* (29) and *NC* (12) and the latter the lowest *PI* (0.086) and the highest *PE* (0.676). The cumulative *PI* was as low as 2.25×10^{-12}, corresponding to a cumulative *PE* of 0.999992, demonstrating the extremely high power of clone identification. In addition, the 248 genotypes could be fingerprinted uniquely with a minimum of seven EST-SSR markers, namely, ARCeSSR075, ARCeSSR095, ARCeSSR141, ARCeSSR266, ARCeSSR277, ARCeSSR304 and ARCeSSR665. The minimum set of markers could be used as core markers with the rest as candidate markers for fingerprinting purposes [31].

Genotypic fingerprints are necessary for the registration and certification of newly released varieties and also for cultivar identification in the process of propagation and commercial usage [22,32]. The advantages of DNA markers over morphological traits support the utility of DNA-based approaches in the accurate description of varieties, including independently derived new candidate varieties [33]. Thus, the fingerprints of the 248 elite genotypes established herein offer a solid technical basis on which the legitimate interests of breeders, growers and the industry can be guaranteed. Moreover, such fingerprints outperform chemical fingerprints which may give incorrect variety identification due to chemical-composition variation arising from age and environment conditions and, along with other DNA-based methods like the bar-coding technique, can act as the best way to authenticate the desired herbal adulterants and prevent the intentional and inadvertent substitution of targeted medicinal herbs [10,34].

3.2. Genetic Structure of the Elite Genotypes

The PCoA and STRUCTURE analysis results are shown in Figure 1. In the PCoA, the first and second principal coordinates explained 46.9% and 19.2%, respectively, of the total genetic variability among the six origins of elite genotypes (Figure 1a). At the first coordinate, the six origins can be divided into two groups, with JNFP independent of the other five origins. This may reflect the fact that JNFP in Hainan Island is isolated from the other mainland origins. In the STRUCTURE analysis, the highest ΔK value was observed at $K = 2$ (Figure 1b), suggesting the most probable division of the entire genotype set into two sub-populations (Figure 1c). Sub-populations 1 and 2 contained 139 and 109 genotypes, respectively. The admixture of genotypes from different geographic origins may indicate a low level of genetic differentiation between origins and a weak genetic structure.

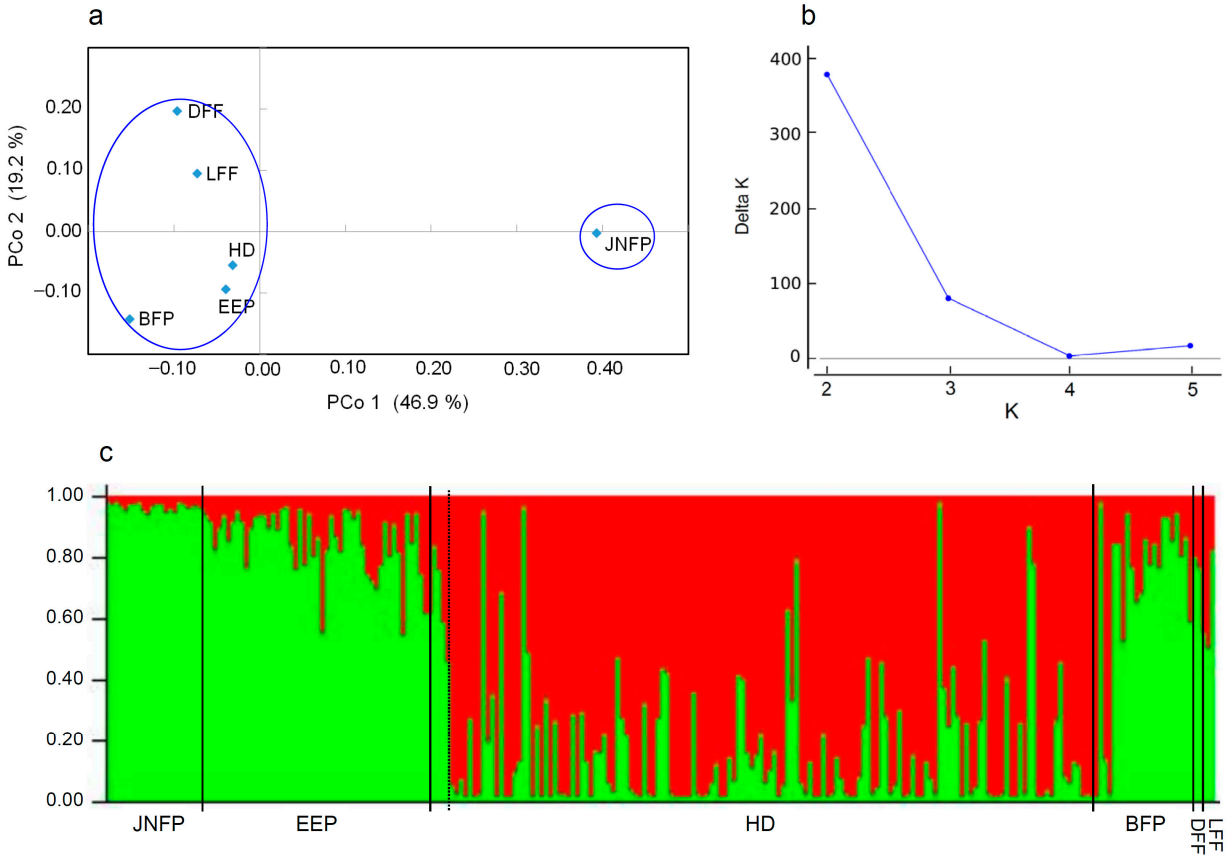

Figure 1. Genetic structure for the six origins and the 248 *Archidendron clypearia* (Jack) I. C. Nielsen elite genotypes based on 15 microsatellite markers: (**a**) principal coordinate analysis (PCoA) for the six origins (blue circle showing the two groups divided at PCo 1); (**b**) ΔK change with different K values for the estimation of optimal ($K = 2$) in the STRUCTRE analysis; (**c**) ancestry coefficients for the 248 elite genotypes determined at $K = 2$, with the origin abbreviations (as described in Table 1) shown on the *x*-axis (green and red color representing ancestry coefficient of a genotype to sub-population 1 and 2, respectively).

Further, the UPGMA dendrogram provided greater details on the genetic relationship among the elite genotypes. Two clusters were divided (Figure 2). Cluster 1 comprised all the 18 and two genotypes of BFP and DFF origins, respectively, plus six HDp- and one EEP-originated genotypes, while Cluster 2 contained all the remaining genotypes that could be further divided into seven main sub-clusters exclusive of the eight relatively independent genotypes (name in black in Figure 2). Sub-clusters 2.1–2.7 were dominated with HDp-, EEP-, JNFP-, EEP-, EEP-, EEP- and JNFP-originated genotypes, respectively.

The same origin of genotypes tended to be clustered closely, e.g., BFP, EEP and HD. Specifically, the majority of genotypes from the same open-pollinating family were clustered together or very closely, indicating the genetic affinity of half-sibs. However, similar to the STRUCTURE analysis, some genotypes from the same origin were mixed with other origins in the UPGMA dendrogram, which may be a result of the low differentiation between geographic origins. As noted, relatively low genetic differentiation was observed between EEP and the most distant JNFP natural populations in *A. clypearia* [7].

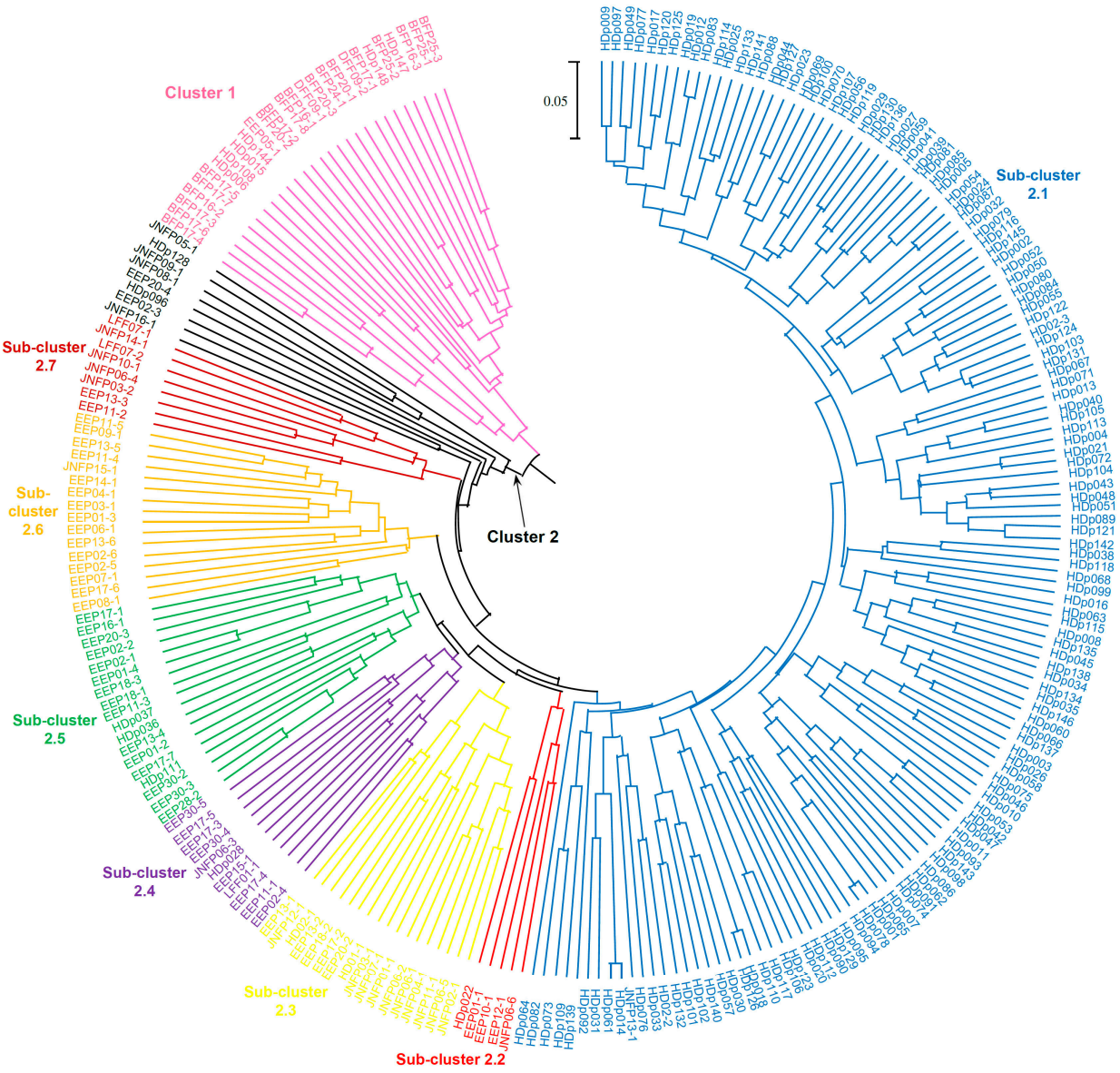

Figure 2. Unweighted pair-group method with arithmetic mean (UPGMA) dendrogram of the 248 *Archidendron clypearia* (Jack) I. C. Nielsen elite genotypes based on their Nei's genetic distance [28]. The genetic distance (0.05) scale bar is shown on the top left of Sub-cluster 2.1.

Genotypes of the same origin were also observed to be divided into different sub-populations and clustered distantly in *E. cloeziana* [18] and *Vitis vinifera* L. [35]. Moreover, the UPGMA dendrogram was somewhat different from the division of sub-populations in the STRUCTURE analysis, with genotypes from different sub-populations grouped closely. Nevertheless, these approaches lead complementarily to a comprehensive understanding of the genetic relationship among elite genotypes [36]. In addition, the UPGMA dendrogram

could discriminate clearly the 248 genotypes from each other, revealing the power of EST-SSR markers for clone fingerprinting and identification.

4. Conclusions

In the present study, a moderate genetic variability was revealed over 248 elite genotypes from six geographical origins of *A. clypearia*. Each elite genotype can be fingerprinted uniquely based on a minimum set of seven microsatellite markers. The same origin of elite genotypes tended to be grouped in a sub-population and clustered closely, but some genotypes from different origins were mixed, indicating a low level of genetic differentiation between origins and a weak genetic structure. The information generated here can contribute to the protection and management of the elite genotype resources of *A. clypearia* and a comprehensive understanding of their genetic structure.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15071168/s1, Dataset: Combination of alleles (fingerprints) of the 248 A. clypearia elite genotypes based on 15 simple sequence repeats (SSR) markers.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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