agriculture

MDPI

Article

Validation of Genome-Wide SSR Markers Developed for Genetic Diversity and Population Structure Study in Grain Amaranth (*Amaranthus hypochondriacus*)

Gautam Vats [1], Dimpi Das [1], Rajat Gupta [1], Akshay Singh [1], Avantika Maurya [1], S. Rajkumar [1], Amit Kumar Singh [1], Rakesh Bharadwaj [2], Sandeep Kumar [2], Surinder Kumar Kaushik [2], Veena Gupta [3], Kuldeep Singh [4] and Rakesh Singh [1,*]

[1] Division of Genomic Resources, ICAR-National Bureau of Plant Genetic Resources, Pusa, New Delhi 110012, India
[2] Division of Germplasm Evaluation, ICAR-National Bureau of Plant Genetic Resources, Pusa, New Delhi 110012, India
[3] Division of Germplasm Conservation, ICAR-National Bureau of Plant Genetic Resources, Pusa, New Delhi 110012, India
[4] International Crop Research Institute for the Semi-Arid Tropics, Hyderabad 502324, India
* Correspondence: rakesh.singh2@icar.gov.in; Tel.: +91-011-25802791; Fax: +91-011-25842495

Abstract: Grain Amaranth is the most promising C_4 dicotyledonous pseudocereal and is distributed globally. It has an excellent nutritional profile and adaptability against a broad range of environmental factors. These traits have renewed the interest of researchers and breeders in exploring this underutilized orphan crop. The present study aimed to validate the genome-wide SSR to assess the genetic diversity among 94 *Amaranthus hypochondriacus* accessions using 57 genomic SSR (g-SSR) markers developed in-house. A total of 36 g-SSRs were recorded as polymorphic and amplified 138 alleles, with an average of 3.83 alleles per locus. Major allele frequency ranged from 0.29 to 0.98, with an average of 0.63 per marker. The expected heterozygosity ranged from 0.03 to 0.81, with an average of 0.46 per locus. Polymorphism information content (PIC) ranged from 0.03 to 0.79, with an average of 0.40, indicating a high level of polymorphism across amaranth accessions. Population structure analysis resulted into two major genetic clusters irrespective of their geographical origin, which suggests there may be sharing of common genomic regions across the accessions. High allelic frequency and heterozygosity levels indicate significant genetic variability in the germplasm, which can be further used in future breeding programs.

Keywords: g-SSR; grain amaranth; PIC; *Amaranthus hypochondriacus*; genetic diversity; PCoA; population structure

check for updates

Citation: Vats, G.; Das, D.; Gupta, R.; Singh, A.; Maurya, A.; Rajkumar, S.; Singh, A.K.; Bharadwaj, R.; Kumar, S.; Kaushik, S.K.; et al. Validation of Genome-Wide SSR Markers Developed for Genetic Diversity and Population Structure Study in Grain Amaranth (*Amaranthus hypochondriacus*). *Agriculture* **2023**, *13*, 431. https://doi.org/10.3390/agriculture13020431

Academic Editor: Rodomiro Ortiz

Received: 27 December 2022
Revised: 9 February 2023
Accepted: 9 February 2023
Published: 12 February 2023

1. Introduction

Grain amaranth (*Amaranthus* spp.) is an underutilized, highly nutritious, cross-pollinated pseudocereal that belongs to the family Amaranthaceae, which comprises approximately 60–70 widely distributed species in wild as well as domesticated forms [1]. Since ancient times, a variety of amaranth species have been cultivated for their diverse use as leafy vegetables, grains, ornamentals, and forage crops [2]. Out of the known species, 55 are considered to have originated in the Americas, with the remaining species possibly being native to Europe, Asia, Africa, and Australia [3,4]. Amaranth represents immortality and derives its name from the Greek word "amarantos" because of its potential for growth and fulfillment, even in unfavorable environmental conditions. Amaranth has been proven to be more nutritious than our traditional staple food, rice, and it contains more than three times the average amount of calcium present in most common used cereals. It is also a rich source of iron, magnesium, phosphorus, and potassium [5]. Grain amaranth is recognized

as a super grain, miracle grain, and grain of the future because of its high nutraceutical value. It has higher content of protein, unsaturated oils, dietary fibers, flavonoids, vitamins, and a well-balanced concentration of the essential amino acids, especially lysine, which ranges from 0.73 to 0.84 percent of the total seed protein content and is often considered as a limiting amino acid in other cereal grains [6–8]. Despite having a good-quality amino acid profile, amaranth protein is gluten-free, making it an excellent diet for patients who have coeliac disease [9]. The regular consumption of amaranth seed or seed oil provides a significant amount of vitamin E and squalene, which benefit people suffering from hypertension or cardiovascular disease by lowering blood pressure and cholesterol levels and improving antioxidant status [10,11]. Squalene has strong antioxidant properties that protect the skin from premature aging by preventing cell damage [12]. Worldwide recognition of amaranth as an excellent crop is due to its extraordinary nutrient profile, capacity to survive in extremely challenging eco-geographic conditions, and lack of any severe diseases [13,14]. Thus, due to the growing demand for food and rising malnutrition, development of amaranth as an alternative crop could prove to be a boon for developing nations that suffer from malnutrition and overpopulation.

Genetic diversity plays a significant role in the development of superior cultivars for crop improvement. Diversity in plant genetic resources provides an opportunity for plant breeders to develop improved cultivars with variable characteristics, including both farmer-preferred and breeder-preferred traits [15]. Diversity can be evaluated by using morphological, cytological, biochemical, and molecular markers. Previously, morphological markers were used for diversity analysis because they were naturally occurring variants of a particular plant species [16,17]. Afterward, differentiation occurs at the cytological and biochemical level in the genotypes of a particular species to be used in genetic diversity assessment [18,19]. With the rapid advancements in sequencing technologies and bioinformatics analysis in recent years, the development of molecular markers has become the method of choice for genetic diversity assessment because it was much easier and more cost-effective [20]. Therefore, evaluating genetic diversity using molecular markers gives more accurate, quick, and authentic results in less time. Among all molecular markers, simple sequence repeat (SSR) markers have been considered more reliable markers for genetic diversity analysis [21,22]. SSRs, also known as microsatellites, are short DNA sequences consisting of tandem repeats of 1-6 nucleotides and distributed in both coding and non-coding regions of the genome. They are highly polymorphic, multi-allelic, co-dominant, highly reproducible, transferable to related species, and easily detectable with polymerase chain reaction (PCR), making them suitable for the understanding genetic diversity in different plant species such as potato (*Solanum tuberosum*) [23], vetiver grass (*Vetiveria zizanioides* L. Nash) [24], rice (*Oryza sativa*) [25], drumstick (*Moringa oleifera* Lam.) [26], kala bhat (*Glycine max* (L.) Merrill) [27], *Tinospora cordifolia* [28], and guava (*Psidium guajava* L.) [29]. They are important in the construction of linkage maps, diversity assessment, marker-assisted selection, and identifying genetic relationships among closely related species [30–32].

To efficiently utilize plant genetic resources, it is necessary to evaluate genetic divergence among and within the crop species [33]. Amaranths exhibit high morphological diversity among their species and display versatile levels of adaptation to a wide range of environmental factors [34]. Different DNA markers have been employed over time for the analysis of intraspecific and interspecific diversity along with hereditary relationships among different species of amaranth due to their agro-economic importance. However, only a few molecular studies with well-established markers such as random amplified polymorphic DNAs (RAPDs) [35], restriction fragment length polymorphisms (RFLPs) [36], amplified fragment length polymorphisms (AFLPs) [37], single nucleotide polymorphisms (SNPs) [38] and simple sequence repeats (SSRs) [39–41] have been performed in *Amaranthus* species. Identification of potential genotypes for different breeding purposes requires an accurate evaluation of the genetic diversity and population structure of any germplasm to achieve the ultimate goal of sustainable agriculture. Therefore, the present investiga-

tion was undertaken to assess the informativeness of the g-SSR markers and screen them against 94 different accessions of *Amaranth hypochondriacus*, consisting of 92 indigenous and four exotic accessions from USA and Russia, to study their molecular diversity and population structure.

2. Materials and Methods

2.1. Plant Materials

The seed materials of 94 diverse grain amaranth accessions belonging to different Indian states (Himachal Pradesh (42), Uttarakhand (23), Uttar Pradesh (1), Madhya Pradesh (3), Arunachal Pradesh (1), Maharashtra (7), and Gujrat (10)) and four exotic accessions (USA (3) and Russia (1)) were obtained from the National Gene Bank, ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi, as depicted in Figure 1 and Table S1. The seeds were sown in the greenhouse at ICAR-NBPGR in three-dimensional seedling trays with 18 cavities, with each cavity having dimensions of 8 cm × 8 cm × 8 cm.

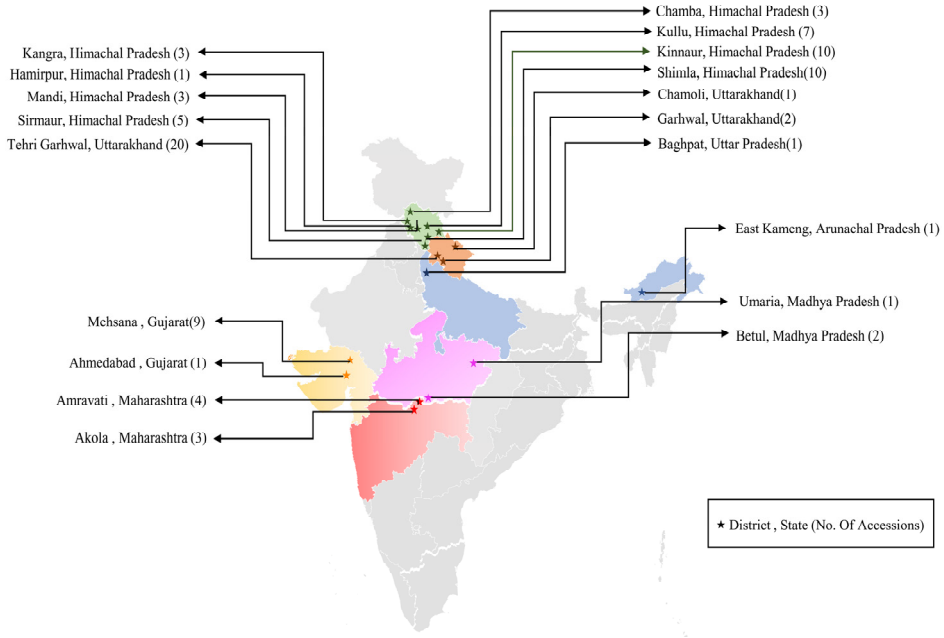

Figure 1. Distribution of grain amaranth accessions across India.

2.2. Plant Genomic DNA Extraction

The fresh and healthy leaves of each accession were collected from 30-day-old seedlings and immediately stored at −80 °C until DNA extraction. The leaves of five seedlings for each accession were pooled for genomic DNA isolation following the Triton-X 100 protocol with slight modifications [42]. The isolated genomic DNA samples were diluted in 1X TE (Tris-EDTA) buffer and stored at a temperature of −20 °C for further use. The quality and quantity of the isolated DNA samples were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The quality of DNA was checked on 0.8% agarose gel, and the final working concentration of each accession was further tuned to 10 ng/µL and stored at 4 °C.

2.3. PCR Amplification and Gel Documentation

The genomic SSR (g-SSR) markers and their PCR primer pairs have been developed using the MISA tool (http://pgrc.ipk-gatersleben.de/misa/, accessed on 15 June 2022) and Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/, accessed on 15 June 2022) software for the development of our in-house database, the Amaranth Genomic Resource Database (AGRDB) (http://www.nbpgr.ernet.in:8080/AmaranthGRD/, accessed on 15 June 2022), using the *A. hypochondriacus* genome version 2.0. A total of 57 g-SSR markers that were

developed in-house were used to assess the genetic variability of the 94 amaranth accessions. The PCR amplification reaction was performed in a total volume of 25 µL, containing 2 µL template DNA (10 ng/µL), 1.5 µL of each forward and reverse primers (10 pmole/µL), and 10 µL of PCR master mix (G-Biosciences, St. Louis, MO, USA) added along with 10 µL of double-distilled water (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to make up the final volume. PCR amplification was carried out using a thermal cycler (G-Storm, Essex, England) according to the following conditions: initial denaturation for 5 min at 95 °C, followed by 30 cycles of denaturation for 40 s at 94 °C, annealing temperature (determined by gradient PCR for each primer pair) for 40 s (Table S2), initial extension for 40 s at 72 °C, and a final extension for 10 min at 72 °C accompanied by cooling temperature of 4 °C. The final amplified PCR products were stained with ethidium bromide and separated on 4% metaphor agarose gel (Lonza, Rockland, ME, USA) along with 100 bp DNA ladder at a constant supply of 120 V for about 3 h in 1 X TAE (Tris-acetate-EDTA) buffer. The pictures of the amplified products on the gel were captured under UV light using a gel documentation system (CLiNX, Shanghai, China).

2.4. Scoring of Markers and Data Analysis

The scoring of PCR-amplified DNA fragments was carried out based on band size (bp) as per Power Marker software format. Different indices of diversity viz., the major allele frequency (M_{AF}), gene diversity or expected heterozygosity, observed heterozygosity, and polymorphism information content (PIC) values for every polymorphic g-SSR marker, were estimated by unbiased estimator using Power Marker v3.5 [43]. The discriminating power of the marker is represented by its PIC value, which is based on the number of amplified alleles and their respective frequency distribution. PIC values were described using the following formula [44].

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2 \ - \sum_{j=1}^{n-1} \sum_{k=j+1}^{n} 2P_{ij}^2 \, P_{ik}^2$$

where P_{ij} and P_{ik} are the frequencies of the *j*th and *k*th alleles for marker *i*.

Percentage Polymorphic Loci (PPL) was calculated using GenAlEx v6.5 [45]. The scored data obtained by selected polymorphic g-SSRs was used to construct a neighbor-joining (NJ) tree based on genetic distances using DARwin v6.0.021 [46] software. The phylogenetic tree was built according to the dissimilarity matrix formed by the application of unweighed neighbor-joining algorithm. Analysis of population structure was performed using the Structure v2.3.4 software [47]. A total of ten runs were carried out for each value of K that was set from 1 to 10 with burn-in iterations of 500,000, accompanied by 500,000 Markov Chain Monte Carlo (MCMC) replications using the admixture model. The Structure Harvester (https://taylor0.biology.ucla.edu/structureHarvester/ accessed on 16 November 2022) was used to obtain the possible number of subpopulations by ΔK methodology.

Analysis of Molecular Variance (AMOVA) is a technique for studying molecular variance within a species. It was performed to calculate the genetic variability among and within the populations using GenAlex v6.5. Principal coordinates analysis (PCoA) is a method of multidimensional scaling (MDS) which uses a similarity matrix or dissimilarity matrix to assign a specific position to each variable in a low dimensional space. In our present study, PCoA was also carried out using GenAlex v6.5 to assess percentage of genetic variance based on dissimilarity indices.

3. Results

3.1. Genetic Diversity Indices for g-SSR Markers

A total of 94 accessions of *A. hypochondriacus* were analyzed using 57 g-SSR markers that were developed in silico in our laboratory. Among them, 36 g-SSRs produced polymorphic alleles, and they were consequently used for molecular characterization (Table S2). For the diversity analysis, parameters such as major allele frequency (M_{AF}), gene diversity

(GD), observed heterozygosity (Ho), and PIC were computed and are summarized in Table 1. A total of 138 alleles were amplified across 36 g-SSR markers, with a mean value of 3.83 alleles per locus. The major allele frequency showed a range of 0.28 to 0.98 with an average of 0.63. The gene diversity (expected heterozygosity) calculated among 36 g-SSR loci varied from 0.03 to 0.81, with a mean of 0.46 per locus. The g-SSR marker AhySSR39451 showed the maximum gene diversity (0.81), while the minimum value (0.03) was recorded in AhySSR31577. The value of observed heterozygosity was lowest in AhySSR31577 (0.016) and the highest in AhySSR5081, AhySSR18586, AhySSR29571 (1.0), with a mean value of 0.61 per locus. The values of polymorphism information content (PIC) showed a range from 0.03 (AhySSR31577) to 0.78 (AhySSR29571) with a mean value of 0.40. Ten g-SSRs markers viz. AhySSR0595, AhySSR5081, AhySSR18586, AhySSR19060, AhySSR23586, AhySSR29093, AhySSR29571, AhySSR43465, AhySSR49996 and AhySSR39451, had PIC values greater than 0.5 for the amaranth accessions, indicating superior discriminatory power as compared to others (Table 1 and Figure S1).

Table 1. Characteristics and genetic diversity indices of 36 g-SSR loci used for genotyping 94 accessions of *A. hypochondriacus*.

S.No.	Marker Name	Scaffold Number	g-SSR Start Position	g-SSR End Position	M_{AF}	Allele No	GD	Ho	PIC
1	AhySSR0595	Scaffolds_1	5688613	5688666	0.4679	3	0.6304	0.9103	0.5557
2	AhySSR3176	Scaffolds_1	23101765	23101806	0.5054	2	0.4999	0.9891	0.375
3	AhySSR4597	Scaffolds_1	35176079	35176178	0.5915	4	0.4977	0.7683	0.391
4	AhySSR0261	Scaffolds_1	2618226	2618270	0.5068	2	0.4999	0.9315	0.375
5	AhySSR1363	Scaffolds_10	22467372	22467407	0.6902	2	0.4276	0.5761	0.3362
6	AhySSR33457	Scaffolds_10	39900	39915	0.7321	2	0.3922	0.5119	0.3153
7	AhySSR37960	Scaffolds_11	9628916	9628945	0.764	3	0.3656	0.427	0.3065
8	AhySSR39451	Scaffolds_11	22234840	22234866	0.2865	8	0.8102	0.8539	0.7845
9	AhySSR38506	Scaffolds_11	14445677	14445715	0.978	2	0.043	0.044	0.0421
10	AhySSR0471	Scaffolds_11	18505355	18505416	0.9773	2	0.0444	0.0455	0.0434
11	AhySSR41001	Scaffolds_12	12761843	12761877	0.8807	2	0.2102	0.0795	0.1881
12	AhySSR39465	Scaffolds_12	40173	40211	0.539	2	0.497	0.2987	0.3735
13	AhySSR12555	Scaffolds_13	146746	146817	0.7987	2	0.3216	0.4026	0.2699
14	AhySSR43989	Scaffolds_13	9524194	9524211	0.4839	5	0.5797	0.9355	0.4904
15	AhySSR43465	Scaffolds_13	6175139	6175180	0.5342	8	0.6538	0.4384	0.6173
16	AhySSR44477	Scaffolds_13	13640854	13640877	0.9477	2	0.0992	0.0814	0.0943
17	AhySSR46467	Scaffolds_14	7295227	7295270	0.5053	2	0.4999	0.9255	0.375
18	AhySSR48966	Scaffolds_15	6889536	6889637	0.5988	3	0.5349	0.8025	0.457
19	AhySSR49996	Scaffolds_15	13907952	13908026	0.5325	8	0.6273	0.9351	0.5736
20	AhySSR0542	Scaffolds_15	11049934	11050033	0.5495	2	0.4951	0.8352	0.3725
21	AhySSR51527	Scaffolds_16	8353654	8353737	0.7443	6	0.4301	0.5114	0.4124
22	AhySSR51061	Scaffolds_16	4284470	4284489	0.7527	2	0.3723	0.4946	0.303
23	AhySSR5081	Scaffolds_2	1020604	1020639	0.3444	10	0.7533	1	0.7189
24	AhySSR8059	Scaffolds_2	22784760	22784831	0.5774	2	0.488	0.0119	0.3689
25	AhySSR11576	Scaffolds_3	16269717	16269792	0.633	2	0.4646	0.6702	0.3567
26	AhySSR12579	Scaffolds_3	23384578	23384688	0.7926	2	0.3288	0.4149	0.2748

Table 1. *Cont.*

S.No.	Marker Name	Scaffold Number	g-SSR Start Position	g-SSR End Position	M_{AF}	Allele No	GD	Ho	PIC
27	AhySSR16096	Scaffolds_4	18892950	18892976	0.5722	4	0.5566	0.8556	0.4796
28	AhySSR18586	Scaffolds_5	10835301	10835348	0.4471	6	0.6828	1	0.6324
29	AhySSR19060	Scaffolds_5	14161515	14161619	0.4718	10	0.6646	0.9577	0.6119
30	AhySSR18091	Scaffolds_5	7461113	7461163	0.6193	2	0.4715	0.7614	0.3604
31	AhySSR23586	Scaffolds_6	20936577	20936639	0.3673	5	0.7097	0.9388	0.6602
32	AhySSR21572	Scaffolds_6	6051583	6051604	0.9506	2	0.0939	0.0741	0.0895
33	AhySSR28598	Scaffolds_8	10843033	10843092	0.6744	2	0.4392	0.6512	0.3427
34	AhySSR29093	Scaffolds_8	14673069	14673119	0.4902	6	0.682	0.7255	0.6407
35	AhySSR29571	Scaffolds_8	17923890	17923961	0.3404	9	0.8077	1	0.7855
36	AhySSR31577	Scaffolds_9	7849897	7849947	0.9828	2	0.0339	0.0115	0.0333
	Mean				0.6286	3.8333	0.4641	0.6075	0.4002

The percentage of polymorphic loci across all the studied accessions was 85.42%. Within the accessions, polymorphic loci percentages ranged from 63.89% to 100.00%. The maximum values of polymorphic loci were detected in accessions from Himachal Pradesh (100%) and with exotic origins (80.56%). The highest values of Shannon's information index (I) (0.883) and expected heterozygosity (He) (0.497) were observed in the population from Himachal Pradesh indicating high genetic variability in accessions from Himachal Pradesh. The maximum value of observed heterozygosity (Ho) (0.583) was recorded in accessions belonging to Madhya Pradesh and with exotic origins (Table 2).

Table 2. Percentage of polymorphic loci detected by 36 g-SSR markers across *A. hypochondriacus* populations, state-wise.

Population	Percent Polymorphism	I	Ho	He
Himachal Pradesh	100.00%	0.883	0.555	0.497
Uttarakhand	97.22%	0.82	0.527	0.48
Maharashtra	91.67%	0.751	0.484	0.459
Gujarat	91.67%	0.715	0.547	0.444
Madhya Pradesh	75.00%	0.587	0.583	0.39
Exotic species	80.56%	0.548	0.583	0.368
Unknown	83.33%	0.651	0.435	0.412
Other	63.89%	0.49	0.431	0.33
Mean	85.42%	0.681	0.518	0.423

3.2. Phylogenetic Analysis of Amaranth Accessions

To further investigate the genetic relationships among the studied amaranth accessions, a neighbor-joining tree was constructed based on 36 g-SSR loci. The results based on genetic distance, as observed in the NJ tree, clustered 94 amaranth accessions into three major genetic clusters (Figure 2). In cluster I, 24 accessions were grouped. Among these accessions, fifteen were from Himachal Pradesh, two accessions each were from Gujarat and the USA, one accession each was from Uttarakhand, Maharashtra, and Russia, and two accessions were of unknown origin. The major cluster II consisted of 48 accessions. Among these, seventeen accessions were from Uttarakhand, seventeen accessions were from Himachal Pradesh, six accessions were from Gujarat, three accessions were from Madhya Pradesh,

two accessions were from Maharashtra, and one accession each was from Arunachal Pradesh and the USA, along with one accession from unknown origin. Cluster III contained 22 accessions. It comprised ten accessions from Himachal Pradesh, five accessions from Uttarakhand, four accessions from Maharashtra, two accessions from Gujarat, and one accession from Uttar Pradesh (Figure 2).

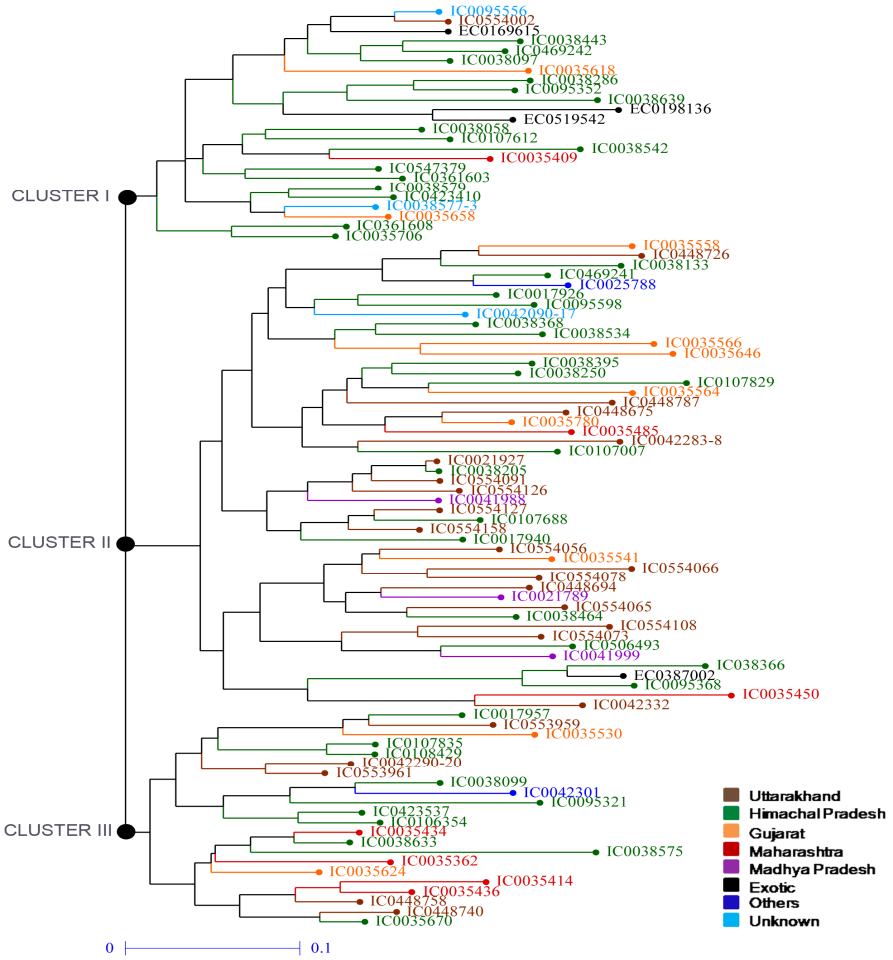

Figure 2. Neighbor-Joining tree analysis across 94 accessions of *A. hypochondriacus.* Three major clusters were observed. Different colors denote the accessions belonging to different states.

3.3. Analysis of Population Structure

The model-based population structure analysis of the 94 studied accessions revealed the presence of two different genetic populations. The maximum value of ΔK was recorded at ΔK = 2, which corresponded to the most possible number of populations in the study (Figure 3). The accessions with a probability score of >0.80 can be considered as genetically pure accessions, while those with a probability score of <0.80 can be considered as admixture. The grouping of amaranth accessions is illustrated in the bar plot diagram obtained using Structure Harvester (Figure 4). Population I contained 46 accessions and population II comprised48 accessions without any admixture. Population I comprised twenty-five accessions from Himachal Pradesh, seven accessions from Uttarakhand, six accessions from Maharashtra, four accessions from Gujarat, two accessions from the USA, and one accession each from Uttar Pradesh and Russia, along with two accessions belonging to an unknown location. Population II included seventeen accessions from Himachal Pradesh, sixteen accessions from Uttarakhand, six accessions from Gujarat, three accessions from Madhya Pradesh, one accession each from Maharashtra, Arunachal and USA along with one accession of unknown origin. If we compare the grouping of population structure with the neighbor-joining tree, Population I correspond to Clusters 1 and 3 of the neighbor-joining

tree, whereas population II corresponds to Cluster 2. This shows that the neighbor-joining tree and population correspond to each other. The only difference is that clusters 1 and 3 are becoming merged together in population 1 during structure analysis.

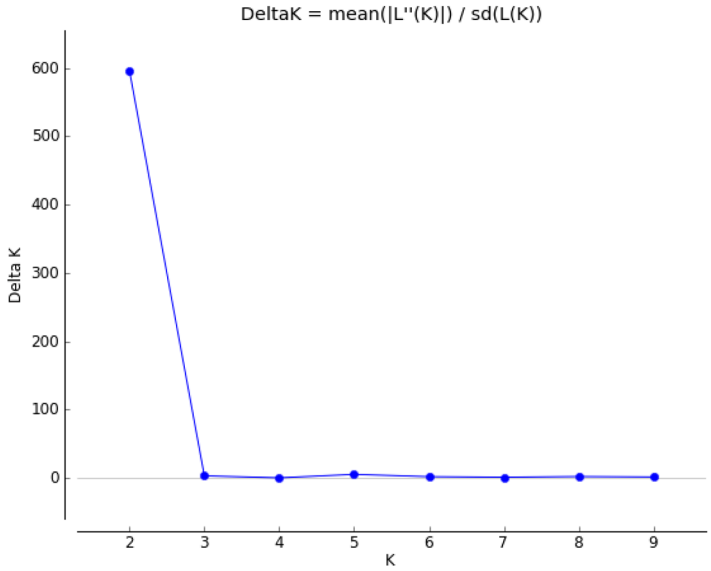

Figure 3. Value of Delta K (ΔK) for determining the optimum number of subpopulations.

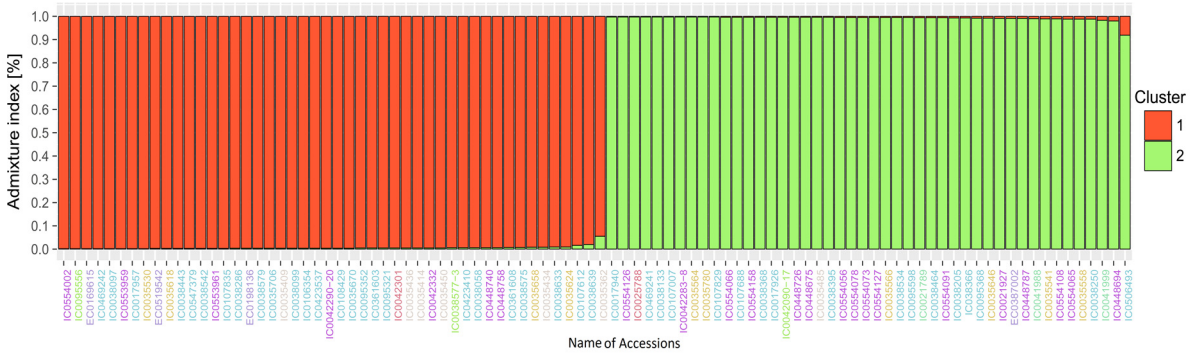

Figure 4. Bar plot generated by population structure analysis of 94 *A. hypochondriacus* accessions based on K = 2. The name of accessions in different colors represents their different states of origin.

3.4. AMOVA and PCoA Analysis

The Analysis of Molecular Variance (AMOVA) was first performed based on geographic populations and then based on the two populations as obtained by the model-based population structure analysis. The genetic variance recorded between the subpopulations in origin-based AMOVA was 11%, whereas within the individuals, 89% variance was recorded (Figure 5a and Table S3). The model-based AMOVA displayed a genetic variation of 8% between the two obtained subpopulations, and a variation of 92% was found within the individuals (Figure 5b and Table S4). There was no significant difference observed among the origin-based and model-based AMOVA analysis.

In addition, the principal coordinate analysis (PCoA) plot displayed the existence of a significant amount of molecular diversity among the analyzed amaranth accessions. The first three axes of the PCoA accounted for a cumulative variation of 27.7%, wherein the first, second and third axes expressed 11.7%, 8.51%, and 7.48% of the total variation, respectively (Table 3). Furthermore, in the PCoA matrix, the amaranth accessions were distributed across the coordinates represented with different colors. The PCoA analysis clearly indicates that accessions from Himachal Pradesh were very distinctly isolated from other states accessions. Similarly, the exotic accessions shared more similarity among themselves and were very

close to Himachal Pradesh accessions (Figure 6), whereas accessions from Uttarakhand were more diverse and shared genetic similarity with accessions from Maharashtra, Gujrat, and Madhya Pradesh.

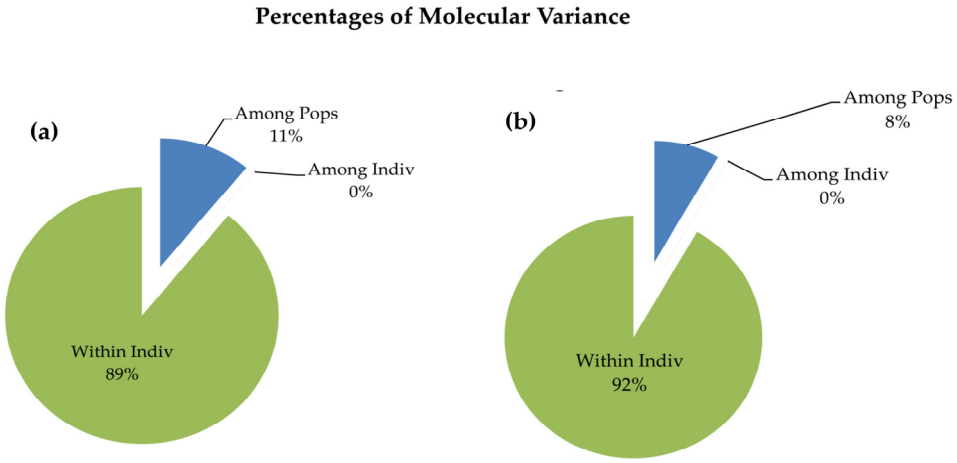

Figure 5. AMOVA analysis depicting variation among populations and within individual accessions. (**a**) The AMOVA analysis based on geographical origin, and (**b**) AMOVA analysis based on model-based population structure analysis.

Table 3. Percentage of cumulative variation explained by the first three axes among amaranth accessions in PCoA analysis.

Axis	Axis-1	Axis-2	Axis-3
Variation (%)	11.72	8.51	7.48
Cumulative variation (%)	11.72	20.23	27.71

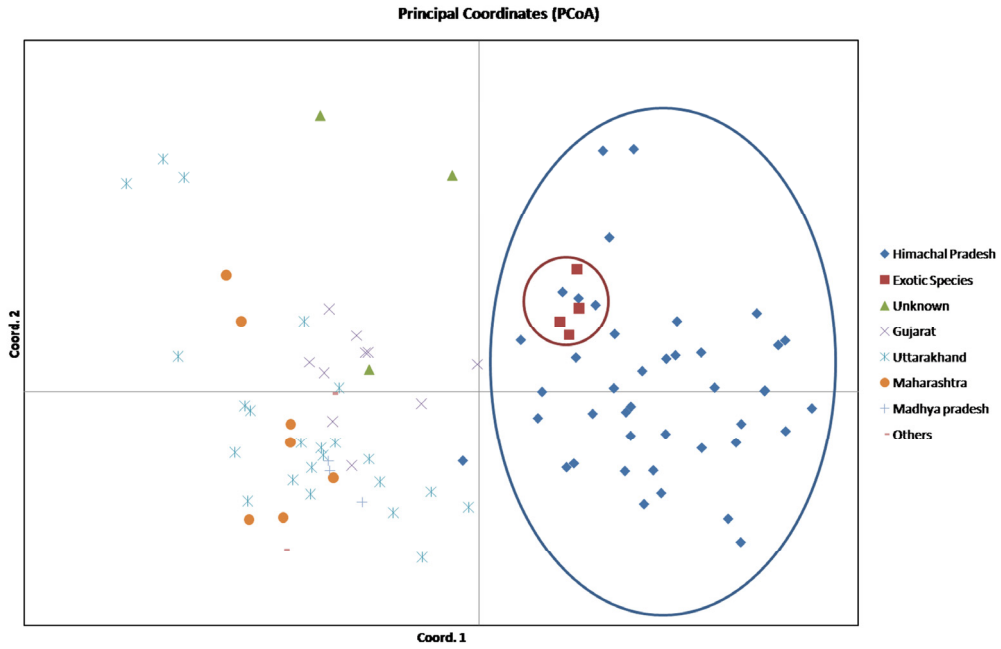

Figure 6. Principal coordinates analysis (PCoA) plot showing the distribution of 94 accessions according to 36 g-SSRs.

4. Discussion

A. hypochondriacus is an ancient paleopolyploid with high nutritional value and excellent agronomic characters [48]. Grain amaranth is a cross-pollinated crop species that

is mainly pollinated by wind. To enhance the use of this crop, information on its genetic diversity needs to be generated. Knowledge of genetic variability within crop species is vital for the potential use of plant genetic resources [49]. Assessment of genetic diversity is considered important in studies related to evolution, population structure, and plant breeding programs [50]. Molecular markers are used to study genetic variability. Over the years, SSRs have proved to be reliable genetic markers for molecular diversity studies, linkage mapping, and marker-aided selection [39,51]. Construction of population structure with the use of molecular data further enables the development of a framework for future breeding programs.

In our present investigation, 36 g-SSR markers were used to analyze 94 accessions of *A. hypochondriacus*. A total of 138 alleles were detected among 36 SSRs across 94 different accessions of amaranth. The mean allelic richness in our study was 3.83 alleles per locus, which was lower than the values obtained in most of the previous studies by Wang et al., 2013 (4.79), Suresh et al., 2014 (11.1), Khaing et al., 2013 (12.9), and Mallory et al., 2008 (4.0) [39,40,52,53]. This difference may be due to the use of different g-SSRs and sample sizes. The values of heterozygosity ranged from 0.01 to 1.00, with a mean value of 0.61 per locus, which is comparable with the studies performed earlier in cultivated relatives of amaranth such as sugar beet (0.61) [54], and quinoa (0.57) [55]. The average value of PIC obtained in our study was 0.40, which is higher than the values obtained previously 0.34 [39] and 0.29 [56]), but lower than others 0.66 [40] and 0.71 [52]. The cause of this difference in PIC values may be due to the variation in sample size considered for the study.

Understanding the population structure is a necessary condition for proper selection of accessions. The model-based structure analysis distributed 94 accessions into two subpopulations. The ΔK at K = 2, meaning all of the accessions were assigned to two subgroups without admixture, suggesting rare genetic connection between the two subgroups based on g-SSR used in this study. Similarly, Suresh et al., 2014 used 11 SSR markers to study population structure among 348 amaranth accessions and classified these into two populations [40]. The clustering analysis based on DNA polymorphism is a better and more trustworthy approach of determining relationships between individuals. As DNA is not affected by several environmental influences, the linkages revealed by DNA markers can give a clear picture of syntenic relations, conservation, and differences between the analyzed individuals of a group or distinct species. A large number of studies on SSR markers in various crops have been reported previously [23–28]. The dendrogram of *A. hypochondriacus* showed three major groups. However, no clear clustering pattern was observed between accessions that were geographically close to each other, which shows that genetic distance has no correlation with geographical distance which is consistent with the results of Wang et al., 2013 [39]. Suresh et al. 2014 also performed clustering analysis based on SSR markers across 348 amaranth accessions, but, similar to our study, no separate groups were observed based on geographical origin. The lack of significant association between genetic divergence and geographical diversity denotes that factors other than geographical origin contribute to genetic diversity such as reproductive nature, genetic sampling error, spontaneous mutation, and natural and artificial selection [57]. This dissociation can also be a result of the cosmopolitan nature of amaranth and activities of human interference such as breeding and resource exchange. Although the neighbor-joining tree and population structure showed a grouping of 94 accessions in to three and two groups, respectively, critical analysis shows that they correspond to each other and that Clusters 1 and 3 are represented by Population 1, while Cluster 2 corresponds to Population 2 with exactly the same number accessions.

The scattered plot generated by PCoA distributed all of the 94 accessions into two distinct groups according to the dissimilarity indices. The total cumulative variation of 20.23% showed less variation as compared to the 34.8% variation among 42 *A. paniculata* accessions [21] and 57.6% variation among 46 pomegranate genotypes [58] reported previously. In the PCoA plot, axis 1 explained a higher proportion of variance (11.72%) compared to axis 2 (8.51%). Furthermore, the grouping pattern of 42 accessions from Himachal Pradesh

showed that these accessions are very distinct from the rest of the accessions representing other states (Uttarakhand, Maharashtra, Gujrat, and Madhya Pradesh) of India. This type of clear distinction was not observed in the cluster analysis or population structure analysis. The four exotic accessions from USA and Russia were very close to each other but were grouped with accessions from Himachal Pradesh. The PCoA also showed that accessions from Uttarakhand are distinct from those from Himachal Pradesh, although geographically both are adjoining states of North India. The Uttarakhand accessions are closer to accessions of Gujrat, Maharashtra, and Madhya Pradesh. Thus, the in-house-developed 36 g-SSRs were found to be effective and informative, and can show that even accessions from neighboring states are very distinct from each other and may represent two different gene pools. Based on PCoA analysis it can be assumed that introduction of grain amaranth might have occurred in Himachal Pradesh because exotic accessions were closer to those from Himachal Pradesh in comparison with other Indian states' accessions. The accessions from Uttarakhand are distinct; this might be due to a different gene pool, or the accessions may have moved from Himachal Pradesh in later stages, become further diversified and moved to western (Maharashtra and Gujrat) and central parts (Madhya Pradesh) of India, before becoming further diversified due to possible cross-pollination with weedy Amaranthus. This may be the reason that accessions from western and central parts of India were not found to share much genetic similarity with exotic and Himachal Pradesh accessions based on PCoA analysis, although this result was not supported by the neighbor-joining tree or population structure study, where intermixing of accessions was observed.

5. Conclusions

In the present study, a set of 36 in-house-developed g-SSR loci were validated through the assessment of genetic diversity and population structure among 94 diverse accessions of *A. hypochondriacus.* The diversity indices represented a substantial amount of molecular diversity among 94 accessions with the in-house-developed g-SSR markers. These markers might distribute the amaranth accessions into different sub-populations. The information related to population structure and diversity analysis of *A. hypochondriacus* proved to be beneficial for further genomic selection, marker-assisted selection (MAS), and genome-wide association studies (GWAS). Based on our study, we also conclude that these 36 polymorphic g-SSR markers are significantly efficient. PCoA studies based on these markers indicated how exotic accessions and Himachal Pradesh accessions were close to each other and distinct from those from other states. Further, this also indicated how grain amaranth has moved to different states of India and diversified. Thus, these markers could be added as new desirable genomic tools for further studies related to the characterization of germplasm banks and future breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13020431/s1, Figure S1: Gel image representation displaying g-SSR profile of 94 accessions of *A. hypochondriacus* using AhySSR0595 marker. Lane M denotes 100 bp DNA ladder; Table S1: List of grain amaranth accessions used in the present study; Table S2: List of g-SSR markers used along with their forward and reverse sequences and annealing temperature; Table S3: Analysis of molecular variance (AMOVA) based on geographic populations using 36 g-SSRs among and within subpopulation of 94 amaranth accessions; Table S4: Model-based analysis of molecular variance (AMOVA) using 36 g-SSRs among and within subpopulation of amaranth accessions.

Author Contributions: R.S. conceived and designed the experiments; G.V., D.D. and R.G. performed data curation and formal data analysis; G.V. performed PCR analysis; R.G. and A.M. performed formal data analysis; D.D. wrote original draft of the manuscript; A.S. performed visualization, reviewed and edited the manuscript; R.S. supervised the study, and provided funding acquisition; V.G. and K.S. provided reagents and material for study; S.R., A.K.S., R.B., S.K., S.K.K. and R.S. writing—review and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Department of Biotechnology, Government of India, grant number "BT/PR23811/AGIII/103/1029/2018". The funder has no role in the design of the study and collection, analysis and interpretation of data and in writing the manuscript.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: All the data and Supplementary Materials are available with the manuscript.

Acknowledgments: We are thankful to the Director, ICAR-NBPGR, New Delhi, for providing necessary facilities to carry out this research work.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Aderibigbe, O.R.; Ezekiel, O.O.; Owolade, S.O.; Korese, J.K.; Sturm, B.; Hensel, O. Exploring the potentials of underutilized grain amaranth (*Amaranthus* spp.) along the value chain for food and nutrition security: A review. *Crit. Rev. Food Sci. Nutr.* **2020**, *62*, 656–669. [CrossRef] [PubMed]
2. Brenner, D.M.; Baltensperger, D.D.; Kulakow, P.A.; Lehmann, J.W.; Myers, R.L.; Slabbert, M.M.; Sleugh, B.B. Genetic Resources and Breeding of *Amaranthus*. *Plant Breed. Rev.* **2010**, *19*, 227–285.
3. Sauer, J.D. The Grain Amaranths and Their Relatives: A Revised Taxonomic and Geographic Survey. *Ann. Mo. Bot. Gard.* **1967**, *54*, 103. [CrossRef]
4. Waselkov, K.E.; Boleda, A.S.; Olsen, K.M. A Phylogeny of the Genus *Amaranthus* (Amaranthaceae) Based on Several Low-Copy Nuclear Loci and Chloroplast Regions. *Syst. Bot.* **2018**, *43*, 439–458. [CrossRef]
5. Nascimento, A.C.; Mota, C.; Coelho, I.; Gueifão, S.; Santos, M.; Matos, A.S.; Gimenez, A.; Lobo, M.; Samman, N.; Castanheira, I. Characterisation of nutrient profile of quinoa (*Chenopodium quinoa*), amaranth (*Amaranthus caudatus*), and purple corn (*Zea mays* L.) consumed in the North of Argentina: Proximates, minerals and trace elements. *Food Chem.* **2014**, *148*, 420–426. [CrossRef]
6. Joshi, N.; Verma, K.C. A review on nutrition value of Amaranth (*Amaranthus caudatus* L.): The crop of future. *J Pharm. Phytochem.* **2020**, *9*, 317–319.
7. Pedersen, B.; Hallgren, L.; Hansen, I.; Eggum, B.O. The nutritive value of amaranth grain (*Amaranthus caudatus*). *Qual. Plant. Plant Foods Hum. Nutr.* **1987**, *36*, 325–334. [CrossRef]
8. Bressani, R.; Gonzáles, J.M.; Zúñiga, J.; Breuner, M.; Elías, L.G. Yield, selected chemical composition and nutritive value of 14 selections of amaranth grain representing four species. *J. Sci. Food Agric.* **1987**, *38*, 347–356. [CrossRef]
9. Rodriguez, J.P.; Rahman, H.; Thushar, S.; Singh, R.K. Healthy and Resilient Cereals and Pseudo-Cereals for Marginal Agriculture: Molecular Advances for Improving Nutrient Bioavailability. *Front. Genet.* **2020**, *11*, 49. [CrossRef]
10. Martirosyan, D.M.; Miroshnichenko, L.A.; Kulakova, S.N.; Pogojeva, A.V.; Zoloedov, V.I. Amaranth oil application for coronary heart disease and hypertension. *Lipids Health Dis.* **2007**, *6*, 1. [CrossRef] [PubMed]
11. Berger, A.; Gremaud, G.; Baumgartner, M.; Rein, D.; Monnard, I.; Kratky, E.; Geiger, W.; Burri, J.; Dionisi, F.; Allan, M.; et al. Cholesterol-Lowering Properties of Amaranth Grain and Oil in Hamsters. *Int. J. Vitam. Nutr. Res.* **2003**, *73*, 39–47. [CrossRef] [PubMed]
12. Huang, Z.R.; Lin, Y.K.; Fang, J.Y. Biological and Pharmacological Activities of Squalene and Related Compounds: Potential Uses in Cosmetic Dermatology. *Molecules* **2009**, *14*, 540–554. [CrossRef]
13. Achigan-Dako, E.G.; Sogbohossou, O.E.D.; Maundu, P. Current knowledge on *Amaranthus* spp.: Research avenues for improved nutritional value and yield in leafy amaranths in sub-Saharan Africa. *Euphytica* **2014**, *197*, 303–317. [CrossRef]
14. Rastogi, A.; Shukla, S. Amaranth: A New Millennium Crop of Nutraceutical Values. *Crit. Rev. Food Sci. Nutr.* **2013**, *53*, 109–125. [CrossRef]
15. Begna, T. Role and economic importance of crop genetic diversity in food security. *J. Agric. Sc. Food Technol.* **2021**, *7*, 164–169.
16. EL-Mansy, A.; Abd El-Moneim, D.; ALshamrani, S.; Safhi, F.; Abdein, M.; Ibrahim, A. Genetic Diversity Analysis of Tomato (*Solanum lycopersicum* L.) with Morphological, Cytological, and Molecular Markers under Heat Stress. *Horticulturae* **2021**, *7*, 65. [CrossRef]
17. Kumar, C.; Singh, S.K.; Pramanick, K.K.; Verma, M.K.; Srivastav, M.; Singh, R.; Bharadwaj, C.; Naga, K.C. Morphological and biochemical diversity among the Malus species including indigenous Himalayan wild apples. *Sci. Hortic.* **2018**, *233*, 204–219. [CrossRef]
18. Bhandari, H.R.; Bhanu, A.N.; Srivastava, K.; Singh, M.N.; Shreya; Hemantaranjan, A. Assessment of genetic diversity in crop plants—An overview. *Adv. Plants Agric. Res.* **2017**, *7*, 279–286.
19. Kumar, P.; Gupta, V.K.; Misra, A.K.; Modi, D.R.; Pandey, B.K. Potential of molecular markers in plant biotechnology. *Plant Omics J.* **2009**, *2*, 141–162.
20. Zhang, Y.; He, Q.; Zhou, X.; Zheng, S.; Wang, Y.; Li, P.; Wang, Y. Genetic diversity and population structure of 93 rice cultivars (lines) (*Oryza sativa* Xian group) in Qinba in China by 3 types of genetic markers. *BMC Genom.* **2022**, *23*, 550. [CrossRef] [PubMed]
21. Kumar, R.; Kumar, C.; Paliwal, R.; Choudhury, D.R.; Singh, I.; Kumar, A.; Kumari, A.; Singh, R. Development of Novel Genomic Simple Sequence Repeat (g-SSR) Markers and Their Validation for Genetic Diversity Analyses in Kalmegh [*Andrographis paniculata* (Burm. F.) Nees]. *Plants* **2020**, *9*, 1734. [CrossRef]

22. Paliwal, R.; Singh, R.; Choudhury, D.R.; Tiwari, G.; Kumar, A.; Bhat, K.C.; Singh, R. Molecular Characterization of *Tinospora cordifolia* (Willd.) Miers Using Novel g-SSR Markers and Their Comparison with EST-SSR and SCoT Markers for Genetic Diversity Study. *Genes* **2022**, *13*, 2042. [CrossRef] [PubMed]
23. Jian, Y.; Yan, W.; Xu, J.; Duan, S.; Li, G.; Jin, L. Genome-wide simple sequence repeat markers in potato: Abundance, distribution, composition, and polymorphism. *DNA Res.* **2021**, *28*, dsab020. [CrossRef]
24. Singh, R.; Narzary, D.; Bhardwaj, J.; Singh, A.K.; Kumar, S.; Kumar, A. Molecular diversity and SSR transferability studies in Vetiver grass (*Vetiveria zizanioides* L. Nash). *Ind. Crops Prod.* **2014**, *53*, 187–198. [CrossRef]
25. Choudhury, D.R.; Singh, N.; Singh, A.K.; Kumar, S.; Srinivasan, K.; Tyagi, R.K.; Ahmad, A.; Singh, N.K.; Singh, R. Analysis of Genetic Diversity and Population Structure of Rice Germplasm from North-Eastern Region of India and Development of a Core Germplasm Set. *PLoS ONE* **2014**, *9*, e113094. [CrossRef] [PubMed]
26. Ganesan, S.K.; Singh, R.; Choudhury, D.R.; Bharadwaj, J.; Gupta, V.; Singode, A. Genetic diversity and population structure study of drumstick (*Moringa oleifera* Lam.) using morphological and SSR markers. *Ind. Crops Prod.* **2014**, *60*, 316–325. [CrossRef]
27. Hipparagi, Y.; Singh, R.; Choudhury, D.R.; Gupta, V. Genetic diversity and population structure analysis of Kala bhat (*Glycine max* (L.) Merrill) genotypes using SSR markers. *Hereditas* **2017**, *154*, 9. [CrossRef]
28. Paliwal, R.; Kumar, R.; Choudhury, D.R.; Singh, A.K.; Kumar, S.; Kumar, A.; Bhatt, K.C.; Singh, R.; Mahato, A.K.; Singh, N.K.; et al. Development of genomic simple sequence repeats (g-SSR) markers in *Tinospora cordifolia* and their application in diversity analyses. *Plant Gene* **2016**, *5*, 118–125. [CrossRef]
29. Kumar, C.; Kumar, R.; Singh, S.K.; Goswami, A.K.; Nagaraja, A.; Paliwal, R.; Singh, R. Development of novel g-SSR markers in guava (*Psidium guajava* L.) cv. Allahabad Safeda and their application in genetic diversity, population structure and cross species transferability studies. *PLoS ONE* **2020**, *15*, e0237538. [CrossRef]
30. Das, M.; Banerjee, S.; Dhariwal, R.; Vyas, S.; Mir, R.R.; Topdar, N.; Kundu, A.; Khurana, J.P.; Tyagi, A.K.; Sarkar, D.; et al. Development of SSR markers and construction of a linkage map in jute. *J. Genet.* **2012**, *91*, 21–31. [CrossRef]
31. Kaur, G.; Joshi, A.; Jain, D. SSR-Marker assisted evaluation of Genetic Diversity in Mungbean (*Vigna radiata* (L.) Wilczek) genotypes. *Braz. Arch. Biol. Technol.* **2018**, *61*. [CrossRef]
32. Moe, K.T.; Zhao, W.; Song, H.S.; Kim, Y.H.; Chung, J.W.; Cho, Y.I.; Park, P.H.; Park, H.S.; Chae, S.C.; Park, Y.J. Development of SSR markers to study diversity in the genus Cymbidium. *Biochem. Syst. Ecol.* **2010**, *38*, 585–594. [CrossRef]
33. Govindaraj, M.; Vetriventhan, M.; Srinivasan, M. Importance of Genetic Diversity Assessment in Crop Plants and Its Recent Advances: An Overview of Its Analytical Perspectives. *Genet. Res. Int.* **2015**, *2015*, 431487. [CrossRef]
34. Lee, J.R.; Hong, G.Y.; Dixit, A.; Chung, J.W.; Ma, K.H.; Lee, J.H.; Kang, H.K.; Cho, Y.H.; Gwag, J.G.; Park, Y.J. Characterization of microsatellite loci developed for *Amaranthus hypochondriacus* and their cross-amplifications in wild species. *Conserv. Genet.* **2007**, *9*, 243–246. [CrossRef]
35. Faseela, K.V.; Salikutty, J. Molecular characterization of amaranth landraces and assessment of interspecific relationships among *Amaranthus* spp. (L.) using RAPD markers. *Indian J. Genet. Plant Breed.* **2007**, *67*, 12–17.
36. Park, Y.J.; Nishikawa, T.; Matsushima, K.; Minami, M.; Nemoto, K. A rapid and reliable PCR-restriction fragment length polymorphism (RFLP) marker for the identification of *Amaranthus cruentus* species. *Breed Sci.* **2014**, *64*, 422–426. [CrossRef]
37. Chandi, A.; Milla-Lewis, S.R.; Jordan, D.L.; York, A.C.; Burton, J.D.; Zuleta, M.C.; Whitaker, J.R.; Culpepper, A.S. Use of AFLP Markers to Assess Genetic Diversity in Palmer Amaranth (*Amaranthus palmeri*) Populations from North Carolina and Georgia. *Weed Sci.* **2013**, *61*, 136–145. [CrossRef]
38. Jimenez, F.R.; Maughan, P.J.; Alvarez, A.; Kietlinski, K.D.; Smith, S.M.; Pratt, D.B.; Elzinga, D.B.; Jellen, E.N. Assessment of Genetic Diversity in Peruvian Amaranth (*Amaranthus caudatus* and *A. hybridus*) Germplasm using Single Nucleotide Polymorphism Markers. *Crop Sci.* **2013**, *53*, 532–541. [CrossRef]
39. Wang, X.Q.; Park, Y.J. Comparison of Genetic Diversity among Amaranth Accessions from South and Southeast Asia using SSR Markers. *Korean J. Med. Crop Sci.* **2013**, *21*, 220–228. [CrossRef]
40. Suresh, S.; Chung, J.W.; Cho, G.T.; Sung, J.S.; Park, J.H.; Gwag, J.G.; Baek, H.J. Analysis of molecular genetic diversity and population structure in Amaranthus germplasm using SSR markers. *Plant Biosyst.—Int. J. Deal. All Asp. Plant Biol.* **2014**, *148*, 635–644.
41. Nguyen, D.C.; Tran, D.S.; Tran, T.T.H.; Ohsawa, R.; Yoshioka, Y. Genetic diversity of leafy amaranth (*Amaranthus tricolor* L.) resources in Vietnam. *Breed. Sci.* **2019**, *69*, 640–650. [CrossRef]
42. Sahu, S.K.; Thangaraj, M.; Kathiresan, K. DNA Extraction Protocol for Plants with High Levels of Secondary Metabolites and Polysaccharides without Using Liquid Nitrogen and Phenol. *ISRN Mol. Biol.* **2012**, *2012*, 205049. [CrossRef]
43. Liu, K.; Muse, S.V. PowerMarker: An integrated analysis environment for genetic marker analysis. *Bioinformatics* **2005**, *21*, 2128–2129. [CrossRef] [PubMed]
44. Botstein, D.; White, R.L.; Skolnick, M.; Davis, R.W. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* **1980**, *32*, 314–331. [PubMed]
45. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* **2012**, *28*, 2537–2539. [CrossRef]
46. Perrier, X.; Flori, A.; Bonnot, F. Data Analysis Methods. In *Genetic Diversity of Cultivated Tropical Plants*; Hamon, P., Seguin, M., Perrier, X., Glaszmann, J.C., Eds.; Science Publishers: Enfield, UK, 2003; pp. 43–76.

47. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959. [CrossRef]
48. Barba de la Rosa, A.; Fomsgaard, I.S.; Laursen, B.; Mortensen, A.G.; Olvera-Martínez, L.; Silva-Sánchez, C.; Mendoza-Herrera, A.; Gonzalez-Castaneda, J.; De León-Rodríguez, A. Amaranth (*Amaranthus hypochondriacus*) as an alternative crop for sustainable food production: Phenolic acids and flavonoids with potential impact on its nutraceutical quality. *J. Cereal Sci.* **2009**, *49*, 117–121. [CrossRef]
49. Brown, A.H.D. Core collections: A practical approach to genetic resources management. *Genome* **1989**, *31*, 818–824. [CrossRef]
50. Fatinah, A.A.; Arumingtyas, E.L.; Mastuti, R. Morphological and genetic variation of *Amaranthus spinosus* L.: An adaptation evidence of climate differences and gene interaction. *Int. J. Biosci.* **2013**, *11*, 205–212.
51. Miah, G.; Rafii, M.; Ismail, M.; Puteh, A.; Rahim, H.; Islam, K.; Latif, M. A Review of Microsatellite Markers and Their Applications in Rice Breeding Programs to Improve Blast Disease Resistance. *Int. J. Mol. Sci.* **2013**, *14*, 22499–22528. [CrossRef]
52. Khaing, A.A.; Moe, K.T.; Chung, J.W.; Baek, H.J.; Park, Y.J. Genetic diversity and population structure of the selected core set inAmaranthus using SSR markers. *Plant Breed.* **2013**, *132*, 165–173. [CrossRef]
53. Mallory, M.A.; Hall, R.V.; McNabb, A.R.; Pratt, D.B.; Jellen, E.N.; Maughan, P.J. Development and Characterization of Microsatellite Markers for the Grain Amaranths. *Crop Sci.* **2008**, *48*, 1098. [CrossRef]
54. Rae, S.J.; Aldam, C.; Dominguez, I.; Hoebrechts, H.; Barnes, S.R.; Edwards, K.J. Development and incorporation of microsatellite markers into the linkage map of sugar beet (*Beta vulgaris* spp.). *Appl Genet.* **2000**, *100*, 1240–1248. [CrossRef]
55. Mason, S.L.; Stevens, M.R.; Jellen, E.N.; Bonifacio, A.; Fairbanks, D.J.; Coleman, C.E.; McCarty, R.R.; Rasmussen, A.G.; Maughan, P.J. Development and Use of Microsatellite Markers for Germplasm Characterization in Quinoa (*Chenopodium quinoa* Willd.). *Crop Sci.* **2005**, *45*, 1618–1630. [CrossRef]
56. Tiwari, K.K.; Thakkar, N.J.; Dharajiya, D.T.; Bhilocha, H.L.; Barvaliya, P.P.; Galvadiya, B.P.; Prajapati, N.N.; Patel, M.P.; Solanki, S.D. Genome-wide microsatellites in amaranth: Development, characterization, and cross-species transferability. *3 Biotech* **2021**, *11*, 395. [CrossRef]
57. Gupta, S.; Kumari, K.; Muthamilarasan, M.; Subramanian, A.; Prasad, M. Development and utilization of novel SSRs in foxtail millet [*Setaria italica* (L.) P. Beauv.]. *Plant Breed.* **2013**, *132*, 367–374. [CrossRef]
58. Patil, P.G.; Singh, N.V.; Parashuram, S.; Bohra, A.; Sowjanya, R.; Gaikwad, N.; Mundewadikar, D.M.; Sangnure, V.R.; Jamma, S.M.; Injak, A.S.; et al. Genome-wide characterization and development of simple sequence repeat markers for genetic studies in pomegranate (*Punica granatum* L.). *Trees* **2020**, *34*, 987–998. [CrossRef]