



Article

Assessment of the Genetic Diversity of *Ulex europaeus* in Maui, California, Hawaii and New Zealand by a Method of Microsatellite Markers [†]

Mika Hozawa *  and Eiji Nawata 

Department of Environmental Engineering, Kyoto University, Kitashirakawa Oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan; nawata.eiji.7c@kyoto-u.jp

* Correspondence: hozawa.mika.x12@kyoto-u.jp; Tel.: +81-90-9606-4385

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Abstract: One of the most serious, invasive, allohexaploid plant species, *Ulex europaeus*, is originally from western Europe and is now spreading to the world by some unknown pathways. Plants often show phenotypic plasticity according to their environment, but elucidating the fact that the differences are derived from environmental or genetic effects is very important for further study. Thus, the aim of this study was to assess the genetic distances among *Ulex europaeus* from four different regions, namely Maui, California, Hawaii and New Zealand. The microsatellite method, which has been frequently used to test the genetic distances of the hexaploid plant species, was recently used for assessment because a normal single nucleotide polymorphism (SNP) often shows genotypic ambiguity on hexaploids. We tested the leaf samples of 37 mother trees from four regions (Maui: 11; California: 4; Hawaii: 7; New Zealand: 15) at five microsatellite loci. After polymerase chain reaction analyses (PCR), dinucleotide-repeat motifs (DRMs) were counted and compared to test the genetic distances of the samples. As a result, a dendrogram and analysis of molecular variance (AMOVA) showed that *Ulex europaeus* sampled in four different regions were genetically very close. If they show any morphological differences, they are inferred to be derived from environmental effects.

Keywords: allohexaploid; genetic distances; invasive species; microsatellite; phenotypic plasticity



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1. Introduction

Phenotypic plasticity is an important trait for the adaptation of introduced *U. europaeus* in many places around the world. In order to explain the mechanism of the adaptation of *U. europaeus*, the genetic assessment of the samples collected in unique sites, such as Maui, California, Hawaii and New Zealand, should have been completed prior to the other assessments. This helped avoid ambiguous discussion about the results, such that the morphological and physiological differences could be derived from the differences in genetic traits. Microsatellite research on human and animal genomes is commonly reported, whereas similar research on plants has to date not been reported very often [1]. *U. europaeus* is a hexaploid species which evolved from the hybridization of a diploid and tetraploid of different *Ulex* lineages [2,3]. As mentioned by multiple authors in previous studies, assessing the genetic differences of the plants of allohexaploid by methods using normal SNP (single nucleotide polymorphism) is not possible [4]. However, the genetic differences of many allohexaploid species also need to be identified in population genetics. For this reason, analytical methods using microsatellites were used and progress is being made towards efficient genetic markers in population genetics [5]. The SSR (simple sequence repeats) method, a representative method using microsatellites, has been used for genotyping plants for over 20 years because it is highly informative and can be experimentally compared [5]. One of the reasons why SSRs is widely used for population

genetics is that it enables the creation of sequence-based linkage maps that visually show the difference.

2. Experiments

2.1. Materials and Methods

Samples were taken in Maui, Hawaii, California and New Zealand from July 2016 to November 2017 (Table 1). Tissue samples of the leaves were taken on FTA[®] Plant Card (Whatman[®] Co., Ltd., Maidstone, UK) and prepared for further analysis according to the protocol by Whatman[®] for PCR.

Table 1. Location and coordinates of *Ulex europaeus* sampled for PCR experiments.

Location	Mother Tree	Latitude	Longitude	Altitude (m)	Habitat
Maui	#22	N20.76	W156.27	1758	Ranch
	#23	N20.76	W156.27	1823	Ranch
	#26	N20.77	W156.26	1767	Ranch
	#27	N20.77	W156.26	1763	Ranch
	#28	N20.77	W156.26	1763	Ranch
	#29	N20.78	W156.25	1773	Ranch
	#30	N20.78	W156.25	1768	Ranch
	#33	N20.79	W156.25	1656	Ranch
	#35	N20.8	W156.28	1060	Forest
	#36	N20.8	W156.28	1067	Forest
	#37	N20.8	W156.28	1046	Forest
California	#40,41,42,43	N37.15	W122.34	33, 33, 34, 31	Fallow land
Hawaii	#49	N19.72	W155.44	2004	Ranch
	#50	N19.72	W155.43	2058	Ranch
	#51	N19.93	W155.41	2015	Ranch
	#52	N19.73	W155.39	1952	Ranch
	#52-2	N19.73	W155.39	1952	Ranch
	#53	N19.74	W155.37	1929	Ranch
	#55	N19.77	W155.36	1980	Ranch
New Zealand	N2-1, 2, 3	S36.88	E174.84	0	Fallow land
	N5-1, 2, 3	S37.86	E175.82	97	Roadside of a ranch
	N8-1, 2, 3	S38.99	E175.76	538	Fallow land with grass
	S1-1, 2, 3	S43.8	E173	623	Ranch
	S4-1, 2, 3	S43.5	E172.52	38	Roadside of a ranch

Five primers (Table 2) were prepared for PCR (polymerase chain reaction) used in the previous research by Hornoy et al. [6]. They chose eight microsatellites' loci from Genetic Identification Services (GIS Inc., Chatsworth, CA, USA) and obtained clear results from 6 loci. We used five of them.

Table 2. Five microsatellite loci used in the experiments.

Locus	Primers	(Primers) (μM)
A110	F: 5'-CTATGGTGAATTTGTGATACAC-3' R: 5'-ACCTTGTGTCATCTTACC-3'	0.35
A125	F: 5'-GCATATACATACCCGAGGTAAG-3' R: 5'-AACCTGATGAAATGCACTATTC-3'	0.26
B4	F: 5'-GGGCTCTGGCTCTGATAC-3' R: 5'-TTGGATTAAACCACTTTCCTC-3'	0.2
B104	F: 5'-GAACCTTATTCACCTGGAATCTG-3' R: 5'-CCCTTTTCTTTCCTTCTTAAC-3'	0.3
B123	F: 5'-AATTTGCCTGACATTGTTACTC-3' R: 5'-AGACCGTGTTTCATTATGGTTAG-3'	0.22

The enzyme, KOD FX Neo of TOYOBO Co., Ltd. was used for PCR experiments. PCR reactions were performed for each locus in 25 μL containing 12.5 μL of buffer, 2.5 μL of dNTP, 9 μL of deionized and sterilized water, 0.4 μL each of primers (F and R) and 0.2 μL of enzyme.

PCR was performed by an initial denaturation step at 94 °C for 2 min, 35 cycles with 10 s denaturation at 98 °C, 30 s hybridization of primers at 60 °C and 1 min elongation at 68 °C, with a final elongation step at 68 °C for 7 min, using iCycler (Bio Rad Laboratories, California, USA). After checking the condition of the bands at electrophoresis with 2% agarose gel, next generation sequencing of the PCR products was conducted by Takara Bio Inc. (Kusatsu, Shiga, Japan).

2.2. Data Analysis

Genetic diversity among and within populations was estimated by the analysis of molecular variance with the software GenAlEx [7]. In addition, a cluster dendrogram was calculated and indicated by software R ver. 3.3.2 [8].

2.3. Estimation of Genetic Distance

All the FASTA data were checked with software ApE (Utah University, free software) and the effective length of the PCR product was manually determined. Only dinucleotide-repeat motifs (DRMs) were focused this time because they have been identified as an enhancer that expresses the gene [9]. Furthermore, as the length of the DNA fragment is often changed, affected by insertion or deletion, as directly counting the repeat numbers was suggested to determine the genotypes of SSR (short sequence repeat) or STR (short tandem repeat) marker more correctly [10]. In this reason, the repeat number of the most common motifs found in five loci, GT/TG, CT/TC, AG/GA, AG/GA, AG/GA, respectively, were counted by each locus using a software SEAVIEW [11] and accordingly amended to the mean effective product size (160 bp this time) for the comparison. By using the data taken, AMOVA was tested to see whether the genetic variation and cluster analysis for making the dendrogram with 1000 bootstraps was performed by using R. ver. 3.3.2 [8].

3. Results and Discussion

3.1. Cluster Analysis

The number of each target dinucleotide repeats were counted using SEAVIEW [11], as shown in Table 3.

Table 3. Dinucleotide repeats number of 36 individuals. M: Maui; C: California; H: Hawaii; N-N: New Zealand North Island; N-S: New Zealand South Island.

Sample NO	Population	Selected Loci				
		A110	A125	B4	B104	B123
22	M	12	16	0	0	10
23	M	0	0	0	0	23
26	M	10	0	0	8	15
27	M	12	0	0	0	0
28	M	0	0	0	2	0
29	M	17	0	0	6	13
30	M	0	0	0	0	5
33	M	5	0	0	8	26
35	M	12	0	27	0	21
36	M	11	0	0	13	0
37	M	14	0	12	0	19
40	C	12	15	0	14	0
41	C	0	0	0	0	19
42	C	9	0	23	0	18
43	C	11	27	0	14	15
49	H	5	0	36	0	20
50	H	14	0	36	0	0
51	H	17	0	0	2	16
52	H	13	0	0	0	19
52-2	H	0	0	0	0	39
53	H	13	48	0	38	15

Table 3. Cont.

Sample NO	Population	Selected Loci				
		A110	A125	B4	B104	B123
55	H	0	0	0	0	17
N2-1	N-N	4	7	11	4	17
N2-2	N-N	0	0	0	4	18
N2-3	N-N	0	2	5	6	14
N5-1	N-N	0	22	0	3	0
N5-2	N-N	0	22	3	7	0
N5-3	N-N	0	22	0	10	14
N8-1	N-N	3	23	0	0	0
N8-2	N-N	15	26	43	3	13
N8-3	N-N	11	21	41	0	0
S1-1	N-S	12	20	4	6	16
S1-2	N-S	18	26	7	15	0
S1-3	N-S	13	0	32	20	0
S4-1	N-S	0	24	37	5	10
S4-2	N-S	12	26	0	0	24
S4-3	N-S	21	11	0	0	0

As the next step, the data from Table 3 were analyzed by software R ver. 3.3.2 [8] for the dendrogram of Figure 1 (1000 boot strap) in order to visualize the correlation data of microsatellite results. The vertical axis was labeled as distance and refers to a distance measure between compounds or compound clusters. The height of the node can be thought of as the distance value between the right and left sub-ranch clusters. When D (distance) and C (correlation) are between a compound cluster, $D = 1 - C$. If compounds are highly correlated, they will have a correlation value close to 1, and the D ($1 - C$) will have a value close to zero. Therefore, highly correlated clusters are nearer the bottom of the dendrogram. As clusters increase in size, their abundance profiles become more general. From the result of the dendrogram (Figure 1), *U. europaeus* sampled in Maui, California, Hawaii and New Zealand show the similarity in genetic distances.

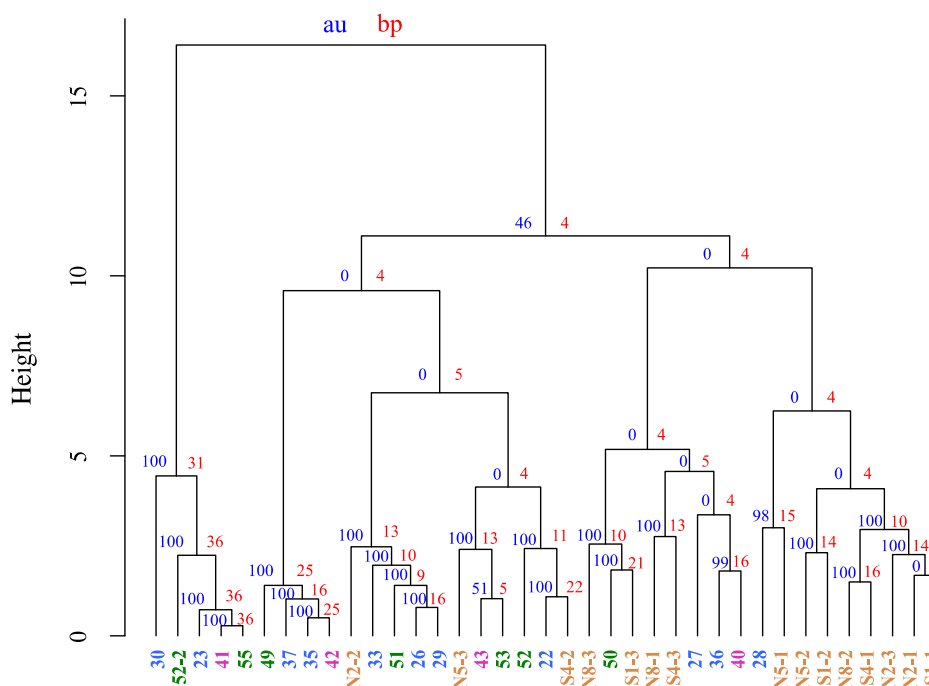


Figure 1. Dendrogram of *Ulex europaeus* sampled in Maui, California, Hawaii and New Zealand after 1000-boot strap. Blue: Maui; green: Hawaii; pink: California; yellow with N: New Zealand North Island; yellow with S: New Zealand South Island. Au, bp are two types of *p*-values (%): au (approximately unbiased) *p*-value and bp (bootstrap probability) value.

3.2. Analysis of Molecular Variance

In addition, the data from Table 3 were analyzed by GenAlEx to see the genetic variation. Table 4 shows the result of AMOVA.

Table 4. Results of the molecular variance of *U. europaeus* sampled in 3 regions for this study. Probability, P (rand $\geq$ data), for PhiPT is based on standard permutation across the full data set.

Source	df	SS	MS	Est. Var.	%
Among Pops	4	2960.2	740.05	27.79	5%
Within Pops	32	17,276.99	539.91	539.91	95%
Total	36	20,237.19		567.69	100%
Stat	Value	P (rand $\geq$ data)			
PhiPT	0.05	0.11			

Though the *p*-value was not significant, the results inferred that the most variation (95%) was detected among populations, and just 5% attributed to variation within four populations. These results showed that the genetic variation mainly occurred among populations: 95% of the total variations. In other words, *Ulex europaeus* which invaded and propagated in Maui, Hawaii, California and New Zealand did not show a large genetic difference among invaded populations. The genetic distance among the 37 samples of different mother trees (11, 4, 7, 15 from Maui, California, Hawaii and New Zealand, respectively) was not significantly large; *Ulex europaeus* of the above four sites were inferred to be introduced from very close places. Though a very stable and accurate method to assess the genetic distance of the plants has to date not been innovated, microsatellites are inferred to be the most useful methods [5]. Whenever the invasiveness of a plant is discussed, the main driver is concluded as its variability. As not all the original places of the invasive species were correctly recorded, records of the introduction of *U. europaeus* to the USA (Hawaii Island, Maui Island and California) and New Zealand were ambiguous. However, if the same plant species with long genetic distance were compared, the variability could be ambiguous. Furthermore, we question the possibility of discussing the results of the phenotypic plasticity of the samples of the same species from many spaciouly different populations without performing any genetic assessments. The results of this investigation were used as the first benchmark before discussing the phenotypic plasticity of *U. europaeus* sampled in the USA (Hawaii Island, Maui Island and California) and New Zealand; their genetic distance was not very large.

4. Conclusions

U. europaeus is known to have originated in western Europe and has spread across the world. However, the introduction cases varied, and some are not very well documented. The genetic variation among *U. europaeus*, even in the place they originate from, has been revealed by recent study [6], and it has become mandatory to distinguish the genetic differences among *Ulex europaeus* in its invaded places far away from western Europe for further studies, such as strategies of invasibility. From the results shown in this study, the genetic distances of *Ulex europaeus* sampled in Maui, California, Hawaii and New Zealand are very close, and these could be the benchmark for further studies.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/IECPS2020-08564/s1>.

Author Contributions: M.H. and E.N. designed the investigation and experiments, analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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