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POPULATION STRUCTURE AND PHYLOGENETIC ANALYSIS OF LABORATORY RABBITS IN TAIWAN BASED ON MICROSATELLITE MARKERS

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Abstract: Laboratory rabbits used in Taiwan are primarily supplied by the Livestock Research Institute (LRI) and the Animal Drugs Inspection Branch (ADIB) of the Animal Health Research Institute. An analysis of the genetic characteristics and structure of these populations would thus be a fundamental step in building a long-term management programme for maintaining stable animal quality and preserving the genetic variation among the populations. In this study, DNA samples were isolated from founders of 5 populations: New Zealand White rabbits (NZW) and Japanese White rabbits (JPN) from the ADIB, NZW and Rex rabbits (REX) from the LRI, and NZW from a private rabbit breeding farm in Ban Ciao (BC). A set of microsatellite markers, 18 in total, was designed for genetic analysis. The average values for the allele number (Na), effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (H_E), and Wright's fixation index (F_{IS}) were 5.50, 2.437, 0.442, 0.568 and 0.232, respectively. These results revealed that this set of microsatellite markers has high diversity and that the major local populations have a tendency toward inbreeding. At the same time, analysis of molecular variance results showed that the laboratory rabbits used in Taiwan have maintained a high level of within-population genetic differentiation (83%). The genetic differentiation among clusters was moderate (F_{ST}=0.18), and Bayesian cluster analysis showed that the most likely number of groups was 4 (K=4). Principal component analysis (PCA) also showed 4 divergent clusters. The LRI and BC NZW populations were not separated when K=4 was used in a Structure software analysis and were also hard to split until principal component 3 in PCA. The individual unrooted phylogenetic tree showed that the 5 populations were separated, except that some individuals from the LRI NZW population overlapped with the ADIB NZW and BC NZW populations. As such, in order to counteract the reduced F_{IS} (0.232) and maximise heterozygosity, the 3 NZW populations could be interbred or have new genes introduced into them. The set of microsatellite markers used herein was useful for studying the relationships and genetic diversities among these rabbit populations of Taiwan. Based on the resulting data, rabbit farms in Taiwan could select parental stocks for planned mating in the future as part of strategies to preserve and restore the rational breeding of laboratory rabbits.

Key Words: genetic monitoring, laboratory rabbit, microsatellite markers, phylogenetic analysis.

INTRODUCTION

The Livestock Research Institute (LRI) of Taiwan's Council of Agriculture (COA) first started raising laboratory rabbits in 1969 for use in nutrition studies. The main varieties of rabbit raised by the LRI at that time were New Zealand White (NZW) and Angora. English Black-ear rabbits and Japanese White rabbits (JPN) were introduced to Taiwan later. In addition, coloured Rex rabbits (REX) were introduced in 1986 to enhance rabbit fur quality for the purposes of Taiwan's fur industry. Meanwhile, a variety of different types of rabbits were sold in the nation's pet shops, but their specific

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breeds were never confirmed. At the same time, the use of rabbit fur and meat have declined in Taiwan in recent years due to increased animal welfare concerns, but NZW and REX have continued to be raised for laboratory use.

In 1978, the LRI began to establish the use of various technologies aimed at raising laboratory rabbits on a large scale to accommodate the increasing demands of researchers in various fields, as well as demands relating to industrial uses such as the toxicological testing of pharmaceuticals. REX were typically used for research in ophthalmology. Meanwhile, NZW, which have thick and obvious ear blood vessels and stable hereditary characteristics, were bred by a wide range of schools, teaching hospitals, and pharmaceutical companies for use as experimental animals (Livestock product plants original information network, Taiwan, http://www.angrin.tlri.gov.tw/rabbit_all.htm). Another minor breed of laboratory rabbit, the JPN, was crossed with many other kinds of rabbit, such as Lop, Angola and Himalayan rabbits, which were first imported into Japan during the 1870s. In subsequent years, other newly imported rabbits such as the Flemish Giant and NZW were also crossed with JPN, and the descendants of all this cross breeding have continued to be called JPN (Fukuta *et al.,* 1996).

In 2015, a total of 20911 rabbits were used in experiments by research institutes in Taiwan (Human care of laboratory animals, annual report, COA, 2002-2015). During the period from 2002-2015, the estimated numbers of laboratory rabbits used in Taiwan ranged from 14000 to 34000 per year. These values accounted for less than 2% of all the laboratory animals used in all of those years except for 2004 to 2006 (Figure 1). At present, the laboratory rabbits used by research institutes in Taiwan are primarily supplied by the LRI, which provides minimum disease NZW, and the Animal Drugs Inspection Branch (ADIB) of the COA's Animal Health Research Institute, which provides conventional clean NZW and specific-pathogen-free (SPF) NZW. These two institutes have supplied anywhere from 43.9% to 93.8% of the total of laboratory rabbits used in Taiwan in previous years (Introduction of production management and quality monitoring of biomedical laboratory animal, 2010). Genetic monitoring of laboratory animals is used to assure the reproducibility of experimental results by ensuring that the animals used in the experiments are genetically defined, as the genetics of the animals can directly affect the results (Fahey *et al.,* 2013). In inbred animals, such monitoring can be utilised to avoid unwanted genetic heterogeneity caused by human error and genetic drift. In contrast with inbred animals, outbred animals consist of closed populations of genetically variable animals that are bred to maintain maximum heterozygosity (Benavides *et al.,* 2001; Chia *et al.,* 2005). As such, the development of a tool that could potentially be used to help realise and maintain genetic variability both within and between populations was required in order to ensure the high quality of outbred lab rabbits in Taiwan.

The LRI and ADIB produce more than 40% of all laboratory rabbits used in Taiwan and are also the main founder breeders in Taiwan, but the genetic information on their rabbit populations is incomplete. In terms of genetic studies, the LRI rabbits have only been subjected to glucose phosphate isomerase (GPI) and 6-phosphogluconate dehydrogenase (PGD) genotyping. Yeh *et al.* (1993) reported that the NZW of the LRI contain a 17.2 kb mitochondrial DNA that became 7 fragments after the enzymatic digestion of *Hind III.* In terms of GPI genotyping, the NZW and REX of the LRI were found to be a single class. In terms of PGD genotypes, the REX had only 1 type with no polymorphisms; however, 3 genotypes, AA, AB, and BB, were found to be present in the LRI's NZW (Chang *et al.,* 1998).

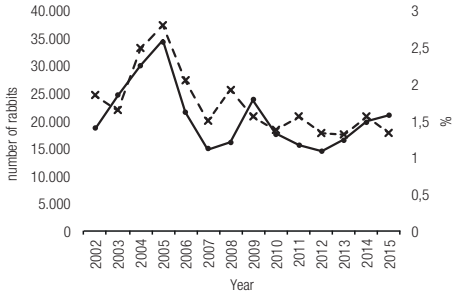

Figure 1: Numbers of procured laboratory rabbit in Taiwan from 2002 to 2015. —●— Number of rabbits. -×- % of total lab animals.

Microsatellite markers were chosen in this study because of their specific characteristics. These markers are abundant and fairly well distributed throughout the whole genome (O'Reilly and Wright, 1995), in addition to being highly polymorphic (O'Connell *et al.,* 1998; Olsen *et al.,* 1998), codominant, and highly reproducible. Compared to isozymes, such as RAPD or AFLP, microsatellite markers yield better results, and are suitable for analyses of individual or population genetics and even for paternity tests (Goldstein and Schlötterer, 1999). Furthermore, while single nucleotide polymorphisms (SNPs) are also highly abundant throughout the genome, large numbers of SNPs are required for precision (with about 6 SNPs being equivalent to one microsatellite) (Toro *et al.,* 2009).

In addition, the detection of SNPs via sequencing techniques is more costly than analyses based on microsatellite markers, and it has been successfully used to investigate genetic diversity in rabbits (Emam *et al.*, 2017).

Based on DNA level detection, microsatellite markers not only constitute a tool for establishing the genetic markers and genetic information of populations, they can also serve as a reference in research relating to animal breeding and phylogenetics. As such, the aims of this study were to establish a set of microsatellite markers for the genetic monitoring of local rabbit populations and use these microsatellite markers to determine the inter- and intra-population genetic variation, as well as the genetic structure, of laboratory rabbits in Taiwan.

MATERIALS AND METHODS

Experiment Animals and Sample Collection

Five founder populations from the LRI, the ADIB and a private rabbit breeding farm in Ban Ciao (BC) were examined in this study. Blood samples were collected from 202 individuals representing the 5 populations, which consisted of NZW (n=35) and JPN (n=8) from the ADIB, REX (n=40) and NZW (n=96) from the LRI, and NZW from the BC (n=23). For each animal, 5 mL of blood were drawn from the marginal ear artery, and then genomic DNA was extracted with Genomic DNA Isolation Reagent (GenePure Technology CO., LTD, Taiwan) using a standard phenol-chloroform protocol. The nucleic acid concentration and purity were determined by absorbance at 260 and 280 nm and the ratio of 260/280 absorbance (Sambrook and Russell, 2001).

Selection of Microsatellite Marker Panel and Genotyping

A set of 18 microsatellite loci were chosen from a gene bank (Table 1), namely, 6L3F8, 12L1E11, 12L4A1, 12L5A6, D3Utr2, D6Utr4 (Korstanje *et al.,* 2003), So130, So133, So144 (Surridge *et al.,* 1997), V193, V235, V344 (Chantry-Darmon *et al.,* 2005), Sat3, Sat4, Sat7, Sat12 (Mougel *et al.,* 1997), A10, and D118 (Estes-Zumpf *et al.,* 2008), for higher allele numbers in our populations. All 202 individuals were genotyped for the 18 microsatellite markers. The genotyping method was modified and conducted as previously described (Schuelke, 2000). The forward primer of PCR was linked to a CAG tag (CAGTCGGGCGTCATCA) on the 5' end and the following thermal cycling was performed with 3 primers: a forward primer with the CAG tag, a reverse primer, and a fluorescence-labelled CAG tag. Each 20 µL reaction contained 50-100 ng of genomic DNA, 0.2 µM of reverse primer, 0.04 µM of forward primer with the CAG tag, 0.16 µM of the CAG tag, 1X of PCR buffer (with 1.5 mM of $MgCl_2$), 0.2 mM of dNTP, and 0.5 U Taq polymerase (TaKaRa Co., Japan). The PCR cycling conditions were as follows: 5 min of denaturation at 95°C followed by 35 cycles of 30 s of denaturation at 95°C, 40 s of annealing at the optimal temperature (Table 1), and 40 s of elongation at 72°C. Additional elongation was then conducted for 7 min at 72°C.

The amplified microsatellite PCR products were analysed with a DNA analyser (ABI PRISM 3730 DNA Analyzer, Applied Biosystem, USA). The allelic sizes of all loci were estimated relative to in-line GeneScan600 LIZ Size Standard marker (ABI PRISM, Applied Biosystem, USA). The fragment size was calibrated and analysed with Peak Scanner Software version 1.0 (ABI PRISM, Applied Biosystem, USA).

Statistical Analysis

For each locus, the commonly derived statistics of the microsatellite genotypic data for each population and across populations included allele frequencies, the observed number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (H_E), and polymorphic information content (PIC), all of which were calculated using the Microsatellite Toolkit (Park, 2001). The Hardy-Weinberg equilibrium test was performed with the GENEPOP program (Raymond and Rousset, 1995), which was also used to estimate *F*-statistics (F_{IT}, F_{IS}, and F_{ST}) (Weir and Cockerham, 1984) for each locus, the pairwise F_{ST} between populations, and the average inbreeding coefficient (F_{IS}). Nei's genetic distance (D_A) (Saitou and Nei, 1987) among populations was measured by Microsatellite Analyzer (MSA) (Dieringer and Schlötterer, 2003). The phylogenetic tree was calculated and drawn with the PHYLIP (Felsenstein, 2002) program using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) and neighbour-joining (NJ) with a bootstrap test of 1000 resamplings of loci with replacement (Felsenstein, 1985). The genetic distances of

Table 1: Molecular characteristics, primer sequences and annealing temperature for 18 microsatellite loci.

Locus	Accession number	Primer sequence (5' 3')	Repeat pattern	Annealing Temp.(°C)
6L3F8	AF421924	F: CTC CTG CCC TGT TCT AT R: CAG GCT GGT CTT ATT AC	$(CA)_{14}$	53
12L1E11	AF421941	F: AGT GGT AGC GCT TTG GTC TG R: GCT CCT TGG GGC ATT TG	$(CA)_{13}$	59
12L4A1	AF421948	F: AGG CAC CGG GTT CTT GAG CAG R: AGC AGG GCC AGC CAC ACT TGA T	$(CA)_{17}$	55
12L5A6	AF421947	F: GGT GTG AAC CAC TAG ATA GAA R: CAA AAT TAG GTC CCT TGT AGT	-	53
D3Utr2	AF421903	F: AGG AAG TGA GGG GAG GTG TT R; ATA ATG TGC TGC CAA AAT AGA AAT	$(CA)_{15}$	53
D6Utr4	AF421916	F: CAG AAG GGC ATT TGT TTT G R: GGT GAT TCT TTC TTC TGC CTC TTA	$(CA)_{16}$	57
Sol30	X79215	F: CCC GAG CCC CAG ATA TTG TTA CCA R: TGC AGC TTC ATA GTC TCA GGT C	$(TC)_{14}A$ $(T)_4(TC)_5$	55
Sol33	X94683	F: GAA GGC TCT GAG ATC TAG AT R: GGG CCA ATA GGT ACT GAT CCA TGT	$(TG)_3CG$ $(TG)_{18}$	63
Sol44	X94684	F: GGC CCT AGT CTG ACT CTG ATT G R: GGT GGG GCG GCG GGT CTG AAA C	$(GT)_{17}$	63
V0193	AJ874531	F: CCA TTT GGG GAG TAA ACC AGT R: CTC TTC TGT GGC GAG ATG TGT	$(TC)_4TT$ $(TC)_{16}(AC)_{13}$	60
V0235	AJ874568	F: GG AAA CTG GTG GGA AAG TTG R: TAA GTC CAG GAT GCA GCA GA	$(GA)_{19}$	58
V0344	AJ874660	F: GGA ATC TGC ACC ACC AAG AT R: AGG TGG GTG GCT ATG TTC AG	$(TG)_{20}(AG)_{19}$	55
Sat3	J03744	F: GGA GAG TGA ATC AGT GGG TG R: GAG GGA AAG AGA GAG ACA GG	$(TC)_{22}$	60
Sat4	M33582	F: GGC CAG TGT CCT TAC ATT TGG R: TGT TGC AGC GAA TTG GGG	$(TC)_{13}N_5$ $(TC)_2TG(TG)_7$	60
Sat7	X99888	F: GTA ACC ACC CAT GCA CAC TC R: GCA CAA TAC CTG GGA TGT AG	$(TG)_{14}$	60
Sat12	X99891	F: CTT GAG TTT TAA ATT CGG GC R: GTT TGG ATG CTA TCT CAG TCC	$(CTAT)_{10}$	58
A10	EF672479	F: TCC CAC TAG AAA CTT TCA AAA C R: CAC GTT AGC ACA GAG TTG TAT C	$(TACA)_3(CATA)_2(CA)_{17}$	58
D118	EF672485	F:AAA TAG TGA CCC TGG CAT GAG R:TGG CAA GAG ATT GTC CTT AGC	$(CT)_{12}CC$ $(CT)_2CC(CT)_2$	58

the proportion of shared alleles (POSA) were used to estimate (Bowcock *et al.,* 1994) and draw a POSA individual phylogenetic tree.

The model-based approach proposed for determining the population structure of the 5 populations was analysed via the software STRUCTURE 2.3.1 (Pritchard *et al.,* 2000), which was used to assess the genomic clustering (K) of the

sample. To obtain a representative value of K for data modelling, 10 independent runs were performed for each value from 1 to 7. The run length was set to 50000 burn-ins, followed by 50000 iterations. The ΔK estimated the most likely number of K that represented the population structure (Evanno *et al.,* 2005). Principal component analysis (PCA) was performed with GENALEX v.6.5 software (Peakall and Smouse, 2012) in order to spatially plot clusters and individuals based on the distance matrix with data standardisation.

A hierarchical analysis of variance was carried out to allow for the partitioning of total genetic variance into components owing to population and individuals. Computations were performed using a hierarchical analysis of molecular variance (AMOVA) procedure, as implemented in the ARLEQUIN 3.5 package (Excoffier and Lischer, 2010).

RESULTS

Genotyping variation, heterozygosity and F-statistic of microsatellite loci

The genetic statistics, including H_E, H_O, PIC, the mean observed Na and the mean effective number of alleles (Ne) indicated the allelic diversity at each locus (removed). There were 99 alleles observed in the 18 microsatellite loci. Polymorphisms at all the microsatellite loci were clearly observed in all 5 populations. The genetic variability statistics of the 18 microsatellite loci are listed in Table 2. The average Na per locus was 5.5. The actual allele numbers ranged from 4 (Sol30, Sol33, Sol44, Sat7, and Sat12) to 11 (V0344). The Ne per locus ranged from 1.377 (12L5A6) to 4.064 (V0344), with an average across loci of 2.437. The PIC values ranged from 0.232 (12L5A6) to 0.690 (V0344), and the average value was 0.470.

The H_E values among the 18 microsatellite loci ranged from 0.305 (12L5A6) to 0.785 (V0235), and the average value of H_E was 0.568. The H_O values among the 18 microsatellite loci ranged from 0.121 (12L5A6) to 0.684 (Sol44), and the average value of H_O was 0.442 (Table 2). All the loci, except for Sol44, significantly departed from the Hardy-Weinberg equilibrium (P<0.01).

Table 2: Characterisation of 18 microsatellite markers used in this study.

Locus	F_{IS}[1]	F_{IT}	F_{ST}	Na	Ne	H_O	H_E	PIC	Exact test of HWE[2]
6L3F8	0.052	0.143	0.096	6	3.294	0.651	0.687	0.638	*
12L1E11	0.104	0.366	0.293	5	2.024	0.461	0.513	0.417	*
12L4A1	0.132	0.212	0.093	5	2.168	0.464	0.532	0.447	*
12L5A6	0.612	0.714	0.264	6	1.377	0.121	0.305	0.232	*
D3Utr2	–0.003	0.290	0.292	6	2.028	0.492	0.489	0.357	*
D6Utr4	0.170	0.270	0.121	5	2.577	0.537	0.643	0.507	*
Sol30	0.629	0.687	0.157	4	2.469	0.219	0.583	0.438	*
Sol33	0.160	0.404	0.291	4	2.011	0.421	0.500	0.406	*
Sol44	–0.164	–0.057	0.092	4	2.294	0.684	0.588	0.447	NS
V0193	0.185	0.210	0.031	6	2.696	0.135	0.576	0.541	*
V0235	0.772	0.805	0.145	6	2.458	0.566	0.785	0.520	*
V0344	0.287	0.325	0.053	11	4.064	0.500	0.612	0.690	*
Sat3	0.111	0.197	0.096	7	2.729	0.566	0.635	0.569	*
Sat4	0.426	0.548	0.213	5	2.187	0.329	0.568	0.412	*
Sat7	0.075	0.285	0.227	4	2.145	0.454	0.491	0.432	*
Sat12	0.091	0.168	0.244	4	2.606	0.553	0.606	0.458	*
A10	0.417	0.534	0.200	6	2.463	0.341	0.598	0.506	*
D118	0.121	0.230	0.323	5	2.281	0.458	0.520	0.443	*
Mean	0.232	0.352	0.180	5.50	2.437	0.442	0.568	0.470	

F_{IS}, F_{IT} and F_{ST}: Wright's F-statistic values; Na: number of observed alleles; Ne: and effective alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; PIC: polymorphism information content; HWE: Hardy-Weinberg equilibrium.

*: represented significant (P<0.01) departure from the Hardy-Weinberg equilibrium. NS: not significant.

The Wright's *F*-statistic values (F_{IS}, F_{IT}, and F_{ST}) for each locus are also shown in Table 2. The average F_{IS} of all the loci was 0.232, and the F_{IS} per locus varied from 0.772 (V0235) to -0.164 (Sol44). The average F_{IT} of all the loci was 0.352, and the F_{IT} per locus varied from 0.805 (V0235) to –0.057 (Sol44). The mean F_{ST} of all the loci was 0.18. This value indicated that around 18% of the total genetic variation was caused by population differences, while 82% of the total genetic variation was due to genetic differentiation among individuals within each population.

Furthermore, all the microsatellite loci in this study were polymorphic, indicating that the loci were suitable for the genetic analysis of lab rabbits in Taiwan. Private alleles were also present in all the populations, and were mostly observed in REX, a population that did not originate from New Zealand (Table 3).

Intra-population Genetic Variability and Hardy-Weinberg Equilibrium Test

The genetic parameters across the 18 loci for the 5 populations are listed in Table 4. H_E varied from 0.382 (JPN) to 0.622 (ADIB), whereas H_0 varied from 0.319 (JPN) to 0.548 (ADIB), and PIC ranged from 0.298 (JPN) and 0.552 (ADIB).

Among these 5 populations, the ADIB and LRI had the highest observed mean number of alleles (MNA) (4.000), followed by the BC (3.944) and REX (3.500), whereas the JPN had the smallest observed MNA (2.278). The positive F_{IS} values for all 5 populations indicated a deficiency of heterozygotes and a sufficiency of inbreeding effect. The deviation from the Hardy-Weinberg proportions within populations (F_{IS}) varied from 0.033 to 0.309. Higher inbreeding effects were found in LRI (0.272) and REX (0.309). For ADIB, LRI, BC, JPN and REX, there were 6, 11, 5, 6, and 13 loci, respectively, that significantly deviated from the Hardy-Weinberg equilibrium ($P<0.05$) (Table 4).

Inter-population Genetic Variation

To estimate the genetic variation of the 5 lab rabbit populations, 3 parameters were evaluated in this study: genetic differentiation (F_{ST}), gene flow (Nm), and genetic distance. The values of Nm and F_{ST} between each test population pair are shown in Table 5. The values of F_{ST} between the population pairs varied from 0.064 (LRI and BC) to 0.289 (JPN and REX). The F_{ST} for each population pair was highly significant ($P<0.001$). The values of Nm between the pairs varied from 0.616 (JPN and REX) to 3.678 (LRI and BC). The highest Nm value was observed between the LRI and BC (3.678), followed by the value between the ADIB and LRI (2.626) and that between the ADIB and BC (1.996). The lowest Nm value was observed between the JPN and REX (0.616), with the value between the JPN and LRI (0.623) and the JPN and ADIB (0.650) being just slightly higher.

Table 3: Size of the private alleles (bp) with the corresponding allele frequencies in the 5 Taiwan lab rabbit populations.

Locus	ADIB	LRI	BC	JPN	REX
6L3F8				118:0.3125	
12L1E					243:0.2125
12L4A1			179:0.0652		172:0.25
12L5A6	271:0.0147		264:0.0217		
D3Utr2			359:0.4125		336:0.375
D6Utr4					189:0.25
Sol44			219:0.0217		
V193			180:0.0217		204:0.025
V235				216:0.4375	
V344	287:0.0333	291:0.0111		273:0.125	
Sat3					162:0.0125
					174:0.0875
Sat4					246:0.1375
D118				297:0.1875	

The populations are: New Zealand White (NZW) of Animal Drugs Inspection Branch, NZW of Livestock Research Institute (LRI), BC: NZW of Ban Ciao rabbit farm, JPN: NZW in ADIB from Japan, REX: Rex rabbit of LRI.

Table 4: Genetic parameter across 18 loci in the 5 lab rabbit populations.

			Mean heterozygosity		MNA		
Population	F_{IS}[1]	PIC[1]	Expected (H_E)	Observed (H_O)	Effective	Observed	Number of loci departure from HWE[2]
ADIB	0.125	0.552	0.622	0.548	2.774	4.000	6
LRI	0.272	0.517	0.575	0.417	2.620	4.000	11
BC	0.164	0.507	0.577	0.487	2.444	3.944	5
JPN	0.033	0.298	0.382	0.319	1.847	2.278	6
REX	0.309	0.477	0.546	0.375	2.501	3.500	13

MNA: Mean number of alleles; PIC: polymorphism information content; F_{IS}: measure of the deviation from the Hardy-Weinberg proportions within subpopulation. HWE: Hardy-Weinberg equilibrium. The population abbreviation as in the Table 3 footnote.

Population Structure Analysis

The degree of structure was quantified via AMOVA. The AMOVA results are summarised in Table 6. The analysis revealed that significant differentiations were found among populations ($P<0.05$), among individuals within populations ($P<0.05$), and among individuals ($P<0.05$). The biggest variation was found among individuals (63%), followed by the variation among individuals within populations (21%). The variations among the populations themselves accounted for 17% of the total variation.

Clustering Based on Genetic Distances, and Population Differentiation Analysis

The Nei (1972) genetic distance of the 5 populations of rabbits in Taiwan was also calculated. A D_A distance matrix was used to build phylogenetic trees with the UPGMA and NJ methods. A phylogenetic tree of the 18 microsatellite loci was constructed with the PHYLIP software using the UPGMA and NJ with bootstrap resampling (n=1000). In the UPGMA tree, the bootstrap values at the nodes showed that the tree was more robust. The results of the UPGMA and NJ (Figure 2) phylogenetic trees revealed highly similar results. According to both trees, the LRI, ADIB, and BC populations were genetically close to each other. The JPN populations was farther from the 3 NZW populations, while the REX population, as expected, was the population farthest from all the others.

PCA was performed on the pair-wise genetic distances among the 5 populations in order to determine the relative positions among the populations. The first (PC1), second (PC2), and third (PC3) principal components accounted for 47.92%, 32.70%, and 13.79% of the total variation, respectively (Figure 3). The PCA results, which were similar to the phylogenetic trees drawn up via the UPGMA and NJ methods, through only PC1 and PC2, showed that the 5 rabbit populations were divided into a major group, consisting of the LRI, ADIB, and BC populations, and the 2 isolated populations of JPN and REX. Adding to PC3, the ADIB was rather far away from LRI and BC, but the tendency of grouping LRI, ADIB and BC was the same.

Structure software using Bayesian clustering of multi-locus genotypes was used to assign individuals to populations, estimate individual admixture proportions, and infer the number of populations (K) for a given sample. Model-based clustering of the microsatellite genotypes revealed that the likelihood variance of the observed data decreased as the predefined number of clusters increased (data not shown). At K=2, two clear clusters were revealed and a given

Table 5: Pairwise estimates of breed differentiation (F_{ST}) (below the diagonal) and gene flow (Nm) (above the diagonal) between each pair of the 5 lab rabbit populations

Population	ADIB	LRI	BC	JPN	REX
ADIB	-	2.626	1.996	0.650	1.244
LRI	0.087*	-	3.678	0.623	0.918
BC	0.111*	0.064*	-	0.715	1.225
JPN	0.278*	0.286*	0.259*	-	0.616
REX	0.167*	0.214*	0.170*	0.289*	-

*Pairwise F_{ST} was significant at $P<0.05$. The population abbreviation as in the Table 3 footnote.

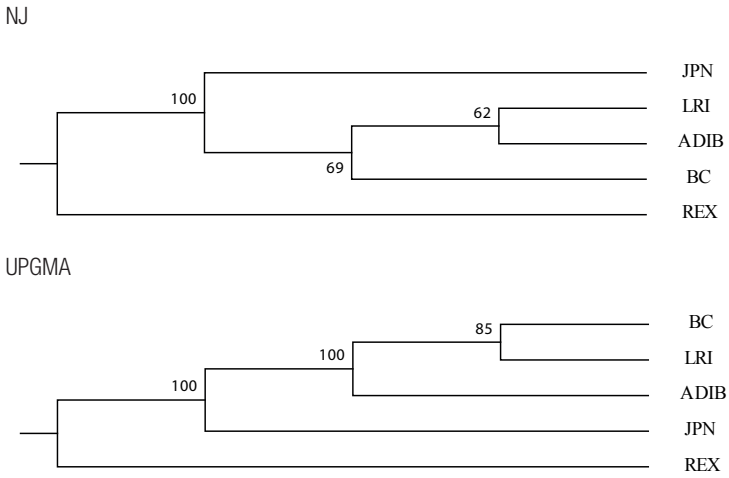

Figure 2: The unweighted pair group method was used with the arithmetic mean (UPGMA) and neighbour joining (NJ) dendrogram summarising genetic relationships among the 5 rabbit populations in Taiwan based on Nei's D_A distances for the 18 microsatellite loci. The numbers on the nodes indicate the percentage bootstrap values generated from 1000 resamplings. Population abbreviation as in the Table 3 footnote.

individual was easily assigned to a population, with the exception of a REX individual. If K=3, the LRI, ADIB, and BC NZW were collected in one cluster. This result was similar to those of the phylogenetic trees. However, some individuals were not clearly assigned to any population, indicating the close relationships among these 3 populations. One clear peak at K=4 was observed in the ΔK distribution. Based on these results, we believed that K=4 was the most likely number of clusters. In addition, the average matrix of membership (Figure 4) demonstrated that the 5 populations sampled could be divided into 4 highly homogenous clusters. The LRI and BC could divide into the same cluster when K=4. In addition, the REX would separate into 2 clusters if K was set at 5.

DISCUSSION

Genetic Variation and Intra-Population Diversity

To establish a genetic database and a means for the genetic monitoring of lab rabbits in Taiwan, we studied the genetic characteristics of the 5 rabbit populations reared by the ADIB, LRI, and a private rabbit farm. A previous report suggested that microsatellite markers used in studies of genetic variation and distance should have no fewer than 4 alleles in order to reduce the standard errors of distance estimates (Barker, 1994), and that such microsatellite markers should have an Ho of between 0.3 and 0.8 in the population (Takezaki and Nei, 1996). We tested over

Table 6: Hierarchical analysis of molecular variance (AMOVA) within and among populations of rabbit.

Source of variation	d.f.	Sum of squares	Variance of component (% total variance)
Among populations	4	278.696	1.087 (17%)*
Among individual within populations	147	1006.323	1.351 (21%)*
Within individuals	152	630.000	4.145 (63%)*
Total	303	1915.020	6.582 (100%)*

*Represented significant: $P<0.05$.

Fixation indices: F_{ST}=0.165, F_{IS}=0.246, F_{IT}=0.370. F_{ST} is the measure of the genetic differentiation among individual within population to total. F_{IS} is the measure of the deviation from the Hardy-Weinberg proportions within population. F_{IT} is the measure of the genetic differentiation within individuals to total. The significance of fixation index was tested with 10000 permutations.

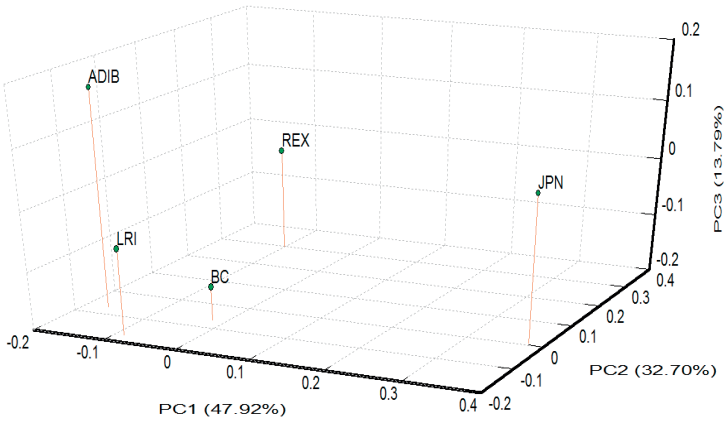

Figure 3: Principal component analysis (PCA) plot of 5 population positions by population genetic distances based on the allele frequencies of 18 microsatellite markers. The first (PC1), second (PC2), and third (PC3) principal component accounted for 47.92%, 32.70%, and 13.79% of the total variation, respectively. The population abbreviation as in the Table 3 footnote.

30 microsatellite makers and finally chose 18 markers, each of which had more than 4 alleles. The Ho values for all these markers were higher than 0.3, with the exception of those for 12L5A6, Sol30, and V0193. The Ne and PIC values across all the loci were 2.437 and 0.470, respectively, which indicated that all 5 rabbit populations in this study expressed lower genetic variability and diversity than rabbit breeds in China (for which the average Ne is 6.625) (Wu *et al.,* 2010). For the ADIB, LRI, BC, JPN, and REX populations investigated in this study, there were 6, 11, 5, 6, and 13 loci, respectively, that significantly departed from the Hardy-Weinberg equilibrium ($P<0.05$). Those deviations were likely caused by the small effective population sizes, the selection, and the sampling size.

The H_E values of these lab rabbit populations from Taiwan (on av. 0.568) were higher than those of domestic rabbit breeds from Tunisia (on av. 0.512) (Ben Larbi *et al.,* 2014) and Egypt (0.525) (Grimal *et al.,* 2012). The H_0 and H_E per population ranged from 0.319 for the JPN to 0.548 for the ADIB and from 0.382 for the JPN to 0.622 for the ADIB. For all 5 populations, the H_0 was lower than the H_E. This indicated that the diversity of the populations had been lost through mating. To rectify this situation, individuals with minimal average co-ancestries could be mated in order to maximise a given population's genetic diversity (Lacy, 1995). The MNA of these lab rabbit populations from Taiwan (on av. 5.50) was higher than that of indigenous rabbit breeds from Tunisia (on av. 3.30) (Ben Larbi *et al.,* 2014) and lower than that of 7 domestic breeds (on av. 10.067) from China (Wu *et al.,* 2010). The Na of the populations from Taiwan was medium. The sample size of the JPN population was much smaller than that of the other populations, and the MNA value for a population is affected by the size of the corresponding sample. Rarefaction is a statistical method that accounts for this effect in order to produce unbiased estimates of allelic richness (that is, the number of alleles) (Kalinowski, 2005). Applying rarefaction, the calculated MNAs of the ADIB, LRI, REX, JPN, and REX were 3.08, 2.93, 2.65, 2.14, and 2.97, respectively, with the value for the JPN still being the smallest.

Significant deviation from the Hardy-Weinberg equilibrium was observed in all 5 populations. The deviation from the Hardy-Weinberg equilibrium in the ADIB and BC populations may have resulted from a relatively small original founding population at the LRI, which would have led to slight inbreeding. Nevertheless, the population of JPN is small. The intra-population inbreeding values (F_{IS}) also supported this hypothesis (Table 2). The overall F_{IS} value (0.232) suggested that a medium level of inbreeding within population was caused by the departure from random mating. This result should be treated seriously when planning the future breeding work for each population.

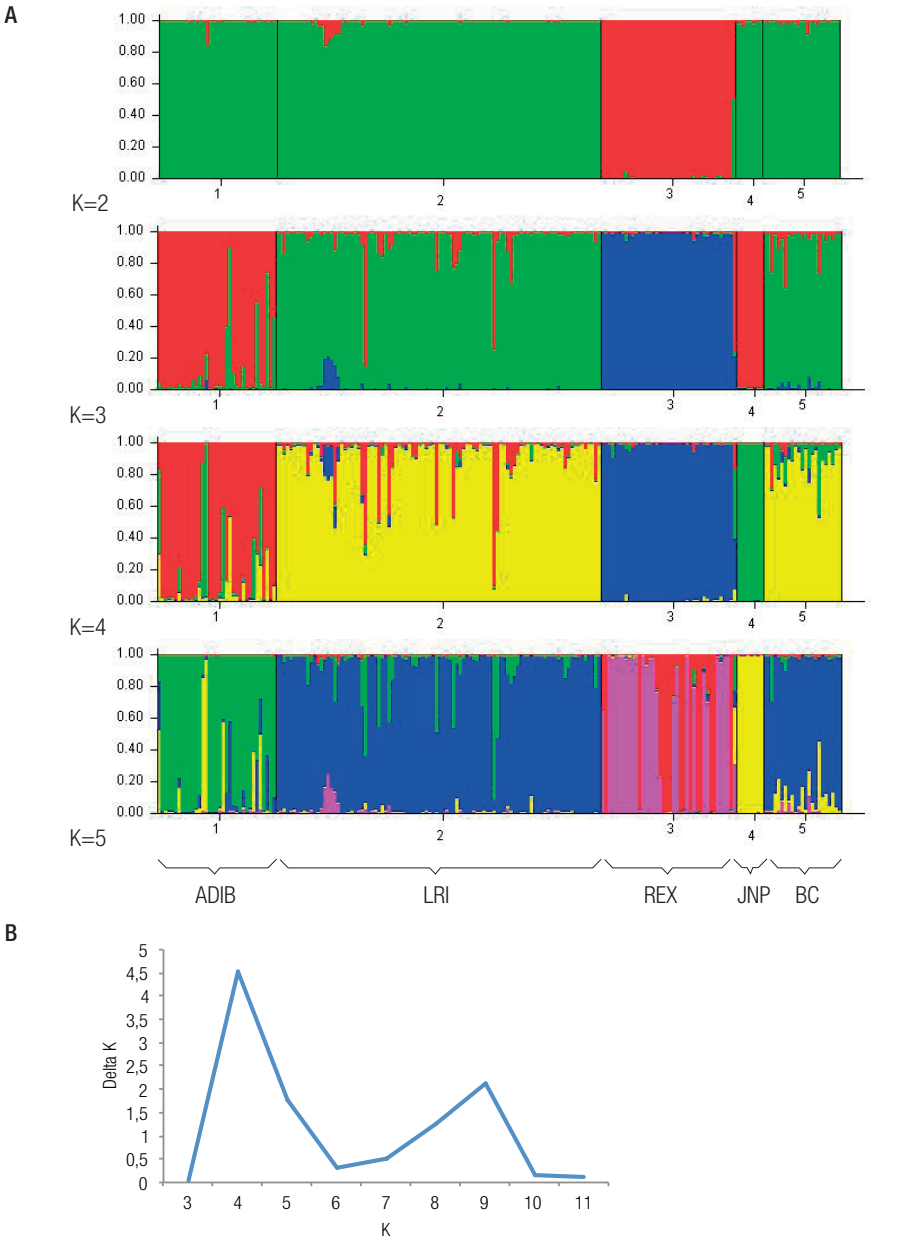

Figure 4: Structure analysis of 5 rabbit populations. Cluster results from a structure analysis of 202 rabbits from 5 populations and based on 18 microsatellite markers. (A) Each genotyped rabbit is represented by a single vertical line divided into K colours, where K is the number of clusters assumed in each structure analysis. Each vertical bar represents an individual rabbit. The colours on each vertical bar represent the probability of the individual belonging to each cluster. (B) The ΔK values with different K value calculated by the Evanno method. At K=4, four clusters are clearly defined representing genetically distinct breed grouping within the rabbits. Population abbreviation as in Table 3 footnote.

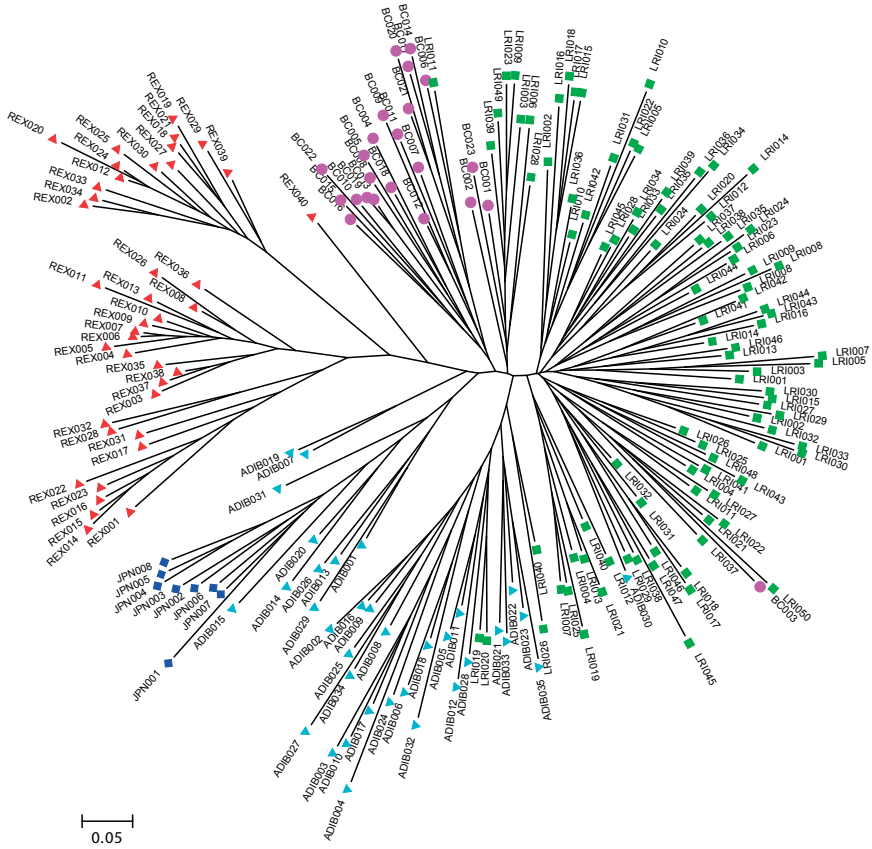

Figure 5: The unrooted individual phylogenetic tree of ADIB, BC, LRI, REX and JPN for Taiwan lab rabbit populations constructed from –ln (shared allele proportion) by 18 microsatellite marker polymorphisms. ▲:ADIB, ●:BC, ■:LRI, ▼:REX, ◆:JPN. Population abbreviation as in Table 3 footnote.

PIC values higher than 0.5 indicate high polymorphism, and markers with this level of polymorphism are highly informative for genetic studies (Moreno *et al.,* 2006). In this study, the total average PIC was 0.470, or relatively close to 0.5, which verified that the 18 microsatellite markers used were useful for studying the genetic relationships and genetic diversities among the rabbit populations in Taiwan.

Genetic Diversity and Inter-Population Relationships

The results of the AMOVA showed that the proportion of the total genetic variation in these lab rabbit populations from Taiwan attributable to population differences was about 17%. In comparison with other studies, the overall F_{ST} value was 0.18, and there was a significant genetic differentiation among the studied populations (P<0.05). Low levels of genetic differentiation have previously been observed among 7 populations in China (on av. 0.099) and 15 populations in Tunisia (on av. 0.011). The F_{ST} among the 5 populations in this study implied that most of the total genetic variation was the result of differences among individuals (83% on AMOVA). A high level of inter-population Nm was the most probable cause of this low level of F_{ST} among the populations in our study. The pairwise F_{ST} results were consistent with the Nm data: lower F_{ST} values were found between the LRI and BC, between the LRI and ADIB,

and between the ADIB and BC, while higher Nm values were found between the LRI and BC, between the LRI and ADIB, and between the ADIB and BC (Table 4).

The genetic relationships among the 5 investigated populations indicated by the NJ and UPGMA methods showed few differences (Figure 2). According to the UPGMA, there was a high percentage of bootstrap value (over 85%) that indicated clear separation. The BC and LRI populations were the closest. However, the percentage of bootstrap value for the LRI, ADIB, and BC clad in the NJ tree was lower (below 70%), which revealed that the 3 NZW rabbit populations were very closely related. These results indicated higher Nm among the 3 NZW rabbit populations. To confirm the genetic differentiations among these 3 NZW populations, the STRUCTURE program clustered the tested rabbit populations. An illustration produced using the STRUCTURE program showed when K=2 (Figure 4), the 3 populations were put into one cluster. The BC and ADIB populations were separated into different clusters when K=3, and the LRI population was represented as a mixture of the BC and ADIB populations. When the K value reached 4 or 5, the BC and ADIB populations were still separated into different clusters, but the LRI population primarily clustered with the BC population, while also having a small ADIB component. It is thus clear that the genetic relationships of the 3 populations were close. According to the phylogenetic tree results, the BC population was closer to the LRI population than to the ADIB population. This may have been because the NZW was the earliest breed introduced by the LRI and because the original founders of the ADIB and BC populations were from the LRI. The introduction date of the ADIB founders might have been earlier than that of the BC founders. In the Structure analysis, the REX population was split into 2 clusters when K=5. It was possible that REX was selected for 2 different traits during the early breeding periods. The Structure program constitutes a benchmark among many genetic clustering programs. However, it was assumed that it contained groups of genotypes maximising Hardy-Weinberg, linkage equilibrium and displaying polymorphism (Pritchard *et al.,* 2000). In order to check the validity of the Structure analysis results, we also conducted an analysis with a non-Bayesian clustering method, FLOCK (Duchesne and Turgeon, 2012), with default conditions, as well as plots using a program called Distruct (Rosenberg, 2004) (data not shown). The greatest difference between the 2 analyses was that the split of REX appeared at K=3 in the FLOCK analysis, not at K=5 as in the Structure analysis. The finding that intra-population heterogeneity was higher than inter-population heterogeneity in some populations indicates a breeding problem for these rabbit populations in Taiwan.

A PCA (Figure 3) was performed according to the genotyping results for the 18 microsatellite loci of the 5 rabbit populations. The PCA results revealed a trend similar to that indicated by the phylogenetic tree results (Figure 2). The REX and JPN populations were found to be relatively far away from the other 3 populations. According to the fixation indices (F_{ST}), the genetic variation between the JPN and the 3 NZW populations was even higher than that between the REX and the 3 NZW populations. The probable causes of this finding could be that the JPN was a highly homozygous breed (H_0=0.319) and the sample size investigated in this study was small (8 samples).

An individual unrooted phylogenetic tree created using the POSA (Figure 5) was provided as an estimator of genetic distance. The results illustrated that individuals from the JPN and REX populations were clustered together; in addition, individuals from ADIB, LRI and BC tended to cluster together, except for a few individuals. Similar results were found in the phylogenetic tree and using PCA, which separated the JPN and REX populations from the ADIB, LRI, and BC populations. The clustering algorithm implied that the Structure analysis provided an alternative method for overcoming the limitations inherent in phylogenetic tree models, and this approach has previously been applied to infer genetic structures in several species (Rosenberg *et al.,* 2002; Parker *et al.,* 2004; Druml *et al.,* 2007; Alves *et al.,* 2015). In this study, the analysis of the population structure detected 4 homogeneous groups (K=4). Only the LRI and BC populations could not be separated. This result was consistent with the findings of the highest Nm value and the lowest F_{ST} value being between the LRI and BC populations. Meanwhile, whether an individual phylogenetic tree analysis or Structure analysis was used, the ADIB, LRI, and BC populations were only mildly separated, indicating that they were very closely related. Otherwise, Structure analysis occurred inter- and intra- populations independently (Alves *et al.,* 2015). The REX population was split into 2 sub-populations at K=5, and this situation was revealed in the unrooted individual phylogenetic tree (Figure 4 and 5). To sum up, the developed microsatellite markers appeared to be suitable for use in the genetic monitoring of laboratory rabbits in Taiwan.

CONCLUSIONS

The set of 18 microsatellite markers used in this study indicated mild separation of the 3 NZW populations in the individual phylogenetic tree and the separation of the REX population into 2 sub-populations in the Structure analysis. These results showed that these markers can be applied in genetic content monitoring for the breeding management of lab rabbits. The analysis of intra-population structure and the establishment of genetic profile using neutral markers provide a general outline of the breeding systems applied to livestock. The genetic diversity of lab rabbits in Taiwan is important for future breed development. It has previously been reported that outbred laboratory animals should maintain the maximum heterozygosity. Our results indicated that there were a total of 99 alleles among the 5 populations; each population had its own unique alleles, and the REX population had 2 sub-populations. These molecular genetic data should be used in the future selection of parental stocks for planned mating as part of strategies to preserve and restore the rational breeding of laboratory rabbits in Taiwan. The application of these molecular genetic data will reduce the chance of inbreeding. According to the microsatellite analysis, the 5 rabbit populations in Taiwan had medium levels of inbreeding, indicating low effective population sizes. Therefore, we recommend the introduction of unrelated rabbits from abroad or from other domestic populations in order to bring new genetic variability to the investigated populations. At the same time, close monitoring of genetic diversity using the presented set of 18 microsatellite markers should be applied.

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