Indian Journal of Animal Sciences **85** (11): 1220–1224, November 2015/Article
https://doi.org/10.56093/ijans.v85i11.53280

Microsatellite analysis after long term selection for egg production in Rhode Island Red chicken

ANANTA KUMAR DAS[1], SANJEEV KUMAR[2] and ABDUL RAHIM[3]

Central Avian Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 20 March 2015; Accepted: 22 April 2015

ABSTRACT

This investigation aimed to analyze microsatellites after long term selection for egg production in the selected line (RIR^S) of Rhode Island Red chicken and its control line (RIR^C) maintained at the institute. Genomic DNA samples isolated from 24 randomly selected birds of RIR^S and RIR^C line were investigated at 24 microsatellite loci. Microsatellite alleles were separated on 6% urea-PAGE and their sizes were estimated with the help of Gel Doc 2000 system. Allelic data was analyzed. Analysis revealed 2 to 7 alleles in RIR^S and 2 to 9 alleles in RIR^C line across 24 loci with their sizes ranged from 84 to 276 bp. Observed number of alleles per locus was 4.04±0.23 in RIR^S and 4.42±0.33 in RIR^C. Allele frequency ranged from 0.083 to 0.667 in RIR^S and 0.042 to 0.833 in RIR^C. Approximately 34.02% of alleles in RIR^S and 39.62% alleles in RIR^C were line specific. The frequencies of the specific alleles ranged from 0.083 to 0.667 in RIR^S and 0.083 to 0.883 in RIR^C. Line specific alleles with higher frequencies can be used in line identification. Corresponding effective number of alleles and Shannon's information index averaged 3.32±0.19 and 1.25±0.06 in RIR^S and 3.66±0.32 and 1.30±0.08 in RIR^C. These diversity estimates indicated that the control line was more diverse than the selected line and certain specific microsatellite alleles were getting fixed in the selected line.

Key words: Allele frequency, Effective number of alleles, Line-specific alleles, Microsatellites, RIR chicken lines, Shannon's information index

Microsatellites are considered as the marker of choice for assessing molecular genetic structure and diversity estimation owing to their presence in the conserved regions of the genome in most cases. Rhode Island Red (RIR) chicken population brought at Central Avian Research Institute in 1980 is well adopted and has acclimatized to Indian climate and backyard system. The flock was genetically improved through 29 generations of selection for egg production up to 40 weeks of age along with some independent culling for egg weights at 28th week of age and being maintained as selected line (RIR^S). Random bred control population (RIR^C) is also being maintained since then. The 2 lines demonstrated significant differences in layer performances (Das *et al.* 2014), but they were not genetically characterized. Therefore, it is an immediate need of the time to explore DNA markers to scan for possibility for faster genetic progress in these chicken lines one of which has undergone for long time selection. Present investigation was carried out to analyze microsatellites after long term selection for egg production in the selected line of RIR chicken and its control line.

MATERIALS AND METHODS

Experimental birds and sampling procedures: The selected and control line of RIR chicken is maintained at the Central Avian Research Institute, by mating the parental female line in individual laying cages artificially inseminating semen collected from the individual sires of respective male line taking records for dam and sire numbers. Birds (24) from the selected and control line of RIR chicken were randomly chosen for this study. Genomic DNA samples were extracted from each 0.1 ml of venous blood by phenol extraction method (Kagami *et al.* 1990), followed by quality checking on 0.7% horizontal submarine agarose gel electrophoresis, purity checking and quantity determination using spectrophotometer. Samples showing intact DNA band and optical density ratio (260 nm: 280 nm) between 1.7 and 1.9 were used in subsequent experiments. PCR ready DNA samples were prepared at a concentration of 50 ng/µl.

Microsatellite markers and primers: A panel of 24 microsatellite markers, recommended by FAO (2011) and/or used by National Bureau of Animal Genetic Resources, Karnal (India) for genetic characterization, was used. The

*Part of Ph.D. thesis of first author, submitted to the Indian Veterinary Research Institute, Izatnagar 243 122 India

Present address: [1]Subject Matter Specialist (Animal Science) (dasugenvet@gmail.com), Howrah Krishi Vigyan Kendra, Jagatballavpur, Howrah, West Bengal. [2]Principal Scientist (skgicar@gmail.com), Avian Genetics and Breeding Division. [3]Ph.D. Scholar (choudhary633@gmail.com), Division of Animal Genetics, Indian Veterinary Research Institute, Izatnagar.

chicken specific primers for the markers were designed and synthesized (0.01 mM). Annealing temperature for each of primers was optimized as per Wimmers *et al.* (2000).

PCR reaction mix and amplification programme: PCR reactions were carried out in 25 µl reaction mix prepared by gently mixing 2.5 µl of 10X Taq buffer with $MgCl_2$, 2.5 mM each of dNTP mix, 0.8 µM each of forward and reverse primers, 0.75 U Taq DNA polymerase and 50 ng template DNA into nuclease free water. PCR amplification was carried out in programmable thermal cycler using PCR programme as initial heat inactivation at 94°C for 5 min, followed by 30 cycles of (i) denaturation at 94°C for 1 min, (ii) annealing at optimized annealing temperature for each microsatellite primer pair for 45 sec and (iii) extension at 72°C for 45 sec, followed by a final extension at 72°C for 5 min and then 4°C forever.

Gel electrophoresis: The molecular sizes of amplified products were adjudged for their probable sizes through 1.4% horizontal agarose gel electrophoresis. For this, approximate 10 µl of PCR product along with 5 µl of 100 bp DNA ladder was loaded and run through electrophoresis at 2 to 5 volts/cm for 60 min. Then the products on to gel were examined and photographed under UV illumination. The microsatellite alleles were then identified by running the amplified products on vertical denaturing polyacrylamide gel electrophoresis (6% urea-PAGE). Approximate 10 µl of PCR product mixed with 6X loading dye was denatured at 95°C for 10 min and snapped immediately on ice for 10 min. Then it was immediately loaded along with 4 µl of 100 bp DNA ladder as molecular size marker on a urea-PAGE gel. The gel was prepared by adding 250 µl of fresh 10% ammonium per sulphate and 30 µl of TEMED in 48 ml of chilled transparent PAGE solution. The PAGE solution was prepared dissolving 18.0 g of urea in to a premix of 10 ml of autoclaved distilled water, 8.7 ml of 5X TBE buffer and 11.3 ml of 30% acrylamide-bisacrylamide (29:1) solution. Then the PAGE solution was filtered through Wattman paper and kept in to refrigerator. The electrophoresis for urea-PAGE was carried out at 5 to 6 volts/cm for 4 to 4½ hours followed by silver staining (Beidler *et al.* 1982).

Determination of microsatellite allele size, data handling and analysis: Molecular sizes of various alleles at different microsatellite loci were determined using the Quantity One software. The observed alleles in each sample at each microsatellite loci and its probable genotypes were recorded. Locus specific alleles were identified according to their molecular sizes. Then the allelic data were analyzed through POPGENE version 1.32 (Yeh *et al.* 1999).

RESULTS AND DISCUSSION

Microsatellite allele profile: Molecular sizes of various alleles ranged from 84 bp at MCW0051 microsatellite (MS) loci in RIR^C to 276 bp at MCW0050 in RIR^C line (Table 1). The present investigation documented various locus specific alleles whose sizes were in accordance to the earlier reports. Romanov and Weigend (2001) documented some similar alleles at MCW0001, MCW0004, MCW0005, MCW0014 and ADL0158 MS loci in Red Jungle Fowls and its related populations. Rajkumar *et al.* (2008) reported some similar alleles at MCW0041, MCW0043, MCW0049, MCW0001, MCW0004, MCW0005, MCW0016, ADL0171, ADL0172, ADL0176 and ADL0267 in RIR, Dahlem Red, WLH-IWD, WLH-IWF, Babcock, Vencobb, Aseel and Desi chickens. Chatterjee *et al.* (2010) resolved some similar alleles at MCW0044, MCW0049, MCW0059, ADL0136, ADL0158, ADL0176 and ADL0210 in Kadaknath, Aseel, Vanraja male and female line, and Dahlem Red chickens. However, the finer difference in the allele sizes might be due to the breed, line or strain difference as well as to the methodologies adopted for their resolution and size estimation.

The number of alleles at various microsatellite loci ranged from 2 to 7 in RIR^S and 2 to 9 in RIR^C chicken line with varied allele frequencies (Table 1). Varied number of observed alleles i.e. 3 to 9 in Aseel, 3 to 8 in Miri and 2 to 7 in Nicobari chicken were also reported (Pandey *et al.* 2002). Some of the alleles in the present study were common whereas some were unique to RIR^S and RIR^C line. The average numbers of alleles (N_o) per locus at these MS loci were 4.04±0.23 in RIR^S and 4.42±0.33 in RIR^C (Table 2). Pandey *et al.* (2002) reported the observed number of alleles in Aseel, Miri and Nikobari chicken for loci MCW0041, MCW0049, MCW0005, ADL0102, ADL0136, ADL0158, ADL0171, ADL0210 and ADL0267 in accordance to the present findings. Jing-Ting *et al.* (2007) reported 4, 5, 6 and 5 numbers of alleles at MCW0004, MCW0044, ADL0136 and ADL0210, respectively in Recessive White and Xianju chicken F_3 populations. Similarly, Saini *et al.* (2008) also reported 3 alleles at MCW0014 in RIR-B strain. Chattopadhyay *et al.* (2009) reported 4 alleles at MCW0016 in RIR-B strain. Chatterjee *et al.* (2010) also reported similar number of alleles at MCW0043 and ADL0102 in Kadaknath, Aseel, Vanraja male and female line, and Dahlem Red chickens as obtained in RIR^C in the present study.

In the present investigation, a total of 97 and 106 alleles were resolved across all MS loci in RIR^S and RIR^C line, respectively with corresponding allele frequencies ranged from 0.083 to 0.667 and 0.042 to 0.833. Out of 97 alleles observed in RIR^S line 13, 45 and 39 numbers of alleles had frequencies more than 0.4, between 0.2 to 0.4 and less than 0.2, respectively. The numbers of alleles in the corresponding frequency ranges were 13, 44 and 49 in RIR^C.

The most frequent alleles were 116 bp sized allele (66.7%) at MCW0051 in RIR^S and 173 bp allele (88.3%) at MCW0059 in RIR^C line. Parmar *et al.* (2007) obtained the overall allele frequencies ranged from 0.009 to 0.704 in the three varieties of Kadaknath chicken.

The present investigation demonstrated some unique alleles, not reported earlier, in the selected and control line of RIR chicken. Out of 97 alleles, 33 (34.02%) and out of 106 alleles, 42 (39.62%) numbers of alleles were line

Table 1. Allele sizes and their frequencies at various microsatellite loci in RIR^S and RIR^C chicken lines

Microsatellite Loci	T_a (°C)	Allele sizes (bp) and respective allele frequency in the parenthesis	
		RIR^S	RIR^C
MCW0041	57.0	148(0.083), 156(0.583), 162(0.333)	148(0.500), 156(0.333), 162(0.167)
MCW0043	52.0	121(0.200), 127(0.400), 133(0.100), 145(0.300)	115(0.083), 121(0.167), 127(0.167), 133(0.250), 136(0.083), 145(0.250)
MCW0044	63.0	133(0.333), 136(0.167), 151(0.333), 160(0.167)	121(0.083), 130(0.250), 136(0.250), 142(0.250), 151(0.167)
MCW0048	55.0	182(0.250), 192(0.250), 226(0.083), 234(0.417)	182(0.167), 192(0.333), 226(0.111), 234(0.333)
MCW0049	63.0	126(0.167), 129(0.333), 132(0.333), 147(0.167)	111(0.167), 114(0.667), 120(0.167)
MCW0050	58.5	234(0.417), 250(0.250), 258(0.083), 276(0.250)	234(0.100), 250(0.400), 258(0.500)
MCW0051	50.5	90(0.083), 105(0.250), 118(0.667)	84(0.333), 90(0.083), 105(0.583)
MCW0059	50.5	140(0.400), 158(0.600)	167(0.167), 173(0.833)
MCW0071	58.5	240(0.400), 244(0.200), 250(0.400)	240(0.400), 250(0.400), 254(0.200)
MCW0075	63.0	180(0.167), 184(0.333) 190(0.167), 196(0.333)	174(0.100), 180(0.200), 184(0.200), 188(0.100), 190(0.200), 196(0.200)
MCW0001	55.0	156(0.250), 160(0.083), 168(0.167), 182(0.250), 190(0.083), 198(0.167)	168(0.250), 174(0.250), 198(0.250), 204(0.250)
MCW0002	57.0	141(0.200), 159(0.300), 175(0.200), 181(0.300)	141(0.417), 159(0.083), 175(0.250), 181(0.167), 195(0.083)
MCW0004	57.0	175(0.300), 181(0.100), 185(0.100), 225(0.200), 229(0.200), 247(0.100)	199(0.083), 205(0.083), 209(0.333), 247(0.083), 259(0.417)
MCW0005	55.0	210(0.083), 216(0.333), 222(0.167), 238(0.167), 244(0.250)	210(0.200), 216(0.300), 238(0.100), 244(0.300), 256(0.100)
MCW0014	60.0	173(0.200), 175(0.200), 177(0.600)	173(0.200), 175(0.200), 177(0.600)
MCW0016	57.0	149(0.417), 163(0.083), 177(0.417), 191(0.083)	145(0.250), 149(0.250), 177(0.417), 191(0.083)
ADL0102	46.5	136(0.200), 146(0.200), 166(0.400), 174(0.200)	136(0.167), 154(0.250), 166(0.083), 182(0.250), 192(0.250)
ADL0136	52.0	122(0.083), 132(0.333), 134(0.167), 150(0.417)	118(0.083), 126(0.083), 132(0.167), 134(0.083), 138(0.083), 148(0.083), 150(0.167), 156(0.167), 162(0.083)
ADL0158	52.0	178(0.083), 184(0.583), 214(0.167), 222(0.167)	164(0.083), 174(0.167), 178(0.083), 184(0.250), 192(0.083), 198(0.167), 214(0.167)
ADL0171	46.5	98(0.250), 112(0.417), 128(0.333)	86(0.083), 98(0.250), 108(0.167), 112(0.333), 142(0.167)
ADL0172	46.5	131(0.333), 147(0.167), 159(0.333), 169(0.167)	131(0.333), 147(0.500), 169(0.167)
ADL0176	55.0	200(0.500), 202(0.400), 236(0.100)	194(0.667), 196(0.250), 236(0.083)
ADL0210	52.0	128(0.083), 130(0.333), 134(0.333), 140(0.167), 150(0.083)	128(0.250), 130(0.417), 134(0.042), 150(0.291)
ADL0267	55.0	98(0.083), 102(0.333), 108(0.083), 110(0.083), 112(0.083), 124(0.167), 130(0.167)	108(0.083), 110(0.167), 112(0.083), 114(0.167), 124(0.250), 130(0.250)
Total alleles resolved		97	106

T_a (°C) denotes optimized annealing temperatures in degree centigrade.

specific for RIR^S and RIR^C, respectively (Table 2). The frequencies of specific alleles ranged from 0.083 to 0.667 in RIR^S and 0.083 to 0.883 in RIR^C (Table 1). Line specific alleles with higher frequencies found in the present investigation could be used in line identification (Tadano *et al.* 2007, Rajkumar *et al.* 2008). Eighteen (75%) out of 24 loci studied exhibited specific alleles, out of which 8 (44.44%) loci in RIR^S and 12 (66.67%) in RIR^C exhibited ≥ 2 specific alleles per locus. Loci ADL0136 and MCW0004 demonstrated the highest number of specific alleles in RIR^S (5 alleles) and RIR^C (6 alleles), respectively. These findings corroborated with the findings of Tadano *et al.* (2007) and Rajkumar *et al.* (2008) in various chicken populations.

Diversity estimates: The MS loci MCW0041, MCW0048, MCW0051 and MCW0016 demonstrated same number of alleles in both RIR^S and RIR^C populations, although with different frequencies; that resulted in varied effective number of allele (N_e) and Shannon's information index (I), which were more in RIR^C than RIR^S (Table 2). The estimates of N_e and I for MCW0059, MCW0005 and ADL0176 were less in RIR^C than RIR^S, although the numbers of alleles were same but the frequencies were different. The number of alleles and their frequencies at MCW0071 and MCW0014 were same in both the populations; the estimates of N_e and I also being the same. The RIR^C population revealed the highest number of alleles and Shannon's diversity estimates for MCW0136, ADL0158, MCW0075, MCW0043, MCW0044, MCW0002, ADL0102 and ADL0171 than those estimated in RIR^S line. The RIR^S population demonstrated the highest number of alleles and Shannon's diversity estimates for ADL0267, MCW0001, MCW0004, ADL0210, MCW0049, MCW0050 and ADL0172 than RIR^C line. The mean effective number of alleles (N_e) in both the populations was less than the mean observed number of alleles (N_o) in accordance to the findings of others (Pandey *et al.* 2002; Rajkumar *et al.* 2008). Varied effective number of alleles i.e. 3.09 in Aseel, 3.39 in Miri and 3.15 in Nicobari chicken were also reported (Pandey *et al.* 2002). Present findings were in accordance with the findings of Rajkumar *et al.* (2008) who reported mean N_e estimate as 3.78 in RIR and 2.69 in Dahlem Red chickens using same panel of 20 microsatellite loci. Hui-Fang *et al.* (2009) reported N_e estimates as 6.20 at ADL0136, 2.325 at ADL0210, 4.976 at ADL0176, 2.138 at MCW0014 and 4.181 at MCW0004 in Qingyuan partridge chicken. Reports of Shannon's diversity estimates were limited.

Table 2. Observed number of alleles, line-specific alleles and diversity estimates at various microsatellite loci in RIR^S and RIR^C chicken lines

MS Locus	RIR^S				RIR^C			
	N_o	N_u	N_e	I	N_o	N_u	N_e	I
MCW0041	3	-	2.1818	0.8877	3	-	2.5714	1.1011
MCW0043	4	-	3.3333	1.2799	6	2	5.1429	1.7046
MCW0044	4	2	3.6000	1.3297	5	3	4.5000	1.5454
MCW0048	4	-	3.2727	1.2650	4	-	3.3061	1.2763
MCW0049	4	4	3.6000	1.3297	3	3	2.000	0.8676
MCW0050	4	1	3.2727	1.2650	3	-	2.3810	0.9433
MCW0051	3	1	1.9459	0.8240	3	1	2.1818	0.8877
MCW0059	2	2	1.9231	0.6730	2	2	1.3846	0.4506
MCW0071	3	1	2.7778	1.0549	3	1	2.7778	1.0549
MCW0075	4	-	3.6000	1.3297	6	2	5.5556	1.7481
MCW0001	6	4	5.1429	1.7046	4	2	4.0000	1.3863
MCW0002	4	-	3.8462	1.3662	5	1	3.6000	1.4241
MCW0004	6	5	5.0000	1.6957	5	4	3.2727	1.3522
MCW0005	5	1	4.2353	1.5171	5	1	4.1667	1.5048
MCW0014	3	-	2.2727	0.9503	3	-	2.2727	0.9503
MCW0016	4	1	2.7692	1.1437	4	1	3.2727	1.2650
ADL0102	4	2	3.5714	1.3322	5	3	4.5000	1.5454
ADL0136	4	1	3.1304	1.2367	9	6	8.0000	2.1383
ADL0158	4	1	2.4828	1.1187	7	4	6.0000	1.8637
ADL0171	3	1	2.8800	1.0776	5	3	4.2353	1.5171
ADL0172	4	1	3.6000	1.3297	3	-	2.5714	1.0114
ADL0176	3	2	2.3810	0.9433	3	2	1.9459	0.8240
ADL0210	5	1	3.7895	1.4452	4	-	3.0968	1.2031
ADL0267	7	2	5.1429	1.7918	6	1	5.1429	1.7046
Mean±SE	4.04 ±0.23	33	3.32 ±0.19	1.25 ±0.06	4.42 ±0.33	42	3.66 ±0.32	1.30 ±0.08

N_o, Observed number of alleles; N_u, Number of unique alleles (line-specific); N_e, expected number of alleles; I, Shannon's information index.

At least 3 to 4 alleles at any microsatellite loci were recommended for estimation of genetic diversity and genetic distances (Hillel *et al.* 2003) and almost all the microsatellite loci excepting MCW0059 (2 alleles) used in this study could be considered useful for evaluation of genetic diversity in chicken breeds. The lower effective number of alleles than the observed number of alleles across the loci in the present investigation indicated that allele frequencies were widely distributed. The estimates of observed and effective numbers of alleles and Shannon's information index indicated that RIR^C was more diverse population than the RIR^S line, could be due to the reason that this population was not subjected to selection.

In light of the above results, it might be concluded that specific alleles of microsatellite loci could be used for line differentiation. The RIR control line was more diverse than its selected line and certain specific microsatellite alleles were getting fixed in the selected line.

ACKNOWLEDGEMENT

The authors sincerely acknowledge the first author's SAC-members and institute fellowship for his Ph.D. programme at Indian Veterinary Research Institute, Izatnagar.

REFERENCES

Beidler J L, Hilliard P R and Rill R L. 1982. Ultrasensitive

staining of nucleic acids with silver. Analytical Biochemistry **126**: 374–80.

Chatterjee R N, Niranjan M, Sharma R P, Dange M and Bhattacharya T K. 2010. Estimation of genetic heterogeneity of chicken germplasm being used for development of rural varieties utilizing DNA markers. *Journal of Genetics* **89**: 33–37.

Chattopadhyay A, Ravi K G, Chaudhary M L and Brah G S. 2009. Genetic diversity analysis in chickens using microsatellite markers. *Indian Journal of Poultry Science* **44**: 163–66.

Das A K, Kumar S, Rahim A and Mishra A K. 2014. Genetic variability in immunocompetence and performance status of Rhode Island Red chicken strains and its crosses. *International Journal of Bio-resource and Stress Management* **5 (2)**: 246–54.

FAO. 2011. Molecular genetic characterization of animal genetic resources. *FAO Animal Production and Health Guidelines*, Rome, No. 9.

Hillel J, Groenen M A M, Tixier-Boichard M, Korol A B, David L, Kirzhner V M, Burke T, Barre-Dirie A, Crooijmans R P M A, Elo K, Feldman M W, Freidlin P J, Maki-Tanila A, Oortwijn M, Thomson P, Vignal A, Wimmers K and Weigend S. 2003. Biodiversity of 52 chicken populations assessed by microsatellite typing of DNA pools. *Genetics of Selection and Evolution* **35**: 533–57.

Hui-Fang L, Jing-Ting S, Wei-Tao S and Kuan-Wei C. 2009. Analysis of genetic diversity in Qingyuan Partridge chickens based on microsatellite markers. *Journal of Animal and Veterinary Advances* **8** (3): 454–8.

Jing-Ting S, Wen-Bin B, Jin-Hua C and Guo-Hong C. 2007. Study on correlation between microsatellite markers and meat quality traits in chicken. *Research Journal of Animal Science* **1**: 107–10.

Kagami H, Nakamura H and Tomita T. 1990. Sex identifica-tion in chickens by means of the presence of the W chromo-some-specific repetitive DNA units. *Japanese Poultry Science* **27**: 379–84.

Pandey A K, Tantia M S, Kumar D, Mishra B, Chaudhary P and Vijh R K. 2002. Microsatellite analysis of three poultry breeds of India. *Asian-Australasian Journal of Animal Science* **15**: 1536–42.

Parmar S N S, Tolenkhomba T C, Thakur M S, Joshi C G, Rank D N, Solanki J V, Srivastava P N and Pillai P V A. 2007. Analysis of genetic relationship among three varieties of indigenous Kadaknath breed using 25 chicken microsatellite markers. *Indian Journal of Biotechnology* **6**: 205–09.

Rajkumar U, Gupta R B and Reddy R A. 2008. Genomic heterogeneity of chicken populations in India. *Asian-Australasian Journal of Animal Science* **21**: 1710–20.

Romanov M N and Weigend S. 2001. Analysis of genetic relationships between various populations of domestic and jungle fowl using microsatellite markers. *Poultry Science* **80**: 1057–63.

Saini S, Chaudhary M L, Brah G S and Ravi-Kumar G V P P S. 2008. Polymorphism analysis in egg type chickens using microsatellite markers. *Indian Journal of Poultry Science* **42**: 27–30.

Tadano R, Nishibori M, Nagasaka N and Tsudzuki M. 2007. Assessing genetic diversity and population structure for commercial chicken lines based on forty microsatellite analyses. *Poultry Science* **86**: 2301–08.

Wimmers K, Ponsuksili S, Hardge T, Valle-Zarate A, Mathur P K and Horst P. 2000. Genetic distinctness of African, Asian and South American local chickens. *Animal Genetics* **31**: 159–65.

Yeh F C, Yang R and Boyle T. 1999. *User manual for POPGENE Version 1.31*, Microsoft Window-based Freeware for Population Genetic Analysis, University of Alberta and Centre for International Forestry Research.