African Journal of Biotechnology Vol. 10(76), pp. 17623-17631, 30 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1896
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Cross-species applicability of chicken microsatellite markers for investigation of genetic diversity in Indian duck (*Anas platyrhynchos*) populations

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Accepted 17 October, 2011

We obtained blood samples of 57 Indian ducks (*Anas platyrhynchos)* belonging to three indigenous duck populations of geographically distinct locations of the country and genotyped them using chicken microsatellite markers. Twenty three of the 30 loci were amplified and 17 loci yielded high success rate (> 91%). Observed and effective number of alleles ranged from 4 to 21 and 1.80 to 13.34, respectively. The observed heterozygosity across populations ranged from between 0.15 and 0.91, with mean (± SE) of 0.63 ± 0.21, while the expected heterozygosity ranged 0.45 to 0.94 with mean (± SE) of 0.72 ± 0.13. The polymorphic information content (PIC) ranged from 0.43 to 0.92 with an average of 0.68. Eleven loci confirmed Hardy-Weinberg equilibrium (P>0.05) and no evidence for linkage disequilibrium was observed among pairs of loci. Dendrogram based on Nei's genetic distance grouped Assam and West Bengal duck populations and separated the Uttarakhand duck population. The results provide evidences of the applicability of chicken microsatellite markers in determining the genetic variations and relationship among three ducks populations in India.

Key words: Chicken microsatellites, Cross-species amplification, *Anas platyrhynchos*, Genetic diversity.

INTRODUCTION

Ducks belong to the order Anseriformes, subfamily Anatinae of the waterfowl family Anatidae. According to the paleontological data, the main radiation of the modern ducks took place during the Miocene, 5 to 23 million years ago (Olson et al., 1985). The Mallard (*Anas platyrhynchos*) is believed to be the wild ancestor of almost all of the varieties of domestic ducks apart from the Muscovy duck (*Cairina moschata*) and they frequently interbreed with their closest relatives in the genus *Anas* (Phillips and John, 1915). Domesticated ducks initially reared for meat, eggs and down, are important genetic reservoirs and essential for facing the future challenges of disease resistance and better quality of meat.

Genetic studies of waterfowl utilizing allozyme electrophoresis and mitochondrial DNA have provided valuable information on their evolutionary history (Cooke and Buckley, 1987). Microsatellite DNA markers consist of tandem repeats between 1 and 6 bp, repeated up to 60 times and referred to as simple sequence loci. These domains were first demonstrated by Hamada and colleagues, during the early eighties (Tautz and Renz, 1984; Tautz, 1989; Smeets et al., 1989). Although microsatellites are very informative and extensively used in forensics, genetic mapping, population genetics, evolutionary studies and investigation of social systems (Chakraborty et al., 1997; Buchlolz et al., 1998; Chowdhury and Bansal, 2001), the need for prior

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sequence information to produce locus specific primer sets remain a major limitation. Therefore, cross species amplification might serve as an alternative way for the genetic characterization of the species of interest if the sequences flanking the repeats of microsatellites are conserved between the species. Previous studies of cross amplification suggested that there was a significant negative correlation between microsatellite performance and evolutionary distances between original species and the taxon being analysed (Moore et al., 1991; Peakall et al., 1998; Dallimer, 1999).

Several early studies have shown the applicability of microsatellite loci among closely related species by means of cross-species amplification (Moore et al., 1991; Primmer et al., 1996; Pang et al., 1999; Baratti et al., 2001; Wilson et al., 2004; Huang et al., 2005; Kupper et al., 2007; Sruoga et al., 2008; Zhou et al., 2009). Chicken microsatellite primers have been tested in other galliformes, turkey (*Meleagris gallopava*) (Levin et al., 1995; Liu et al., 1996) and Himalayan Monal (Thakur et al., 2011). Few studies have also developed molecular markers for ducks (Buchlolz et al., 1998; Maak et al., 2000, 2003; Paulus and Tiedemann, 2003; Stai and Hughes, 2003; Huang et al., 2005). However, this study was conducted with the aim of developing heterologous microsatellite markers for ducks, and to investigate their application in assessing genetic diversity and relationship among three ducks populations of India. Universal heterologous microsatellite primers for birds will play a major role in studies on bird evolution, phylogeny and genetic divergence of birds of different taxa.

MATERIALS AND METHODS

Blood sample collection and DNA isolation

Blood samples from 57 unrelated birds of three indigenous duck populations (Assam, n = 21; Uttarakhand, n = 18 and West Bengal, n = 18) were collected on FTA® Classic-Cards from different geographical locations of the country (Figure 1) and DNA was isolated as described by Smith and Burgoyne (2004).

Amplification of microsatellite loci using multiplex PCR

A total of 30 microsatellite primers used in 'European Chicken Biodiversity Project' (AVIANDIV, Weigend et al., 1998-2000) were tested in this study. Polymerase chain reactions were performed on an Applied Biosystem thermal cycler (2700 and 2720) using Qiagen Multiplex PCR Kit (Cat# 206143). PCR reaction for each multiplex panel of six loci was set up in a 15 µl reaction volume containing 7.5 µl of 2x Qiagen multiplex PCR master mix, 0.50 µl of 10 µM of each primer pair (3.0 µL for 6 loci), 1 µl of DNA elutant (approx. 20 ng) and 3.5 µl of RNase-free water. The amplification conditions were as follows: initial heat-activation of Hot Start Taq DNA polymerase at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at a specific temperature (Table 1) for 90 s, extension at 72°C for 60 s, with a final extension at 60°C for 30 min. Approximately, 5 µl of PCR products were loaded onto 2% agarose gel and a negative PCR control was kept throughout the procedure. Fluorescence based genotyping was performed on ABI 3130 Genetic Analyzer (Applied Biosystem) and fragment size of each allele was analysed using GeneScan 500 (-250) LIZ by GeneMapper Software (Version 3.7, Applied Biosystem). Data of 17 loci (Table 1) was subjected for statistical analysis.

Data analysis for polymorphic microsatellite markers

Success rate of each locus was calculated and loci generating high success rate was subjected to further analysis. The observed (Na), effective number of alleles (Ne), observed (Ho) and expected heterozygosity (He) estimates were computed after Nei (1973), as executed in POPGENE Software (Yeh et al., 1999). Wright's fixation index (Fis), the measurement of heterozygote deficiency was calculated (Wright 1978). Using allelic frequencies, polymorphic information content (PIC), a measure of marker's informativeness, and predicted null allele frequencies was calculated with the Cervus (ver. 3.0) computer programme (Kalinowski et al., 2007). Departure of microsatellite loci from Hardy-Weinberg equilibrium (HWE) was tested using the exact probability test (Haldane, 1954) in the software TFPGA. P-value for HWE deviation was calculated using conventional Monte Carlo method. An unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) based on Nei's genetic distances (DA) was used to construct the dendrogram using TFPGA program. In order to reduce the dimensionality of the data, the correspondence analysis was carried out using GENETIX ver. 4.02 (Belkhir et al., 2004). Linkage disequilibrium (LD) test between pairs of loci was performed in FSTAT (Goudet, 1995).

RESULTS

Initially, 30 chicken microsatellite loci were tested for amplification in Indian ducks and seven of them (LEI0166, MCW0020, LEI0192, MCW0222, MCW0284, MCW0014 and LEI0094) did not amplify or show weak gel band. The remaining loci were amplified successfully and six loci showed low success rate; MCW0248 (47.05%), MCW0034 (55.8%), MCW0206 (58.82%), MCW0183 (70.58%), ADL0112 (85.29%) and MCW0330 (85.29%). Therefore, data of these loci was excluded from the analysis. PCR cycling conditions to amplify the microsatellites in ducks were the same as used in our earlier study on RJF (Mukesh et al., 2011), while intensive re-optimization of PCR conditions could give better success rate. Finally, 17 loci data that showed a success rate >91% was subjected to further analysis.

Polymorphism of markers and genetic diversity statistics

All the 17 microsatellite markers were polymorphic across three duck populations and the summary of the diversity measures are presented in Table 2. The mean (± standard deviation) observed number of allele was 8.0 ± 3.72 in Assam duck population while it was 4.11 ± 1.69 in West Bengal duck population. The mean effective number of alleles ranged between 4.21 ± 2.81 and 2.81 ± 1.30 for Assam duck population and West Bengal population, respectively. Mean observed heterozygosity was lower than the mean expected heterozygosity in

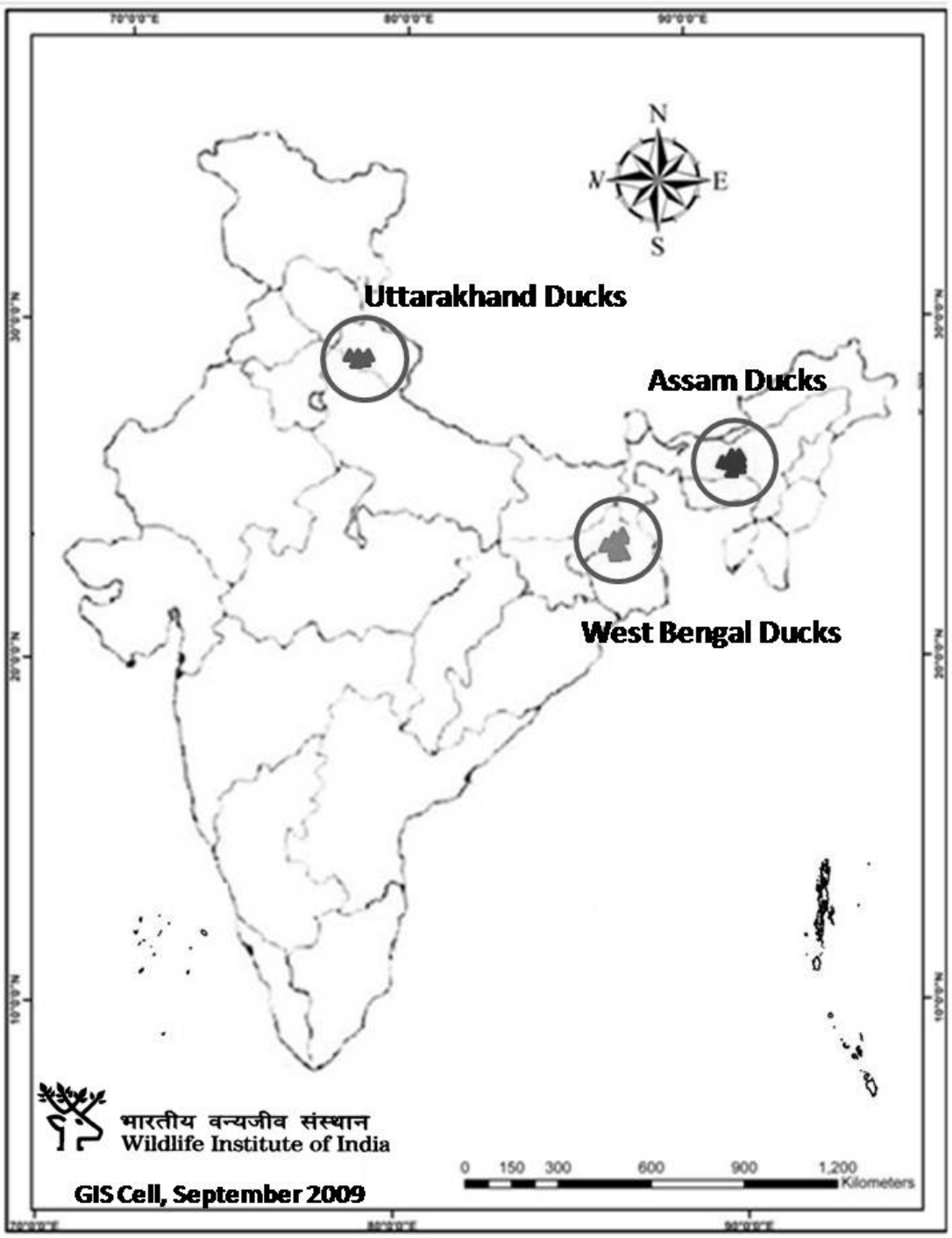

Figure 1. Map of India showing the sampling locations of three indigenous duck populations in the country.

Assam and Uttarakhand duck population, while it was higher in West Bengal duck population. Also, the mean expected heterozygosity ranged between 0.61 ± 0.20 and 0.80 ± 0.10 in West Bengal and Uttarakhand duck populations, respectively, while mean observed heterozygosity was 0.62 to 0.64 in Assam (± 0.24), Uttarakhand (±0.30) and West Bangal (± 0.31), respectively.

Locus wise genetic diversity estimates are depicted in Table 3. Altogether, 171 alleles were observed across 17 loci, which showed a reasonable amount of polymorphism in the chicken microsatellite markers. The number of observed alleles ranged from four (MCW0037 and MCW0103) to 21 (ADL0278), with overall mean number of alleles per locus was 10.05. The observed number of

Table 1. Characteristics of chicken microsatellite markers.

Marker	GenBank accession number	Chromosome	Primer Sequence *(5′ → 3′)* (forward and reverse)	Allele range†	Allele range‡	T_A (°C)
ADL0268	G01688	1	CTCCACCCCTCTCAGAACTA CAACTTCCCATCTACCTACT	102 - 116	92 - 122	60
MCW0037	L43676	3	ACCGGTGCCATCAATTACCTATTA GAAAGCTCACATGACACTGCGAAA	154 - 160	152 - 158	64
MCW0295	G32051	4	ATCACTACAGAACACCCTCTC TATGTATGCACGCAGATATCC	88 - 106	78 - 100	60
MCW0067	G31945	8	GCACTACTGTGTGCTGCAGTTT GAGATGTAGTTGCCACATTCCGAC	176 - 186	161 - 201	60
MCW0104	L43640	13	TAGCACAACTCAAGCTGTGAG AGACTTGCACAGCTGTGTACC	190 - 234	190 - 206	60
MCW0111	L48909	1	GCTCCATGTGAAGTGGTTTA ATGTCCACTTGTCAATGATG	96 - 120	86 - 112	60
MCW0216	AF030586	13	GGGTTTTACAGGATGGGACG AGTTTCACTCCCAGGGCTCG	139 - 149	117 - 161	60
MCW0081	L43636	5	GTTGCTGAGAGCCTGGTGCAG CCTGTATGTGGAATTACTTCTC	112 - 136	94 - 150	60
LEI0234	Z94837	2	ATGCATCAGATTGGTATTCAA CGTGGCTGTGAACAAATATG	216 - 364	214 - 306	60
MCW0103	G31956	3	AACTGCGTTGAGAGTGAATGC TTTCCTAACTGGATGCTTCTG	266 - 270	268 - 274	64
MCW0098	L40074	4	GGCTGCTTTGTGCTCTTCTCG CGATGGTCGTAATTCTCACGT	261 - 265	253 - 263	60
MCW0069	L43684	E60C04W23	GCACTCGAGAAAACTTCCTGCG ATTGCTTCAGCAAGCATGGGAGGA	158 - 176	144 - 194	60
MCW0016	L40041	3	ATGGCGCAGAAGGCAAAGCGATAT TGGCTTCTGAAGCAGTTGCTATGG	162 - 206	142 - 196	60
MCW0078	L43686	5	CCACACGGAGAGGAGAAGGTCT TAGCATATGAGTGTACTGAGCTTC	135 - 147	129 - 159	60
MCW0123	L43645	14	CCACTAGAAAAGAACATCCTC GGCTGATGTAAGAAGGGATGA	76 - 100	56 - 98	60
MCW0165	L43663	23	CAGACATGCATGCCCAGATGA GATCCAGTCCTGCAGGCTGC	114 - 118	98 - 128	60
ADL0278	G01698	8	CCAGCAGTCTACCTTCCTAT TGTCATCCAAGAACAGTGTG	114 - 126	92 - 142	60

† Weigend et al. (1998); ‡ this study.

Table 2 Genetic diversity estimates of three indigenous duck populations of the country

Population	Sample size	Mean observed number of alleles (Na)	Mean effective number of alleles (Ne)	Mean observed Heterozygosity (Ho)	Mean expected Heterozygosity (He)
Assam Ducks	21	8.0 ± 3.72	4.21 ± 2.81	0.62 ± 0.24	0.70 ± 0.16
Uttarakhand Ducks	18	5.0 ± 1.27	3.94 ± 1.25	0.62 ± 0.30	0.80 ± 0.10
West Bengal Ducks	18	4.11 ± 1.69	2.81 ± 1.30	0.64 ± 0.31	0.61 ± 0.20

Table 3. Overall genetic diversity estimates for 17 microsatellite markers.

Locus	Na[a]	Ne[b]	H_O[c]	H_E[d]	F_{is}[e]	F_{it}	F_{st}	PIC	*P*-value[f]	F (Null)†
ADL0268	9	1.80	0.26	0.45	0.05	0.39	0.36	0.43	0.0001	0.32
MCW0037	4	2.44	0.87	0.59	-0.52	-0.49	0.01	0.50	0.001	0.01
MCW0295	9	4.18	0.76	0.77	-0.12	0.002	0.11	0.72	0.7061	-0.01
MCW0067	14	4.87	0.57	0.80	0.27	0.40	0.17	0.77	0.0635	0.16
MCW0104	8	3.43	0.62	0.72	0.01	0.05	0.03	0.66	0.7085	0.06
MCW0111	9	4.52	0.84	0.79	-0.21	0.001	0.17	0.74	0.0326	-0.06
MCW0216	11	4.98	0.81	0.83	-0.05	0.07	0.12	0.77	0.2133	-0.02
MCW0081	17	7.18	0.53	0.87	0.19	0.28	0.10	0.85	0.000	0.24
LEI0234	10	2.47	0.52	0.60	0.06	0.19	0.13	0.57	1.000	0.03
MCW0103	4	2.18	0.15	0.55	0.59	0.72	0.32	0.48	0.000	0.5
MCW0098	5	2.22	0.42	0.55	0.34	0.39	0.08	0.49	0.4571	0.10
MCW0069	9	4.77	0.81	0.80	0.02	0.09	0.07	0.76	1.000	-0.01
MCW0016	9	4.21	0.55	0.77	0.24	0.37	0.16	0.74	0.2894	0.15
MCW0078	8	3.32	0.70	0.88	-0.31	-0.09	0.16	0.65	0.0136	-0.14
MCW0123	15	10.27	0.91	0.91	-0.02	0.05	0.08	0.89	0.5575	-0.008
MCW0165	9	3.28	0.55	0.70	0.06	0.10	0.04	0.64	0.073	0.11
ADL0278	21	13.34	0.61	0.94	0.19	0.25	0.07	0.92	0.093	0.20
Mean	10.0588	4.67	0.63	0.72	0.03	0.15	0.12	0.68		
St. Dev	4.4926	3.05	0.21	0.13						

[a]Observed number of alleles; [b]effective number of alleles; [c]observed heterozygosity; [d]expected heterozygosity; [e]Inbreeding coefficient; [f]HWE -p value;† predicted null allele frequencies.

alleles for all 17 loci exceeded the effective number of alleles, which ranged from 1.80 (ADL0268) to 13.34 (MCW0330) with mean of 4.67. H_E values were higher than H_O values for all 17 loci except for MCW0037, MCW0111 and MCW0069. Mean observed heterozygosity over 17 loci was 0.63 ± 0.21, which was lower than the expected heterozygosity 0.72 ±0.13. Observed (Ho) and expected heterozygosity (He) ranged from 0.15 (MCW0103) to 0.91 (MCW0123) and from 0.45 (ADL0268) to 0.94 (ADL0278), respectively. With exception of loci ADL0268, MCW0103 and MCW0098, PIC values were higher than 0.5 for all the 17 loci examined in this study, which is normally considered as informative in population-genetic analyses (Botstein et al., 1980). The mean PIC value in our samples was 0.68. All the 17 loci were tested for any deviation from Hardy-Weinberg equilibrium and six markers (ADL0268, MCW0037, MCW0111, MCW0081, MCW0103 and MCW0078) deviated from Hardy-Weinberg equilibrium ($P<0.05$). The mean inbreeding coefficient (F_{IS}) was 0.03 and the overall mean F_{IT} and F_{ST} were 0.15 and 0.12, respectively. High predicted null allele frequencies for some loci and deviation of HWE may be due to inaccuracies that may have occurred in scoring of genotypes. No significant linkage disequilibrium was observed among the tests for each pair of loci (all adjusted P values > 0.0007).

Genetic distance and relationship among three indigenous duck populations

Nei's genetic distance of three indigenous duck populations (Table 4) showed that Assam duck population is genetically least distant (MGD = 0.06) to West Bengal duck population when compared to Uttarakhand duck population. Dendrogram based on Nei's genetic distance indicated that Assam duck population and West Bengal

Table 4. Nei's unbiased measures of genetic identity and genetic distance.

Duck population	Assam	Uttarakhand	West Bengal
Assam	*****	0.52	0.93
Uttarakhand	0.64	*****	0.36
West Bengal	0.06	1.02	*****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

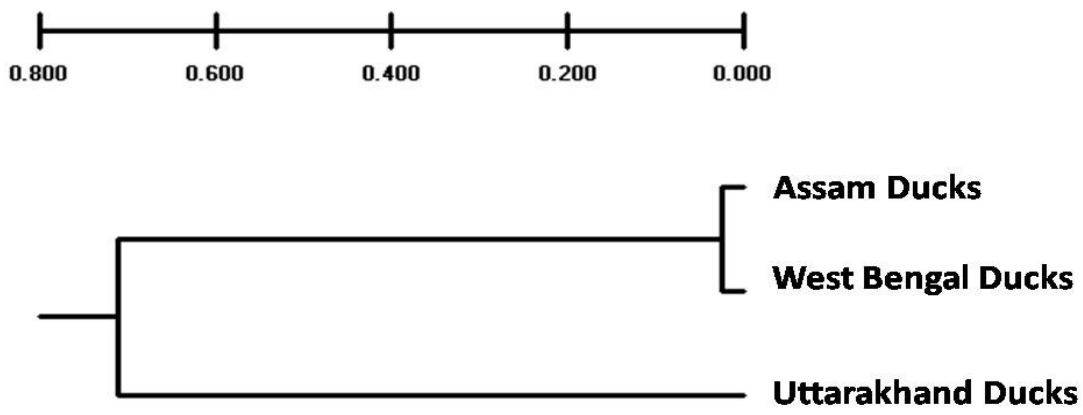

Figure 2. UPGMA dendrogram of the three ducks populations using Nei's unbiased (1978) distance data.

duck population formed one group, while Uttarakhand duck population formed a separate branch from the two populations (Figure 2). The correspondence analysis also showed a similar pattern and connectivity between the Assam duck and West Bengal duck populations. The three axis of the correspondence analysis cumulatively contributed 100% of the total variance. Three-dimensional view along the three axes is shown in Figure 3.

DISCUSSION

In this study, 23 chicken microsatellite loci were transferred on Indian ducks through cross species amplification and 17 of them showed high amplification success rate (>91%). We report that Ho for nearest all loci was quite high in all the three indigenous duck populations. It is considered that loci are highly polymorphic and informative for genetic studies when PIC >0.5 (Botstein et al., 1980; Vanhala et al., 1998). PIC value was higher than 0.5 in 14 loci with the highest value 0.92 (ADL0278). Therefore, these loci could be employed for further genetic studies on ducks. Hardy-Weinberg equilibrium describes the expected frequencies of genotypes in a population under random mating. In this present study, 11 loci were confirmed to Hardy-Weinberg equilibrium (P>0.05), while six loci deviated from it.

Furthermore, null alleles are a common cause of apparent deviations from HWE at microsatellite loci (Pemberton et al., 1995). Null alleles most often occur because of mutations in one or both primer binding sites, sufficient to prevent effective amplification of the microsatellite allele. This problem is particularly common when the microsatellite loci are cloned in one species and used in other species with the identical primers. Allele scoring errors might also be the cause of HWE departure and presence of null allele for one or more loci. This study shows the presence of ample polymorphism in three ducks populations of India. In addition, dendrogram based on Nei's genetic distance and correspondence analysis revealed that Northeastern and coast-line ducks are found to be different from the main land ducks and this was in accordance with their geographical distribution in India.

This study was the part of a national project on conservation genetics of red jungledowl (Gallus gallus) conducted by Wildlife Institute of India, Dehradun, and this is our third successful attempt for developing cross-species chicken microsatellite markers. Earlier, we transferred 15 chicken microsatellites in Himalayan monal (*Lophophorus impejanus)* (Thakur et al., 2011) and 18 chicken microsatellites in domestic pigeon (*Columba livia* var. *domestica)* (Mukesh et al., 2011). For this study, our aim was to examine the applicability of chicken microsatellites in ducks and their use in assessing the genetic variation and relationship among Indian duck populations. The findings of this study would be further utilized to compare the structure of repeat motifs and study the evolution of microsatellites across

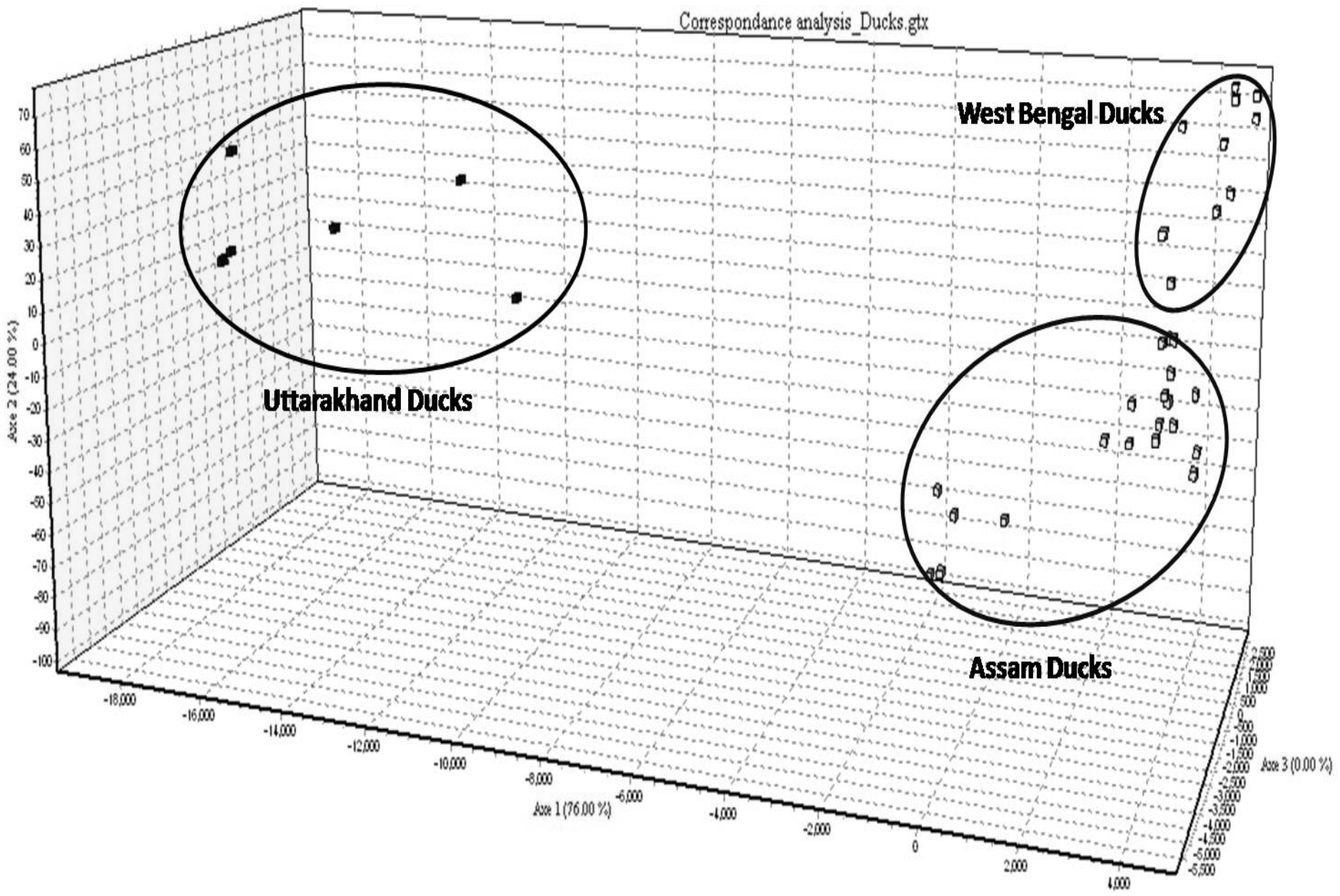

Figure 3. The correspondence analysis of the three ducks populations of India.

these species; Himalayan monal, ducks, pigeon and red jungle fowl. Therefore, this would provide further insight into the evolution of microsatellites and genetic divergence of birds of different orders in the class Aves.

ACKNOWLEDGEMENTS

The study was funded by Wildlife Institute of India, Dehradun, India. We thank the Director, Dean, Dr. S.P. Goyal and Shri Sandeep K. Gupta, WII for their encouragement and support. We are grateful for the help received from the forensic laboratory members and acknowledge two anonymous reviewers for reading the manuscript. Special thanks to Pramod and Shivam for their help during sample collection.

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