Genetic bottleneck studies in five duck (*Anas platyrhynchos*) populations of India

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Received: 20 September 2009; Accepted: 5 July 2010

ABSTRACT

The data of 24 microsatellite loci were generated for 4 indigenous duck populations and 1 commercial breed (Khakhi Campbell). The data were subjected to statistical analysis to test for heterozygosity excess or deficiency since any bottlenecked population would undergo transient heterozygosity excess. Three tests, viz. Sign-rank test, Standardised differences test and Wilcoxon test were utilised in each of the three models of mutations, IAM, SMM and TPM. SMM, which is the most suited model for evolution of microsatellites did not reveal any bottleneck in the last few generations. In our present study the heterozygosity excess is not evident in any of the 5 duck populations and thus the duck populations of India under study are in mutation drift equilibrium. The rejection of null hypothesis in the duck populations is attributed to heterozygotic deficiency.

Key words: Ducks, Genetic bottleneck, Microsatellite, Heterozygosity excess

The total duck population of India is 10 million and it ranks second in the world after Indonesia. In India, the ducks are mainly concentrated along eastern and southern coastline. Out of total egg produced in India 5.3% comes from ducks. Indigenous ducks contributes 91% of the total eggs produced by ducks while 9% are produced by improved varieties/strains. Indigenous ducks produce about 130–140 eggs per annum, and they are a good dietary source of proteins.

The characterization and conservation of ducks assume prime importance as they provide food security to the rural folk. These locally adapted ducks are important biodiversity resources as they are important genetic reservoirs and are essential for facing the future challenges of disease resistance and better quality of meat. The microsatellites have been the markers of choice for diversity analysis and relationship among different species of livestock and poultry (Vijh *et al.* 2007, Ahlawat *et al.* 2007, Vijh *et al.* 2008). The genetic data can be used to infer about the demographic history of populations, which helps to take informed decisions in the management of the populations. The population bottleneck and their effect on genetic variation is a matter of concern for the biologists. It is extremely important to detect historical

bottlenecks, their magnitude and pattern of genetic changes associated with founder events/bottlenecks.

In this paper authors have tried to find out whether any/all of the five populations of Ducks, viz. West Bengal, Orissa, Khakhi Campbell, Jharkhand and Tamil Nadu have undergone a recent reduction in the effective population size, a recent colonisation event or a genetic bottleneck. We have used three tests, ie; sign test, standardised differences test and Wilcoxon test for this purpose using IAM, SMM and TPM models of microsatellite evolution.

MATERIAL AND METHODS

The blood samples (240) from 5 duck populations (48 samples from each population) were collected from unrelated birds. The samples were collected from Nadia, 24 Parganas and Hooghly districts of West Bengal; Bhubaneswar, Cuttack, Puri, Kendrapara districts of Orissa, Ranchi and Jamshedpur districts of Jharkhand; Kendrapara and Thiruvullar districts of Tamil Nadu. The Khakhi Campbell samples were taken from Bisra Agricultural University, Ranchi. The DNA was isolated and amplified using PCR. The conditions for PCR were optimised for all the 24 loci. The amplified products were genotyped using 3100 Avant Automated DNA Analyzer. The data were extracted and subjected to statistical analysis. The allele numbers and heterozygosity were estimated from the data generated using the software Bottleneck.

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The program *BOTTLENECK* computes for each population sample and for each locus the distribution of the heterozygosity expected from the observed number of alleles (k), given the sample size (n) under the assumption of mutation-drift equilibrium. This distribution is obtained through simulating the coalescent process of n genes under 2 possible mutation models, the IAM and the SMM. This enables the computation of the average (H_{exp}) which is compared to the observed heterozygosity (H_{obs} , in the sense of Nei's *gene diversity*) to establish whether there is a heterozygosity excess or deficit at this locus. In addition, the standard deviation (SD) of the mutation-drift equilibrium distribution of the heterozygosity is used to compute the standardized difference for each locus ($(H_{obs}-H_{exp})/SD$). The distribution obtained through simulation enables also the computation of a P-value for the observed heterozygosity.

Once all loci available in a population sample have been processed, the three statistical tests are performed for each mutation model as explained in Cornuet and Luikart (1996) and Luikart *et al.* (1997a, b) and the allele frequency distribution is established to see whether it is approximately L-shaped (as expected under mutation-drift equilibrium) or not (recent bottlenecks provoke a mode shift).

The population bottlenecks induce a transient excess of heterozygosities. Any population, which has experienced a recent bottleneck, will show higher than the expected (equilibrium) heterozygosity for a large majority of loci. A simple test for this shall be (observed - expected) heterozygosity across all loci in a population sample. The Sign-test, Standardised difference test and Wilcoxon Rank test were carried out using software Bottleneck (Piry *et al.* 1999).

Sign-test

The probability distribution was established using 1000 simulations under all the possible models [Infinite Allele Model (IAM), Stepwise Mutation Model (SMM) and Two Phase Model (TPM)].

The probability ($Pr_L(\cdot)$) of having ℓ loci among L , for which there is a heterozygosity excess, utilising usual rationale in which the drawing of additional locus is considered as a Markovian process in which states are the number of loci showing an heterozygosity excess (0,1,2,..... L). Starting with zero locus and a probability distribution $Pr_0(\ell)$ equal to (1,0,0,....0) the recurrence relationship was estimated for $m=1$ to L .

$Pr_m[\cdot] = [(1-p_{lm}) pr_{m-1}(\cdot)] + [p_{lm} pr_{m-1}(\cdot - 1)]$
where, p_{lm} is the probability of heterozygosity excess at locus m .

$$\text{Let } Q^L = \sum_{\ell=0}^L pr_L(\ell)$$

where, Q is the probability of getting at least ℓ_0 loci with heterozygosity excess. In case Q is less than 0.05, the null hypothesis (mutation drift equilibrium) is rejected in favour

of overall heterozygosity excess and a recent genetic bottleneck.

Standardised differences test

The difference between the observed (H_o) and the expected heterozygosity (H_e) is divided by a s (standard deviation) of the corresponding distributions of heterozygosities, we can get standardised deviates. The test consists in comparing the statistics T_2 to an $N(0, 1)$ distribution.

$$T_2 = \frac{(-)0.5}{L} \sum_{\ell=1}^L [\Delta \ell \sqrt{\sigma \ell}]$$

With $\Delta = (H_o - H_e)$ for the ℓ th locus and σ is the standard deviation of the distribution of H at the ℓ th locus. If the alternative hypothesis is heterozygosity excess (One tailed test, we will reject the null hypothesis of population at mutation drift equilibrium) at 5% level if T_2 values are larger than or equal to 1.645 (value from table of normal distribution)

Wilcoxon rank test

The Wilcoxon rank test (Luikart 1997) is more powerful than the previous 2 tests, i.e. Sign Rank test and standardised differences test.

If $n > 8$, Z_+ the distribution is approximately normal and the mean and variance shall be calculated as:

$$\mu = \frac{n(n+1)}{4}$$

$$\sigma^2 = \frac{n(n+1)(2n+1)}{24}$$

$$\text{Sum of } Z \text{ values} = Z_{(+)} + Z_{(-)} = \frac{n(n+1)}{2}$$

$$\text{Test statistic } W \text{ is calculated as } W = \frac{Z_+ - \mu}{\sigma}$$

The significant can be tested as supposing w is test statistics

Prob ($W \leq w$) ≤ 0.05 : Reject the null hypothesis; for one tailed test.

Prob ($W \leq |w|$) $= 2 * \text{Prob} (W \leq w) \leq 0.05$; for two tailed test.

For each locus, the average heterozygosity and standard deviation was estimated through the above simulation processes with a level of precision controlled by the number of simulation replications.

The heterozygosity were calculated using the formula of Nei (1987) for each locus

$$\text{heterozygosity} = \frac{n}{n-1} [1 - \sum (p_i)^2]$$

where n , number of haploid genomes; p_i , allele frequencies.

RESULTS AND DISCUSSION

For microsatellite loci, allele number and frequency distribution in the natural populations result from an equilibrium between mutations and genetic drift. The mutation drift equilibrium depends on the mutation rate and the effective population size. Whenever there is bottleneck in a population the effective population size is significantly reduced. There is also a correlative and progressive reduction of the allele number and heterozygosity. As noted by Nei *et al.* (1975) and Denniston (1978), allelic diversity is reduced faster than the heterozygosity during a bottleneck. This results in consequential transient deficiency in the number of alleles found in a sample of individuals (Maruyama and Fuerst 1985). This implies that the observed number of alleles in the sample is less than the number of alleles expected from the observed heterozygosity under the assumption that the population is in mutation drift equilibrium. These computations rely on the assumption that loci evolve according to IAM (Maruyama and Fuerst 1985). Every mutation under IAM produces a new allele that is different from all existing ones (Kimura and Crow 1964).

The heterozygosity excess or deficit can occur after a recent change of the effective population size but also if heterozygote have a selective advantage or a disadvantage.

The comparison between the observed and expected homozygosity has already been in wide use to detect selection (Watterson 1978). The values of n_l (sample size in haploid genomes), H_e the heterozygosity observed, k_0 (No. of alleles observed) for the data are given in the Table 1 for the 5 populations.

In West Bengal ducks the number of alleles at various loci varied from 3 to 26 (Table 1). The expected number of loci with heterozygotic excess was 13.99 and 14.22 (for IAM and TPM) in the finite sample size and assuming mutation drift equilibrium (Table 2). The number of loci actually observed with heterozygosity excess were 17 and 8 for both IAM and TPM respectively. There is no significant difference between the observed and expected values for heterozygosity excess. The SMM gave the expected and observed values as 14.33 and 3 respectively. The difference is significant but revealed heterozygosity deficiency. The West Bengal ducks population has thus not undergone recent bottleneck rather there is a heterozygote deficiency. In Orissa ducks the expected number of loci with heterozygosity excess were 13.60, 13.93 and 14.14 for IAM, TPM and SMM respectively, whereas number of loci actually observed with heterozygosity excess were 17, 11 and 7 respectively. These values are non-significant in both IAM and TPM, whereas it is significant in SMM favouring heterozygote deficiency. In Khakhi Campbell ducks the expected number of loci with

Table 1. The values of n (sample size in haploid genomes), H_e (heterozygosity observed), k_0 (No. of alleles observed)

Locus	West Bengal			Orissa			Khakhi Campbell			Jharkhand			Tamil Nadu		
	n	k_0	H_e	n	k_0	H_e	n	k_0	H_e	n	k_0	H_e	n	k_0	H_e
APH01	96	4	0.371	96	3	0.157	96	3	0.491	96	4	0.335	96	2	0.081
Caud01	96	13	0.811	96	9	0.709	96	5	0.554	96	9	0.717	96	5	0.665
Caud10	96	5	0.584	96	3	0.381	96	2	0.100	96	3	0.207	96	3	0.383
Caud17	96	12	0.868	96	10	0.744	96	4	0.397	96	9	0.852	96	5	0.595
Caud13	96	6	0.692	96	5	0.671	96	5	0.527	96	3	0.369	96	8	0.763
Caud23	96	6	0.623	96	6	0.724	96	5	0.648	96	7	0.701	96	6	0.668
Caud25	96	8	0.774	96	4	0.423	96	3	0.524	96	6	0.623	96	2	0.449
Caud33	96	4	0.525	96	4	0.520	96	4	0.359	96	4	0.527	96	2	0.504
APH10	96	5	0.469	96	4	0.553	96	3	0.446	96	3	0.456	96	3	0.194
Caud16	96	7	0.614	96	5	0.500	96	3	0.534	96	4	0.627	96	4	0.566
Caud19	96	10	0.848	96	20	0.938	96	8	0.603	96	17	0.899	96	17	0.877
Caud31	96	8	0.682	96	8	0.716	96	7	0.522	96	5	0.420	96	8	0.794
APH09	96	6	0.773	96	6	0.795	96	3	0.590	96	7	0.740	96	8	0.691
Caud26	96	6	0.628	96	4	0.593	96	4	0.568	96	5	0.620	96	6	0.674
Caud24	96	26	0.940	96	21	0.941	96	14	0.772	96	22	0.928	96	22	0.934
Caud35	96	7	0.501	96	9	0.548	96	6	0.687	96	8	0.686	96	5	0.551
APH03	96	3	0.381	96	3	0.554	96	4	0.642	96	6	0.604	96	2	0.379
APH07	96	5	0.541	96	6	0.568	96	10	0.774	96	7	0.626	96	4	0.523
Caud22	96	5	0.390	96	3	0.194	96	4	0.499	96	3	0.408	96	2	0.021
Caud27	96	6	0.587	96	4	0.579	96	5	0.626	96	6	0.616	96	4	0.618
Caud04	96	7	0.703	96	9	0.741	96	6	0.764	96	7	0.672	96	8	0.759
Caud11	96	4	0.608	96	5	0.498	96	4	0.624	96	4	0.590	96	3	0.294
Caud32	96	7	0.521	96	5	0.497	96	4	0.466	96	6	0.469	96	3	0.523
MCW328	96	5	0.230	96	2	0.418	96	2	0.041	96	3	0.041	96	4	0.569

heterozygosity excess under mutation-drift equilibrium were 13.09 (IAM), 13.68 (TPM) and 14.04 (SMM). The corresponding observed values were 17, 12 and 7 respectively. The values are nonsignificant in IAM and TPM, but it is significant with heterozygote deficiency in SMM. In Jharkhand ducks the expected numbers of loci with heterozygosity excess were 13.70, 14.02 and 14.29, respectively, whereas the observed numbers of loci with heterozygosity excess were 18, 10 and 3, for IAM, TPM and SMM respectively. The difference between expected and observed for heterozygosity excess was non-significant in IAM and TPM, whereas is significant for heterozygote deficiency in SMM. The expected number of loci with heterozygosity excess in Tamil Nadu Ducks were 12.84 (IAM), 13.31 (TPM) and 13.68 (SMM) while the corresponding observed values were 20, 16 and 5 respectively (Table 2). The sign test gave significant value under Infinite Allele Model, while it was non-significant in 2 Phase Model. However, the sign test was significant in step-wise Mutation Model with Heterozygote deficiency.

The statistic T_2 (Standardized difference test) is equal to 1.046, -2.248 and -8.715 respectively for IAM, TPM and SMM models in West Bengal. All the values are less than 1.645 (value from table of normal distribution) and thus accept the null hypothesis of mutation-drift equilibrium. In Orissa duck population the standardised difference test provides the T_2 statistics equal to 1.956, -0.803 and -5.936 for the 3 models of IAM, TPM and SMM respectively. The

values are significant for IAM but not significant for the other 2 models for heterozygotic excess. In Khakhi Campbell the test provide the T_2 values of 1.853 (IAM), -0.801 (TPM) and -5.356 (SMM). The values are smaller than the table values of normal distribution for TPM and SMM, and are higher in IAM. The standardised difference test provides the T_2 statistics equal to 1.291, -1.540 and -6.863 for the three models of IAM, TPM and SMM respectively in Jharkhand duck populations. The deviations are not significant for the population in all the three models. In Tamil Nadu duck population the test provides the T_2 statistics of -2.747 and 0.694 respectively for SMM and TPM. Both the values are smaller than 1.645 and accept the null hypothesis of mutation drift equilibrium. However, the null hypothesis is rejected under IAM where T_2 value is 2.751.

Wilcoxon Rank test which is a non-parametric test gave probability values of 0.11396, 0.06043 and 0.00001 respectively for IAM, TPM and SMM in West Bengal Ducks. The values are more than 0.05 for IAM and TPM and thus accept the null hypothesis of mutation-drift equilibrium, where this hypothesis is rejected for SMM (0.00001), which is most stringent and the deviation is in the favour of heterozygosity deficiency. The test gave the probability values of 0.02693 (IAM), 0.66344 (TPM) and 0.00533 (SMM) in Orissa Ducks. In Khakhi Campbell ducks the Wilcoxon test gave the probability values of 0.01787, 0.83337 and 0.00481 for the three mutation models. Since the values are less than 0.05 for IAM and SMM, the null hypothesis is

Table 2. Test for Null Hypothesis under 3 microsatellite evolution models (* rejection of Null Hypothesis/Bottleneck)

States/Khakhi Campbell	IAM		TPM		SMM	
	Expected	Observed	Expected	Observed	Expected	Observed
<i>Sign Test (No. of loci with heterozygosity excess)</i>						
West Bengal	13.99	17	14.22	8	14.33	3
Orissa	13.60	17	13.93	11	14.14	7
Khakhi Campbell	13.09	17	13.68	12	14.04	7
Jharkhand	13.70	18	14.02	10	14.29	3
Tamil Nadu	12.84	20*	13.31	16*	13.68	5
<i>Standardized differences test (T_2 values)</i>						
West Bengal	1.046	-2.248 ⁺	-8.715 ⁺			
Orissa	1.956*	-0.803	-5.936 ⁺			
Khakhi Campbell	1.853*	-0.801	-5.356 ⁺			
Jharkhand	1.291	-1.540	-6.863 ⁺			
Tamil Nadu	2.751*	0.694	-2.747 ⁺			
<i>Wilcoxon Rank Test (Probability of Heterozygosity Excess)</i>						
West Bengal	0.11396	0.06043	0.00001 ⁺			
Orissa	0.02693*	0.66344	0.00533 ⁺			
Khakhi Campbell	0.01787*	0.83337	0.00481 ⁺			
Jharkhand	0.05264	0.13553	0.00001 ⁺			
Tamil Nadu	0.00109*	0.34488	0.01378 ⁺			

•Bottleneck (rejection of null hypothesis of mutation drift equilibrium).

•Negative values in standardised differences test signifies heterozygotic deficiency.

•*Deviation significant but with heterozygotic deficiency.

Fig. 1 (a-e). The mode-shift test using graphic representation in 5 duck populations.

rejected in these 2 models, but accepted for TPM (0.83337). Thus the population of Khakhi Campbell has not undergone recent bottleneck and the population is in mutation drift equilibrium. The Wilcoxon Rank test gave the probability values of 0.05264 (IAM), 0.13553 (TPM) and 0.00001 (SMM) in Jharkhand Ducks. The null hypothesis is accepted for both IAM and TPM but rejected by SMM in favour of heterozygosity deficiency. In Tamil Nadu ducks the Wilcoxon Rank test gave the probability values of 0.00109, 0.34488 and 0.01378 and thus the null hypothesis is rejected under IAM and SMM while it is accepted under Two Phase Model (value >0.05).

The 3 tests utilised in the study are based on the property of the bottlenecked populations which develop a transient heterozygosity excess. If the loci evolve under strict one step mutation model the heterozygosity excess and deficiency can occur depending on the variability of the locus and the time that has passed since the bottleneck began. The IAM and SMM represent the two extreme models of mutation (Chakraborty and Jin 1992) while TPM is an intermediate one. Most of the microsatellite loci evolve in an intermediate manner and that intermediate models of mutations reveal very little or no heterozygosity deficiency in bottlenecked populations (Cornuet and Luikart 1996). The population expansions cause exclusively reveal heterozygotic deficiency. Thus detecting a significant heterozygosity excess under SMM can be taken as evidence that a population has been recently bottlenecked.

The strict SMM which is the most conservative model for testing for a significant heterozygosity excess caused by bottlenecks, as in some conditions it can produce a heterozygosity deficiency and because the heterozygosity excess is always lower than other mutation models (Cornuet and Luikart 1996) and type I error rate for the three statistical tests applied is slightly lower in SMM compared to IAM. In our present study the heterozygosity excess is not evident in any of the five duck populations and thus the duck populations of India under study are in mutation drift equilibrium. The rejection of null hypothesis in the duck

populations is attributed to heterozygotic deficiency.

These tests for heterozygosity excess can detect the bottleneck for only a short duration of time after a bottleneck has been initiated. A bottleneck of $N_e = 50$ is likely to be detectable for 25–250 generations (0.25–2.5 times $2N_e$) after the initiation of a population reduction. Thus only recent historical population declines are detectable.

In the field of conservation the most important type of bottleneck to detect is a severe and rapid decline from large N_e . Severe population declines also are a sort of bottleneck and can be detected by the three tests as done in the study. The duck populations that have been severely bottlenecked are important to identify because of several reasons like inbreeding depression, loss of genetic variation, fixation of deleterious alleles as well as increased demographic stochasticity all of which can reduce adaptive potential and the probability of population persistence (Lande 1994, Mills and Smouse 1994, Frankham 1995a).

The fourth test utilised to test the bottleneck is Mode-shift. This is a qualitative method for estimation of bottleneck. The non-bottleneck populations that are near mutation-drift equilibrium are expected to have a large proportion of alleles at low frequency. The graphical method (Luikart *et al.* 1998) utilises allelic classes and proportion of alleles. The graphs thus plotted for the 5 duck populations are shown in Fig. 1(a-e). None of the five populations exhibit a mode shift rather a normal L shaped distribution is observed, suggesting no bottleneck in indigenous ducks and outgroup. The shift mode test can detect the recent bottleneck up to 40–80 generations while the quantitative test of Cornuet and Luikart (1996) can detect bottleneck up to 250 generations and are thus more sensitive. The duck populations of India thus have a large effective population size and have shown no evidence of severe reduction in effective population size in immediate past.

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