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Molecular characterization of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) stocks in India

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Introduction

In India, rainbow trout was introduced by British more than 100 years ago (Agarwal 2006) for recreational purposes. The fish is being cultured in both government and private farms of different coldwater states of India, mainly in Jammu and Kashmir, Himachal Pradesh, Sikkim, Arunachal Pradesh and Uttarakhand, and also in Nilgiri hills of south India for breeding and rearing purposes. In recent past, growth and production rate of this fish in various farms has been reduced. Microsatellite markers in combination with recent statistical approaches represent a useful tool for genetic characterization which ultimately supports the management of cultured stocks. These markers have been successfully used to evaluate the wild and farm stocks of rainbow trout in western Australia (Ward *et al.* 2003); resident and anadromus forms in the Walla Walla river (Narum *et al.* 2004); domesticated strains of rainbow trout in USA (Silverstein *et al.* 2004); strains in northern and eastern Europe (Gross *et al.* 2007) and three groups of different origin in north of Iran (Yousefian *et al*. 2012). Hence, the present study was carried out to assess the genetic variability in different stocks of rainbow trout in India using microsatellite markers.

Materials and methods

A total of 226 caudal fin samples of rainbow trout were collected from five different regions, namely Dachigam (RTDK, 48, 32°43′29″N, 76°17′07″E; Oct, 2013); Bairangana (RTBU, 42, 30°26′36″N, 79°17′01″E; Aug, 2013); Champawat (RTCU, 42, 29°17′49″N, 80°06′04″E; Aug, 2013); Patlikul (RTPH, 46, 31°39′03″N, 77°21′52″E; Sep, 2013) and Munnar (RTMK, 48, 10°05′21″N, 77°19′39″E; Dec, 2013) (population code, number of samples collected, coordinate locations and duration of collections are provided in parenthesis), and preserved in 70% ethanol (Merck Bioscience, Darmstadt, Germany). Genomic DNA was isolated from fin tissue samples using phenol-chloroform-isoamyl alcohol protocol (Sambrook and Russell 2001).

Fifteen microsatellite markers (Rexroad *et al.* 2002) were amplified each in 10 μL reaction containing 50 ng template DNA, 200 μM of each dNTPs, 5 pM of each primer, 1.0 mM $MgCl_2$, 10 mM Tris (pH 9.0), 50 mM KCl, 0.01% gelatin and 0.5 U of *Taq* DNA polymerase using GeneAmp 9700 thermocycler (Applied Biosystem, Austin, USA). PCR conditions were as follows: 94°C for 4 min followed by 34 cycles of 94°C for 30 s; annealing temperature 58–64°C for 35 s (table 1) and 72°C for 60 s, with a final extension at 72°C for 10 min. Amplified products were resolved through 6% denaturing polyacrylamide gel with 7.5 M urea and 1× TBE buffer in a vertical gel electrophoresis apparatus (Hoeffer, Holliston, USA). The gels were visualized and allele patterns were analysed after staining with ethidium bromide in AlphaImager® EP (Alpha Innotech, San Leandro, USA) gel documentation system. A DNA ladder pBR322/Hae III (Thermo Fisher, Waltham, USA) was used in every gel for size detection of the alleles.

Genetic polymorphism within five stocks were measured by the number of alleles (N_a), observed (H_o) and expected heterozygosity (H_e), the effective number of alleles (N_e) and Shannon's diversity index (I) using GDA ver. 1.1 (Lewis and Zaykin 2008). Presence of null alleles were estimated using Micro-Checker ver. 2.2 (Van-Oosterhout *et al.* 2004). Polymorphism information content (PIC) were calculated using CERVUS ver. 3.0 (Kalinowski *et al.* 2007). For each locus, F_{is} (inbreeding coefficient), F_{st} as well as R_{st} were calculated using FSTAT ver. 2.9 (Goudet 1995). Genotypic

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Table 1. Details of microsatellite loci, allelic richness, heterozygosity etc. across five different stocks (primers were adopted from Rexford *et al.* 2002).

Locus	Repeat motif	Accession #	Sequence of primers	Size (bp)	T_a (°C)	N_a	H_e	H_o	PIC	F_{is}	F_{st}	R_{st}
*OMM1001**	$(TGA)_9$	AF346665	F:GGTCGGCTGTCGTAATTGGTT R:GTCTGATGAGTTGCGGACCAC	260–290	58	4.8	0.68	0.04	0.75	0.94	0.14	0.41
OMM1007	$(TCA)_{15}$	AF346669	F:CATAGTTTTCCTGGTTCAC R:CCCTTAACTGACGCTATT	140–200	58	4.2	0.61	0.51	0.82	0.16	0.31	0.84
OMM1008	$(GAT)_{11}$	AF346670	F:GATCCTTTGGGAGATTAACAG R:CACCACAGTTGCTACTGCC	240–288	58	5.8	0.77	0.61	0.78	0.21	0.04	0.12
OMM1009	$(CA)_{42}$	AF346671	F:ACTGGAATCCAATAACAACCC R:CGGAGGTTTGATGAGTCATT	236–292	58	8.0	0.81	0.46	0.84	0.43	0.05	0.10
OMM1012	$(CA)_{14}$	AF346673	F:TGCACTTCCGCTTCT R:ATAGGACAGGGTAATGGG	260–308	60	5.6	0.75	0.64	0.81	0.15	0.11	0.36
OMM1013	$(AG)_{22}$	AF346674	F:GAGGGAGCCAGAGATATTGAG R:GGTCCTCCATCGATTCAAA	200–320	58	9.0	0.82	0.31	0.88	0.63	0.08	0.49
*OMM1016a**	$(GACA)_7$ $(GGCA)_{10}$	AF346676	F:CTCAGAGCCTCTCAGTCTTA R:CGGATGAGGGATATGTC	138–186	60	5.8	0.70	0.34	0.80	0.51	0.17	0.51
OMM1016b	$(AG)_{27}$	AF346676	F:AGCGAGCGAGCGAAAGA R:CATGTCACGGCTGAGATACA	106–132	58	4.2	0.64	0.49	0.67	0.24	0.13	0.37
*OMM1018**	$(AG)_{13}$ $N3(GA)_4$	AF346677	F:CGAGGGTCTGTCTAACGGGTGG R:TCTGCACCTTCACACTAGCGCTCA	210–260	60	5.6	0.71	0.18	0.77	0.74	0.10	0.07
OMM1019	$(AG)_{24}$	AF346678	F:CCAGCAGTAAACCTTAGGTG R:GTCAAAGGAGACGTAGAGCTT	152–230	58	6.2	0.74	0.93	0.84	−0.26	0.15	0.38
OMM1020	$(AG)_{20}$	AF346679	F:CCTGTGAGTGTTAATTCGACCTGT R:GGTCTTACCTCAACATCGGTGA	110–174	58	4.8	0.69	0.74	0.74	−0.07	0.11	0.51
OMM1021	$(AG)_2(AC)$ $4(TC)_{26}$	AF346680	F:GTAACGACCCTTGACCCGGAACTA R:CAGATCCTTCTAACGCGCAGACA	156–214	62	5.8	0.75	0.50	0.80	0.34	0.09	0.47
OMM1025	$(AG)_{18}$	AF346682	F:CGCCATTGTAGTCTCGTC R:AGTCCGCTATGTTGTTATGTC	286–342	58	6.8	0.79	0.63	0.86	0.20	0.11	0.48
OMM1034	$(TCTA)_{23}$	AF346684	F:ACCCCCGCCCAGTCGTCTCTCT R:TTGGGGGTGTCTTGCTAATTGCCT	200–320	64	7.8	0.80	0.46	0.89	0.43	0.12	0.85
OMM1035	$(TAGA)_{31}$	AF346685	F:CTGCTGCTGAGAGATGTGTTT R:CGCCTCATTTCAACTACTGT	160–220	60	7.0	0.80	0.80	0.82	−0.004	0.05	0.02
Average across all populations (data after '±' indicates standard error)						6.09± 0.36	0.74 ± 0.02	0.51±0.06	0.80	0.31 ± 0.08	0.12 ± 0.01	0.52 ± 0.06

T_a, annealing temperature; N_a, mean number of alleles per locus; H_e, average expected heterozygosity per locus; H_o, average observed heterozygosity per locus; PIC, polymorphic information content; F_{st}, R_{st}: allele frequency, allele-size based correlation; *locus indicating nonsignificant HWD at 0.1% level.

Table 2. Summary statistics for genetic variation of 15 microsatellite loci used in five rainbow trout stocks.

Population		Locus																
		OMM1 001	*OMM10 07*	*OMM10 08*	*OMM1 009*	*OMM10 12*	*OMM1 013*	*OMM10 16A*	*OMM10 16B*	*OMM1 018*	*OMM1 019*	*OMM1 020*	*OMM1 021*	*OMM1 025*	*OMM1 034*	*OMM1 035*	Mean	S.E. (±)
RTDK	N_a	6.00	4.00	6.00	9.00	4.00	12.00	7.00	4.00	7.00	12.00	9.00	7.00	6.00	6.00	7.00	7.07	0.65
(Dachigam, 48)	N_e	4.57	2.80	5.36	6.60	3.20	7.28	3.89	2.80	5.06	9.02	7.82	5.17	4.19	4.57	5.38	5.18	0.47
	H_o	0.06	0.35	0.79	0.34	0.13	0.43	0.43	0.50	0.17	1.00	0.48	0.47	0.35	0.71	0.92	0.47	0.07
	H_e	0.78	0.64	0.81	0.85	0.69	0.86	0.74	0.64	0.80	0.89	0.87	0.81	0.76	0.78	0.81	0.78	0.02
	F	0.92	0.46	0.03	0.60	0.82	0.51	0.41	0.22	0.78	−0.12	0.45	0.42	0.54	0.09	−0.13	0.40	0.08
	P HW	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.001	0.000		
RTBU	N_a	4.00	4.00	6.00	8.00	7.00	9.00	4.00	4.00	4.00	5.00	4.00	5.00	6.00	9.00	8.00	5.80	0.50
(Bairangana, 42)	N_e	2.80	1.82	5.17	5.58	5.57	5.38	2.95	2.73	2.72	3.13	2.78	3.84	3.96	5.24	5.98	3.98	0.36
	H_o	0.05	0.33	0.59	0.56	0.97	0.27	0.20	0.39	0.10	0.85	0.95	0.60	0.85	0.23	0.79	0.52	0.08
	H_e	0.64	0.45	0.81	0.82	0.82	0.81	0.66	0.63	0.63	0.68	0.64	0.74	0.75	0.81	0.83	0.72	0.03
	F	0.92	0.26	0.27	0.31	−0.19	0.67	0.70	0.38	0.84	−0.25	−0.48	0.19	−0.14	0.72	0.06	0.28	0.11
	P HW	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
RTCU	N_a	4.00	5.00	5.00	7.00	5.00	9.00	7.00	4.00	5.00	4.00	4.00	6.00	8.00	9.00	8.00	6.00	0.48
(Champawat, 42)	N_e	2.30	4.33	3.92	4.56	3.13	6.51	4.64	3.02	3.05	3.18	3.19	4.11	6.20	4.71	6.08	4.20	0.33
	H_o	0.00	0.83	0.76	0.34	0.41	0.19	0.33	0.61	0.06	0.82	0.79	0.63	0.82	0.59	0.79	0.53	0.08
	H_e	0.57	0.77	0.74	0.78	0.68	0.85	0.78	0.67	0.67	0.69	0.69	0.76	0.84	0.79	0.84	0.74	0.02
	F	1.00	−0.08	−0.02	0.56	0.40	0.77	0.59	0.09	0.91	−0.20	−0.16	0.16	0.03	0.25	0.06	0.29	0.10
	P HW	0.000	0.010	0.483*	0.000	0.000	0.000	0.000	0.326*	0.000	0.000	0.012	0.000	0.000	0.000	0.000		
RTPH	N_a	5.00	4.00	6.00	7.00	6.00	8.00	5.00	5.00	6.00	6.00	3.00	5.00	7.00	10.00	8.00	6.07	0.45
(Patlikul, 46)	N_e	3.63	2.57	4.38	3.74	3.96	5.10	3.64	3.44	3.22	5.34	2.81	3.40	4.95	7.70	6.33	4.28	0.36
	H_o	0.00	0.53	0.60	0.27	0.67	0.26	0.22	0.62	0.19	1.00	0.71	0.63	0.74	0.53	0.98	0.53	0.08
	H_e	0.72	0.61	0.77	0.73	0.75	0.80	0.73	0.71	0.69	0.81	0.64	0.71	0.80	0.87	0.84	0.75	0.02
	F	1.00	0.13	0.22	0.63	0.11	0.68	0.70	0.12	0.72	−0.23	−0.11	0.10	0.07	0.39	−0.16	0.29	0.10
	P HW	0.000	0.182*	0.012	0.000	0.209*	0.000	0.000	0.096*	0.000	0.000	0.009	0.028	0.000	0.000	0.000		
RTMK	N_a	5.00	4.00	6.00	9.00	6.00	7.00	6.00	4.00	6.00	4.00	4.00	6.00	7.00	5.00	4.00	5.53	0.38
(Munnar, 48)	N_e	3.10	2.41	3.83	7.71	4.89	5.01	2.36	2.16	4.55	2.89	2.59	4.02	4.54	3.88	3.00	3.80	0.37
	H_o	0.10	0.52	0.33	0.79	1.00	0.40	0.52	0.31	0.40	1.00	0.75	0.15	0.40	0.23	0.54	0.50	0.07
	H_e	0.68	0.59	0.74	0.87	0.80	0.80	0.58	0.54	0.78	0.65	0.61	0.75	0.78	0.74	0.67	0.70	0.03
	F	0.85	0.11	0.55	0.09	−0.26	0.51	0.10	0.42	0.49	−0.53	−0.22	0.81	0.49	0.69	0.19	0.29	0.11
	P HW	0.000	0.114*	0.000	0.000	0.000	0.000	0.084*	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.008		

N_a, number of different alleles; N_e, number of effective alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F, fixation index; P HW, probability of HWE; *not significant; parenthesis contain sample collection site and number of samples collected.

linkage disequilibrium, deviation from Hardy–Weinberg equilibrium and population differentiation was computed using GENEPOP ver. 4.0 (Rousset 2008).

Analysis of molecular variance (AMOVA) was computed to enumerate the differences among stocks using ARLEQUIN ver. 3.5 (Excoffier and Lischer 2010). The genetic distance and genetic similarity index following Nei (1978) were also estimated using ARLEQUIN ver. 3.5 and data matrix was used to construct UPGMA dendrogram under MEGA ver. 5.05 (Tamura *et al.* 2011). Genetic differentiation was also depicted by two-dimensional plots from multivariate data analysis, principal component analysis (PCA) of the allele frequency matrix in SPSS ver. 19 (SPSS, Armonk, USA). Additionally, the Bayesian program STRUCTURE ver. 2.3.4 (Pritchard *et al.* 2000) was used to infer the most likely number of genetic clusters (K) (between 1 to 10 with 10 iterations at each K). The most likely value of K was selected following Evanno *et al.* (2005). A series of simulations were run for 10^6 burn-in generations with 5×10^6 MCMC repeats for final sampling of data. Heterozygote excess to analyse any inbreeding depression was calculated using Wilcoxon signed-rank test, two models of mutation-drift equilibrium i.e. infinite alleles model (IAM) and stepwise mutation model (SMM) using 1000 replicates under BOTTLENECK ver. 1.2.02 (Piry *et al.* 1999).

Results

We screened variation across 15 microsatellite loci of rainbow trout to quantify genetic variation within and among five major cultured stocks in India. All 15 markers were successfully amplified in all the stocks and two loci (*OMM1001* and *OMM1018*) had detected significant frequency of null allele through Micro-Cheker but their inclusion in analysis did not affect stock structure prediction. Therefore, those two loci were not removed from analysis.

The mean number of allele frequency observed across all 15 microsatellite loci was 6.09 (table 1) which was more than the effective number of alleles (4.29). The PIC showed that most of the loci were highly polymorphic with an overall mean of 0.80. The average expected gene diversity ranged from 0.61 (*OMM1007*) to 0.82 (*OMM1013*) with an overall mean of 0.74 over all loci (table 1). The average observed heterozygosity and expected heterozygosity across different stocks varied from 0.47 to 0.53 and from 0.70 to 0.78, respectively (table 1). Of the 15 studied loci across five populations, five showed significant deviations from Hardy–Weinberg equilibrium (HWE) (i.e. $P>0.01$) (table 2). There was no significant detection of linkage disequilibrium between any pair of loci when analysed using GENEPOP ver. 4.0. The F_{is} for the investigated loci, within population was 0.31 and ranged from -0.004 (*OMM1035*) to 0.94 (*OMM1001*). Three loci revealed negative F_{is} values (table 1) which may be a result of heterozygote deficiency.

AMOVA revealed that the genetic variance among five stocks were significant at each level in which most variance was observed within individuals (63.02%), but it was only 13.17% between the stocks. F_{st} and R_{st} were used to estimate stock structure of rainbow trout. Both F_{st} and R_{st} revealed that the stocks of rainbow trout were significantly different ($P < 0.05$). The pairwise F_{st} ranged from 0.07 (between RTCU and RTPH) to 0.16 (between RTBU and RTMK). Overall R_{st} value (0.52) was higher than F_{st} (0.12) and most of the loci showed higher level of R_{st} than F_{st} except *OMM1018* and *OMM1035* (table 1).

The dendrogram constructed using Nei's genetic distance matrix (figure 1a; table 3) revealed a clear separation of RTMK (Munnar, Kerala) of South India from farmed stocks of northwestern India. Again in North-Western stocks, the RTDK (Dachigam, Kashmir) stock remained isolated from the rest of other group, namely, RTPH (Patlikul, Himachal Pradesh), RTBU (Bairangana, Uttarakhand) and RTCU (Champawat, Uttarakhand). The ΔK showed a clear peak at the true value ($K = 3$). Evanno's method also indicated three STRUCTURE clusters (figure 1b). Cluster 1 grouped individuals of RTDK, cluster 2 grouped mostly all samples of RTBU, RTCU and RTPH stocks and RTMK was assigned to cluster 3. Overall, these results agreed with the population relationships depicted by first two factors of PCA (figure 2). The first and second factor explained 62.4 and 20.1% of allelic variance, respectively, and clearly differentiated five stocks. Concisely, stocks of RTDK and RTMK were discriminated from each other and rest of the populations, whereas intermixing was observed among RTBU, RTCU and RTPH stocks (figure 1b).

The heterozygosity excess method was followed to analyse historical bottlenecks. Test for heterozygosity excess were significant ($P < 0.01$) under both mutation models (IAM and SMM). In bottleneck analysis, we found limited evidences for recent reduction in population size using stepwise mutation model. However, the mode shift test did not detect any significant distortion of allele frequency and showed a normal 'L' shaped distribution (figure 3) which is a typical property of a population in equilibrium.

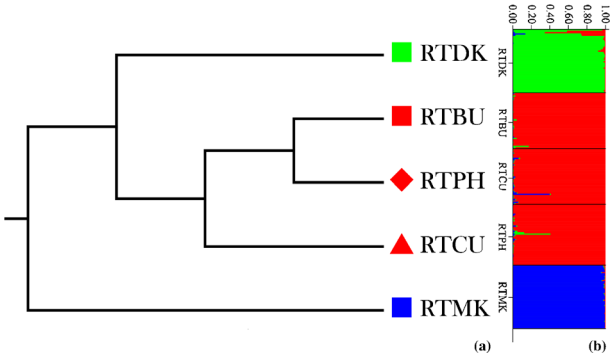

Figure 1. (a) Phenogram (UPGMA) constructed based on Nei's genetic distance among stocks. Genetic distance was computed using microsatellite genotype data in ARLEQUIN ver. 3.5. (b) Structure analysis using the most likely K value (K =3) and individuals are represented in three major clusters.

Table 3. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among stocks.

Farm stocks	RTDK (Dachigam)	RTBU (Bairangana)	RTCU (Champawat)	RTPH (Patlikul)	RTMK (Munnar)
RTDK (Dachigam)	–	0.5304	0.5619	0.5445	0.4290
RTBU (Bairangana)	0.6342	–	0.7042	0.7248	0.4640
RTCU (Champawat)	0.5764	0.3507	–	0.7192	0.5921
RTPH (Patlikul)	0.6079	0.3218	0.3296	–	0.5801
RTMK (Munnar)	0.8462	0.7679	0.5241	0.5446	–

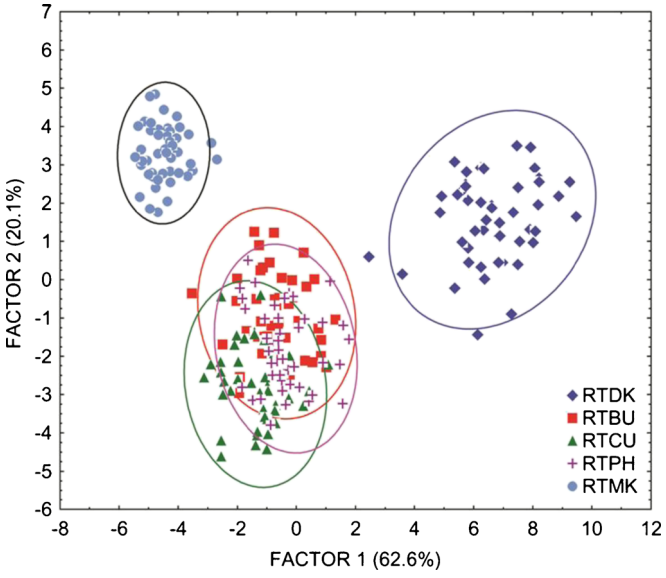

Figure 2. PCA based on variance-covariance parameters and sample sizes in the five stocks of rainbow trout. Samples are projected in first two major axis; consisting of factor 1 with a maximum of 62.6% total variance and factor 2 with 20.1% variance.

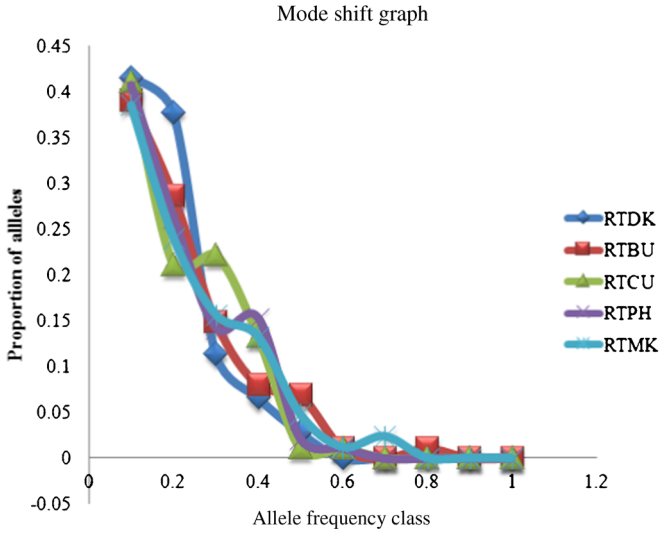

Figure 3. Mode shift graph showing 'L-shaped' distribution and indicating the absence of bottleneck in rainbow trout stocks.

Discussion

In the present study, an attempt was made for the first time to evaluate the genetic variability of different stocks of rainbow trout which was implanted mainly in the high altitude rivers a few decades ago. Fifteen microsatellite loci were selected for characterization of five different geographically isolated farm stocks. PCR amplification of 15 loci was successfully achieved in 226 individuals. These microsatellite markers were originally developed by Rexroad *et al.* (2002). All the markers had high PIC (0.80) and mean allele number (6.09), indicating a high allelic variability. The observed allele sizes of the present study were comparable with the allele size observed by Rexroad *et al.* (2002) at respective loci. Thus, the selection of markers had shown their ability to characterize the rainbow trout stocks in India. Gross *et al.* (2007) and Ward *et al.* (2003) also studied the genetic diversity of rainbow trout in north and eastern Europe, and western Australia, respectively using some microsatellite markers developed by Rexroad *et al.* (2002). They had also observed high allelic richness in different populations.

The RTMK (Munnar, Kerala) stock from extreme south of India is quite distinct from other four stocks examined from western and central Himalayas (figures 1&2). This stock is being maintained isolated since its introduction and the stock also has low level of genetic variation which may be due to large geographic barrier and less gene flow. The RTDK (Dachigam, J&K) stock of northwestern India forms a separate cluster from others (Bairangana, Champawat and Patlikul). As per the information collected from different State Fisheries Department, the RTDK (Dachigam, J&K) and RTPH (Patlikul, Himachal Pradesh) farm are the major hatcheries for seed production and propagation to other areas of north India (Uttarakhand) and northeast India (Sikkim and Arunachal Pradesh). Thus, there are mainly three different founder stock (Kerala, Kashmir and Patlikul) among these five stocks which are forming three different clusters and may have different origin.

In the present study F_{st}, R_{st} and AMOVA analysis revealed significant differences among five farmed stocks with overall F_{st} value of 0.12 and about 12% of the genetic variation was gained due to interpopulation differences. However, the average level of genetic differentiation among five stocks in the present study is comparable to the reported value of $F_{st} = 0.089$ among three domesticated strains of rainbow trout in USA (Silverstein *et al.* 2004) but lesser than the value reported among strains ($F_{st} = 0.14$) of northern and eastern Europe (Gross *et al.* 2007) and between four populations of western Australia ($F_{st} = 0.192$) (Ward *et al.* 2003). In

the present study, higher value of R_{st} than F_{st} was observed, which predicts a role of mutation rather than genetic drift in the differentiation of present stocks of rainbow trout.

On the basis of present study, the observed heterozygosity in terms of average of all loci across the stocks was less when compared with the expected heterozygosity. This may be attributed to inbreeding effect (overall F_{is} was estimated to be 0.31); highest inbreeding coefficient was found in RTDK population (0.40). The average observed heterozygosity in the present study was 0.51 as against expected heterozygosity (H_e) of 0.74 (table 1). Silverstein *et al.* (2004) reported genetic variation among three domesticated strains of rainbow trout in USA with an average heterozygosity of 0.72. Gross *et al.* (2007) observed average $H_o = 0.67$ and $H_e = 0.76$ in domesticated strains of rainbow trout reared in Finland, Denmark, Sweden, Norway, Estonia and Poland. Though the genetic variability in the present study was less in comparison with Silverstein *et al.* (2004) and Gross *et al.* (2007), the stocks were not in genetic bottleneck as observed in Wilcoxon test under BOTTLENECK ver. 1.2.02, where all the stocks showed an L-shaped curve (figure 3). Garza and Williamson (2001) reported that number of alleles may decrease up to 2–3 alleles per locus in case of chronic bottleneck that last multiple generations. In the present study, the mean number of alleles was 6.09 with a range from 5.53 to 7.07 across the rainbow trout stock. This finding indicates high allelic variability that still persists across the different stocks, hence the chances are less likely that stocks are under bottleneck.

Though the present stocks have considerable amount of genetic variability, there is need for pedigree breeding strategies so that future chances of inbreeding can be minimized using many pairings and equalization of family size to maximize effective population size and thereby the effects of drift. There is also a possibility for better genetic improvement of the existing stocks by introducing newly selected strains and enhancing aquaculture productivity in the coldwater regions.

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