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Identification of Microsatellite DNA for Population Genetic Analysis in *Tor tor*

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ABSTRACT

Microsatellites markers are the most prevalent and adaptable genetic marker with varieties of applications in conservation biology, population genetics and evolutionary biology. These are the arrangements of DNA sequences, consisting of tandemly repeating mono-, di, tri and tetranucleotide units, which are distributed throughout the genomes of most eukaryotic species. Microsatellites are codominant in nature, easily typed, highly polymorphic and Mendelian inherited. These all properties make them very suitable for the study of population structure and pedigree analysis and also capable for detecting differences among closely related species. Polymerase chain reaction (PCR) for microsatellites can be automated for identifying simple sequence repeat polymorphism. Small amount of blood samples or alcohol preserved tissue is adequate for analyzing them. Most of the microsatellites are noncoding, and therefore variations are independent of natural selection, all properties make microsatellites ideal genetic markers for fisheries management and conservation genetics. In this review We aimed at isolating microsattelites suitable to quantify the genetic variability in *Tor tor.*

Keyword: *Tor tor,* microsattelite marker, PCR

INTRODUCTION

Mahseer inhabits both rivers, lakes ascending to rapid streams with rocky bottoms for breeding. They are omnivorous, eating not only algae, insects, frogs, other fishes, but also fruits that fall from trees overhead. The first Species of this group were scientifically described by Hamilton- Buchanan in 1822.

Classification of Mahseer

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Teleostomi
Super order	:	Ostariophysi
Order	:	Cypriniformes
Sub order	:	Cyprinoidei
Family	:	Cyprinidae
Sub family	:	Cypriniae
Genus	:	*Tor, Neolissochilus, Naziritor*

Introduction about *Tor tor*

Common name*: Red-finned Mahseer or Barbeau tor.*

Maximum length-52cm, common length-17.5cm, length at first maturity-36cm

Maximum published weight-9000gm

Maximum reported age- 10 years

Color- Silvery green

Habitat-Inhabit river and lakes of Asia, Pakistan, India, Bangladesh, Nepal and Bhutan.

Tor tor Grow better in a river with a rocky bottom. Travels towards head water at the start of the rainy season and downstream at the end of the rainy season. Omnivorous, feed on filamentous algae.

Taxonomic classification

Phylum	:	Vertebrata
Subphylum	:	Craniata

Class	:	Teleostomi
Subclass		Actinopterygii
Order	:	Cypriniformes
Suborder	:	Cyprinoidei
Family	:	Cyprinidae
Genus	:	*Tor*
Species	:	*Tor tor*

Fig.1.1 *Tor tor*

It is valued as commercial and aquaculture level. Molecular genetic markers are heritable characteristics associated with the identification and characterization of a specific genotype, crucial for both management and conservation programs. These markers have several applications in aquaculture genetics and breeding programs, including the characterization of genetic diversity both within and between populations. They have been used for various objectives such as to determine the species-diagnostic markers, population genetic structures, phylogenetic relationships and quantitative trait linkage studies, etc. (Smith and McVeagh,2004; Faria and Miyaki, 2006).

Biological diversity is 'the variety of life' which refers to variation at all levels of biological organization. The genetic variation in and between populations is the outcome of several factors, such as mutation rate, breeding size of the population, breeding strategy, migration and, above all, selection. The most common measures of genetic diversity are heterozygosity, allelic diversity, and proportion of polymeric loci (Nei *et al.*, 1975; Leberg, 1992). Heterozygosity is important to the well being of natural and artificial populations (Orr and Sohal, 1994). Habitats with greater biodiversity are more resilient and recover from various disturbances.

The methodology is currently undergoing rapid evolution with the development of various molecular markers for characterizing genetic variation at the DNA level within individuals, organisms, population, species, and genera. Traditional techniques, which directly or indirectly investigated gene products like blood groups and enzymes. The studies of morphometric and meristic characteristics, are being completed by the data from new molecular techniques (Beadmore *et al*., 1997)

Molecular genetic markers are heritable characteristics associated with the identification and characterization of a specific genotype, crucial for both management and conservation programs. These markers have several applications in aquaculture genetics and breeding programs, including the characterization of genetic diversity both within and among populations. They have been used for various objectives, to determine the species diagnostic markers, phylogenetic relationships, quantitative trait linkage studies, genetic population structure and construction of genetic linkage map.

The variation in intraspecific level is measured by means of population genetics in which the levels of genetic variation are quantified and its distribution pattern is across the distribution range of species. Traditional techniques, which directly or indirectly investigated gene products like blood groups and enzymes. The studies of morphometric and meristic characteristics are being completed by the data from new molecular techniques.

The present study was aimed at identification of microsatellite markers in *Tor tor*. This study was undertaken to identify polymorphic microsatellite marker that could be used to determine genetic variation within the population of the species of the same family. The outcome of this study will provide microsatellite markers to be utilized in genetic improvement programs and planning, conservation as well as management of natural fishery resources of these economically important fish species.

MATERIALS AND METHODS

Materials

Raw materials: Fish (blood sample and muscles samples). The blood samples were collected from the fish (*Tor tor)* with a needle and syringe and fixed in 95% alcohol in 1:5. The blood samples were transported to the laboratory at room temperature and stored at 4^{o}C till analysis, primers, and markers.

Chemicals

0.5M Tris-Cl, EDTA, Alcohol, Bromophenol blue dye, High TE working solution, Incubation buffer, Proteinase K, RNAase Buffer, Saturated phenol, Chlorofom: Isoamyle alcohol, Sodium acetate, Acryl amide, Bis-acrylamide, APS, Boric EDTA, and Agarose.

Steps involvedinthe analysis

- Isolation of genomic DNA from the Ethanol fixed blood and muscles sample

- Determination of quality and quantity of isolated DNA
- Polymerase chain reaction (PCR)
- Visualization of Microsatellites using PAGE and silver staining
- Study of microsatellite band patterns

Total genomic DNA was extracted from the ethanol fixed blood of these two species of Genus *Rit*a, with proteinase-K and phenol-chloroform method which removes proteins and other cellular components of the nucleic acids and pure genomic DNA was obtained.

Determined the quality and quantity of isolated DNA by the help of agarose gel electrophoresis. Analyzed the molecular weight of the desired sequence with the PCR. A total of 27 microsatellite primers of six resource species was used to screen suitable primers for *Tor tor* species. Visualization of Microsatellites was performed by the using PAGE and silver staining. Gel band pattern images were captured and saved in the computer with the help of the BIOVIS gel software. Molecular weight of each microsatellite band was calculated comparing the distance run by the standard molecular weight bands through the BIOVIS-ID analysis software. Each individual was genotyped; each microsatellite locus as homo or heterozygote. The individual having single band was designate as homozygote and with two bands heterozygote

RESULTS AND DISCUSSION

Identification of Microsatellite markers

In cross-species amplification of microsatellite primers seventeen primer pairs from four resource species were tested for identification of homologous microsatellite loci in Tor tor (table 1). Successful amplification was observed in Tor tor with twelve primer (71%) pairs of related species ie., Five from *Labeo rohita*(71%), one from *Barbus barbus** (25%),five from*Cyprinus carpio** (100%) and one from*Compostma enomalum** (100%).

Table1: Primers of Microsatellite loci tested for cross species amplification in *Tor tor (Family Cyprinidae)*

S.No.	Family	Resourse species	No. of primer pair tested	Primer pairs	Gene bank accession no.	Successful amplified loci in species
1	Cyprinidae	*Labeo rohita**	7	*R12**	AJ507524	5
				*R6**	507522	(71%)
				Lr36*	AM269526	
				Lr32*	231187	
				Lr45*	269535	
				Lr29*	231178	
				Lro44*	AM184162	
2	Cyprinidae	*Barbus barbus**	4	Barb54*	U57659	1
				Barb62*	U 51664	(25%)
				Barb59*	51663	
				Barb37*	51654	
3	Cyprinidae	*Cyprinus carpio**	5	*MFW01**	-	5
				*MFW07**	-	(100%)
				*MFW17**	-	
				MFW26	-	
				*MFW02**	-	
4	Cyprinidae	*Compostma enomalum**	1	*Ca12**	AF277584	1 (100%)

The optimum annealing temperature was determined through experimental standardization for each primer pair. The optimum annealing temperatures, to obtain scorable band pattern in *Tor tor* for all the twelve primers, differed from that reported for the respective primer pairs in the resource species.

Table 2: Successful microsatellite primers in *Tor tor*

S.No.	Primer	Primer sequences(5'-3')	T1 (^{0}c)	T2 (^{0}c)	Nature of Locus
01	*R12**	CTATTCCTGTGCAGACCTTC GATACACGTCCAGTTTCACC	-	50	Polymorphic
02	*Lr36**	CTTGTTCACTGCACAGACACC AAGGTTCAGATTGCCTCCTG	-	50	Polymorphic

S.No.	Primer	Primer sequences(5'-3')	T1 (^{0}c)	T2 (^{0}c)	Nature of Locus
03	*Lr32**	AAATCAGAGAGGGAAGGACAGA CATTGATTGGTTGAGCACCTAC	-	50	Polymorphic
04	*Lr45**	GTGAGGCTCTCTGTTTGGTG AACGCAGCCAACCTAACGTA	-	50	Monomorphic
05	*Lr29**	CCCACGCAAACTCCTGTT GGAACAAGGCCAGAGCTTTA	-	50	Monomorphic
06	*Barb62**	CA CAAAAATGGATTCATATATTT GT ACACGAGCATATGGACAA	58	48	Polymorphic
07	*MFW1**	GTCCAGACTGTTCATCAGGAGG AGGTGTACACTGAGTCACGC	55	50	Polymorphic
08	*MFW7**	TACTTTGCTCAGGACGGATGC ATCACCTGCACATGGCCACTC	55	50	Polymorphic
09	MFW17*	CAACTACAGAGAAATTTCATCCA GAAATGGTACATGACCTCAAG	55	50	Polymorphic
10	*MFW26**	CCCTGAGATAGAAACCACTGCA CACCATGCTTGGATGCAAAAG	55	50	Polymorphic
11	*MFW2**	CACACCGGGCTACTGCAGAG GTGCAGTGCAGGCAGTTTGC	55	50	Monomorphic
12	*Ca12**	GTGAAGCATGGCATAGCACACAGGAAAGTGCCAGGCATACAC	55	50	Monomorphic

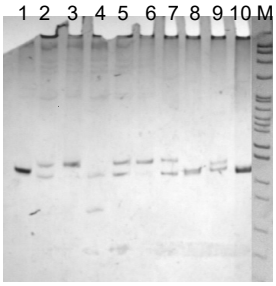
T1 (ºC) - Annealing temperature in resource species T2 (ºC) - Annealing temperature in *Tor tor*

In *Tor tor* out of seven primers of *Labio rohita** only five (R12*, *Lr36**, *Lr32*, Lr45*, Lr25**) yield amplified product, out of which *Lr36** and *Lr32* and R12** are polymorphic *Lr45**and *Lr29** are monomorphic.

Among five primers of *Barbus barbus* one primer (barb62) amplified that is polymorphic. In case of Mfw all primers (*MFW1*, MFW7*, MFW17*, MFW26*, MFW2**) amplified. In *Compostoma enomalum,* primer (*Ca12**) monomorphicly amplified and rest are not amplified.

Table3: Number of alleles and allele size range at microsatellite loci of *Tor tor*

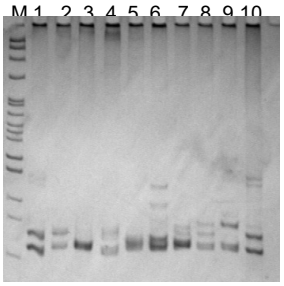
S.No.	Loci name*	No. of alleles In *Tor tor*	Product size range	Figure
Polymorphic loci				
1.	*Lr36**	10	128-143	a
2.	*Lr32**	10	92-106	b
3.	*R12**	6	134-175	c
4.	*Barb62**	5	98-120	d
5.	*MFW1**	7	134-172	e
6.	*MFW17**	9	109-174	f
7.	*MFW26**	5	110-177	g
	*MFW7**	7	174-210	h
Monomorphic loci				
9.	*Ca12**	1	156	I
10.	*Lr45**	1	119	j
11.	*Lr29**	1	132	k
12.	*MFW2**	1	233	l



Lr36 (a)

1. 131,131
2. 138,128
3. 138,138
4. 139,139
5. 140,129
6. 141,141
7. 143,129
8. 132,132
9. 133,133
10. 133,133

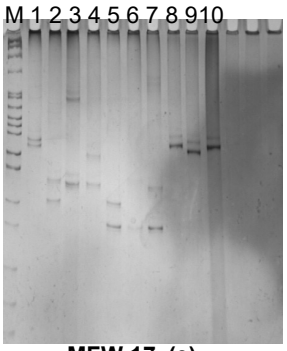
M



Lr32 (b)

M

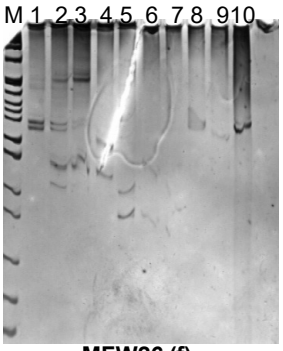
1. 101,93
2. 103,96
3. 95,95
4. 92,92
5. 95,95
6. 95,98
7. 96,96
8. 95,95
9. 95,95
10. 106,94



MFW-17 (e)

M

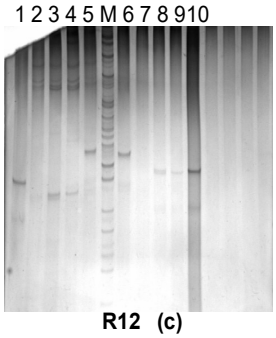
1. 174,174
2. 123,132
3. 135,135
4. 134,134
5. 122,109
6. Blank
7. 109,109
8. 167,167
9. 161,161
10. 167,167



MFW26 (f)

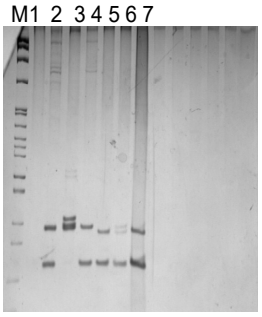
M

1. 177,172
2. 125,125
3. Blank
4. 132,132
5. 110,110



R12 (c)

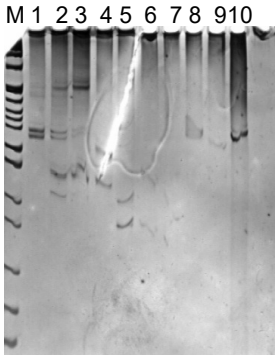
1. 144,144
2. blank
3. 134,134
4. 136,136
5. 175,175
M
6. 172,172
7. blank
8. blank
9. blank
10. 154,154



Barb-62 (d)

M

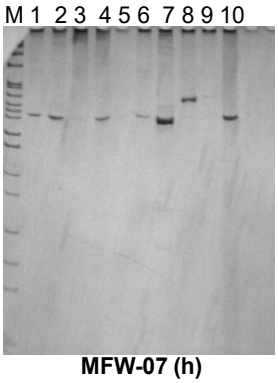
1. 120,98
2. 121,121
3. 120,99
4. 117,99
5. 120,120
6. 118,99



MFW26 (g)

M

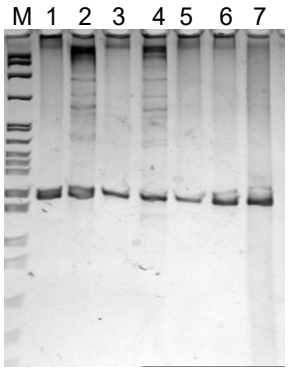
1. 177,172
2. 125,125
3. Blank
4. 132,132
5. 110,110



MFW-07 (h)

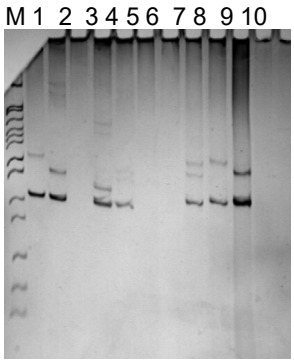
M

1. 184,184
2. 174,174
3. Blank
4. 178,178
5. Blank
6. 182,182
7. 174,174
8. 210,210
9. Blank
10. 179,179



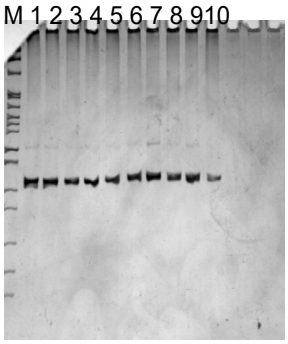
Ca12 (i)

M
1. 156
2. 156
3. 156
4. 156
5. 156
6. 156
7. 156



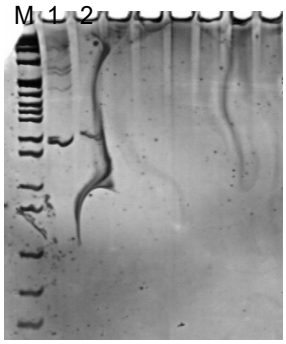
Lr-45 (j)

M
1. 119
2. 119
3. Blank
4. 119
5. 119
6. Blank
7. Blank
8. 119
9. 119
10. 119



Lr-29 (k)

M
1. 133
2. 133
3. 133
4. 133
5. 133
6. 133
7. 133
8. 133
9. 133
10. 133



MFW02 (l)

M
1. 233
2. 233

Fig:2a-l Microsatellite loci and different genotypes observed in *Tor tor.* M- Standard molecular weight marker pBR322 DNA/ MspI digest.

Number of alleles at microsatellite loci

A total of twelve microsatellite loci was amplified out of seventeen in *Tor tor*, in which polymorphic locus was eight and monomorphic was four, the rest not amplified. And the numberof alleles in polymorphic condition ranges from 5 -10 and for a monomorphic number of allelesare 1 different primers.

DISCUSSION

Microsatellite loci are abundant and distributed throughout the eukaryotic genome and each locus is characterized by known DNA sequences. These sequences consist of both unique DNA and a repetitive DNA motif. Many microsatellite loci despite their high rates of repeat evolution are quite conserved in their flanking regions and hence can persist unchanged over a long evolutionary time. Out of the seventeen primers,onlyeight amplified a polymorphic locus in *Tor tor.* The number of alleles in the species was different for different primers.

In *Tor tor* at locus, allele size generated by cross species amplification turned out to be larger than those from the resource species. Similar observation was also made by Yue etal at several loci, when the primers were cross primed with congeneric species and species from different family.

These data indicated that the cross species amplification might be locus specific,. Comparing the data of Tor species, it was surprising to note that the same primers were successful in amplification of microsatellite loci in the target species. Zardoya also reported that homologous microsatellite locus could persist for about 300 million years in turtle and in fishes and their flanking regions are highly conserved.

The optimum annealing temperature to get scramble bands in *Tor tor* slightly differed from that reported for their respective primer pairs in resource species. This may be due to slight variation occurring as a result of mutation at the primer sequences. Zardoya and Gulbusera also reported necessities of PCR conditions for the study species in cross species amplification tests.

The present result identified eight polymorphic microsatellite loci and four monomorphic in *Tor tor*. The polymorphisms at some of these loci indicate their potential for use in population genetic studies of *Tor tor* species.

CONCLUSION

Tor tor is important as esteemed table fishes. The present result identified eight polymorphic microsatellite loci namely, *Lr36*, Lr32*, Barb62*, MFW01*, MFW7*, MFW17*, MFW26*,* R*12*,* and four monomorphic loci namely *Ca12*, Lr45*, Lr29*, MFW2** in *Tor tor.* The observations showed that the primer from *Labio rohita** (which belongs to the same family Cyprinidae),

*Barbus barbus** (Family Cyprinidae), *Cyprinus carpio** (Family Cyprinidae) and Campastma anomalum* (family Cyprinidae) can be used to identify microsatellite loci in *Tor tor*. DNA sequencing of the identified microsatellite loci confirmed the presence of repeat motif.Our results suggested that these identified microsatellite can be used for population genetic analysis and to assess or monitor genetic variation. The availability of conserved microsatellite markers is important for gene mapping, marker assisted selection and evolutionary studies.

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APPENDIX - I

Reagents required for DNA Isolation

Stock Solutions:

1. 0.5 M Tris-Cl (pH 8.0)

Tris base	-	3.028 g
Distilled water	-	40.0 ml

Adjust the pH to 8.0 using 1N HCl

Makeup final volume to 50ml

Store at 4^0C.

2. 0.5 M Tris-Cl (pH 8.3)

Tris base	-	3.028 g
Distilled water	-	40 ml

Adjust the pH to 8.3 using 1N HCl

Makeup final volume to 50ml

Store at 4^0C.

3. 0.5 M EDTA (pH 8.0)

Na_2 EDTA. $2H_2O$	-	9.34 g
Distilled water	-	40 ml

Adjust the pH to 8.0 using 0.5M NaOH.

Make up final volume to 50ml.

Store at roo

4. 0.5 M Tris-Cl (pH 7.5)

Tris base	-	3.028 g
Distilled water	-	40 ml

Adjust the pH to 7.5using 1N HCl

Make up final volume to 50 ml.

Store at 4^0C.

5. 5X TAE

Tris base	-	12.10 g
0.5 M Na_2 EDTA. $2H_2O$ (pH 8.0)		- 5.0 ml
Glacial Acetic acid	-	2.85 ml

Make up the solution to 500 ml with distilled water.

Store at room temperature.

6. Bromophenol Blue dye

Bromophenol blue	-	2.5 mg
Sucrose	-	40.0 mg

Dissolve in 1 ml distilled water.

Autoclave it and store at 4^0C.

Working Solutions

1. High TE

Stock 0.5 M Tris-Cl (pH 8.0)	-	20 ml
Stock 0.5 M Na_2 EDTA. $2H_2O$ (pH 8.0)	-	8 ml

Makeup the solution to 100 ml with distilled water.

Autoclave it, cool it down to room temperature and store at 4^0C.

2. Incubation buffer

0.5 M Tris-Cl (pH 8.0)	-	2 ml
0.5 M EDTA (pH 8.0)	-	2 ml
Distilled water	-	96 ml

Autoclave it, cool it down to room temperature and store at 4^0C.

3. Proteinase K

Proteinase K	-	10 mg
Autoclaved distilled water	-	500 ^{0}l

Dissolve Proteinase K in autoclaved distilled water.

Store at -20^0C.

4. RNAase Buffer

0.5M Tris-Cl (pH 7.5)	-	0.2 ml
NaCl (0.292 g in 10 ml)	-	0.3 ml
Distilled water	-	9.5 ml

Autoclave it, cool it down to room temperature and store at 4°C.

5. RNAase

RNAase	-	10 mg
RNAase buffer (autoclaved)	-	1 ml

Dissolve RNAase in RNAase buffer.

Keep the tube in boiling water for 15 minutes.

Allow to cool at room temperature

Store at -20^0C.

6. Saturation of Phenol with Tris-HCl (pH 8.0)

Reagents required

Water saturated Phenol	-	500ml
0.5 M Tris-HCl (pH 8.0)	-	1000ml
(60.56g of Tris base in1000 ml)		
0.1 M Tris-HCl (pH8.0)	-	1500ml

(For 300 ml of 0.5M Tris-HCl (pH8.0) add 1200 ml of DDW.)

7. Chloroform: Isoamyl alcohol (24:1 V/V)

Chloroform	-	96 ml
Isoamyl alcohol	-	4 ml

8. 3M Sodium acetate (pH 5.2)

Sodium acetate - 12.4 g

Distilled water - 20ml

Adjust the pH to 5.2 using glacial acetic acid

Makeup final volume to 50 ml

Autoclave it, cool it down to room temperature and store at 4^0C.

9. TE buffer

Stock 0.5 M Tris-Cl (pH 8.0) - 2.0 ml

Stock 0.5 M Na_2 EDTA.$2H_2O$ (pH 8.0) - 0.02ml

Make up the solution to 100 ml with distilled water.

Autoclave it, cool it down to room temperature and store at 4^0C.

10. 0.5X TAE (Gel running buffer)

5 X TAE (stock) - 25 ml

Distilled water - 225 ml

Make fresh every time it is required.

Reagents required for PAGE

1. 20% Acrylamide

Acrylamide - 1.9gm

Bis-acrylamide - 0.1gm

Makeup the final volume to 10 ml with distilled water.

2. 10% Ammonium persulphate

APS - 100 mg

Makeup the final volume to 1ml with distilled water.

3. 5X Tris Boric EDTA

Trisbase - 27 gm

Boric acid - 13.7gm

0.5 M EDTA - 12.5 ml

Makeup the final volume to 500 ml with distilled water.

4. 1X Tris Boric EDTA

5X TBE - 60 ml

DDW - 240 ml

5. Agarose (For sealing of 2 gels)

50 mg / 5 ml (for one set of 2 gels)

Table. 4 resource species with annealing temperature

S No.	Name of locus	Primer	Primer sequence (5'→3')	Ta (˚C) Annealing Tempature in Resource Species
1		Mfw*1	GTCCAGACTGTTCATCAGGAGGAGGTGTACACTGAGTCACGC	50
2		R12*	CTATTCCTGTGCAGACCTTC	50
			GATACACGTCCAGTTTCACC	
3	Tor tor	Lr 36*	CTTGTTCACTGCACAGACACC	50
			AAGGTTCAGATTGCCTCCTG	
4		Lr 32*	AAATCAGAGAGGGAAGGACAGA	50
			CATTGATTGGTTGAGCACCTAC	
5		Lr 45*	GTGAGGCTCTCTGTTTGGTG	50
			AACGCAGCCAACCTAACGTA	
6		Lr 29*	CCCACGCAAACTCCTGTT	50
			GGAACAAGGCCAGAGCTTTA	
7		MFW7*	TACTTTGCTCAGGACGGATGC	50
			ATCACCTGCACATGGCCACTC	
8		MFW17*	CAACTACAGAGAAATTTCATC CA	50
			GAAATGGTACATGACCTCAAG	
9		MFW26*	CCCTGAGATAGAAACCACTG CA	50
			CACCATGCTTGGATGCAAAAG	
10		MFW2*	CACACCGGGCTACTGCAGAG	50
			GTGCAGTGCAGGCAGTTTGC	
11		Lro 44*	CTGTGACCGGAGAGGAATGT	50
			GAACGAGGAGAGGACGAATG	

12	Barb 62*	GGCACAAAAATGGATTCATATC ATTT GTACACGAGCATATGGACAA	50
13	Barb 59*	CTGTATCCATCACATAGGCTGATA CATGATTTAATAGAACACACAC	50
14	Barb 54*	TTGTTTTGATTCACACTGAG CA TACCATCTGCTGCTGCTTC	50
15	Barb 37*	AAATACGCTCTCCTCATTAATTT GTACAAAAGCAAAAATAAATTA	50
16	R 6*	TATCCTGGCTGAAAACTTTG CTACAGGAACAACCATCACC	50
17	Ca 12*	GTGAAGCATGGCATAGCACACAGGAAAGTGCCAGGCATACAC	50

Kits used

1. Silver staining of DNA in PAGE gels
   - Amersham Biosciences.
2. PCR product purification kit
   - QIAGEN PCR product purification kit.