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FIELD OF INVENTION:

This invention relates to a Microsatellite DNA marker used for identifying disease resistant populations of *Penaeus monodon*.

This invention also relates to a method of developing microsatellite DNA marker.

BACKGROUND OF THE INVENTION:

Reliable methods for estimating genetic diversity within and between species are crucial for studying populations, classifying germplasm, breeding and identifying genes conferring particular traits. A comparison of the common DNA marker systems has been reviewed by Rafalski et al. 1996. Among the various methods reviewed were-random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and arbitrarily primed PCR (AP-PCR) or DNA amplification fingerprinting (DAF). All these four methods provide good discrimination within and between species and allow analyses of whole genomes without prior DNA sequence information. Though the advantage of these methods is through the generation of multiplexed DNA fingerprints, among disadvantages are: generation of dominant markers, revelation of few alleles and usual requirement of careful optimization to assure reproducibility. On the other hand, methods that detect co-dominant polymorphisms at simple sequence repeat (SSR) microsatellite loci offer good reproducibility and generate large numbers of detectable alleles. SSR repeats are arrays of mono-, di-, tri-, tetra-or penta-nucleotide units widely dispersed throughout animal and plant genomes that display high levels of genetic variation based on differences in the number of tandemly repeating units at a locus.

Recently, many groups are also trying to develop population specific various DNA markers in various shrimp system. Among these Garcia et al. 1996, reported a population-specific DNA fragment (B20) in *Penaeus vannamei* shrimp. This fragment, found using the randomly amplified polymorphic DNA (RAPD) procedure, was present in Population 2 but not in populations 1 and 4. Marine shrimp, *Penaeus vannamei*

specific microsatellite markers are also been developed. Random amplified polymorphic DNA (RAPD) analysis was used to amplify the genome of black tiger shrimp (*Penaeus monodon*) to detect DNA markers and assess the utility of the RAPD method for investigating genetic variation in wild *P. monodon* in Thailand. The RAPD analysis of brood stocks from three different locals, Satun-Trang, Trat, and Angsila, revealed different levels of genetic variability in the samples. Recently *P. monodon* specific microsatellite markers have also being developed by others.

The viral diseases of cultivated penaeid shrimp in Asia and elsewhere have been summarized in several reviews. An excellent disease manual has also been published and this includes information on the types of shrimp viruses, their host ranges and degrees of severity. These reviews described recent developments for five viruses affecting the cultivation of *P.monodon*. These five viruses are: white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvo-like virus (HPV), infectious hypodermal and hematopoeitic necrosis virus (IHHNV) and monodon baculovirus (MBV). Among these, WSSV is the most life threatening one in shrimp culture that cause mass mortalities of cultured shrimp within 2-7 days (often reaching 80-100% of affected populations). Shrimp aquaculture industry in India has also been affected by WSSV among populations of Indian tiger shrimp (Penaeus monodon) in commercial hatcheries and farms. A number of groups in India are working on pathogens and have developed diagnostic methods for these viruses. As per literature search, no such report has come out so far about disease resistant DNA markers and their application to develop disease free brood stock of *Penaeus monodon*.

We have collected one population of shrimp that are growing well even in the areas severely affected by viral disease. This population has no clinical symptoms of viral disease, absence of virus in gill tissue confirmed by PCR method and have therefore been designated by us as disease resistant. We have also collected disease susceptible population those died due to viral disease has clinical symptoms of virus infection and also shows virus positive by PCR. We are studying the genetic diversity between these two populations of *P. monodon* using various microsatellites as DNA markers.

Despite of serious efforts the major problems due to viral diseases is continued in shrimp aquaculture worldwide. No such remedy has come out so far though various commercial kits are available to identify the pathogens in shrimp. Prevention is one of the better approach should be main focus at this stage to control the disease in shrimp. The main drawbacks in the existing state of art is that no such useful DNA markers have been reported so far to study the genetic diversity in the breeding program of disease resistant *P.monodon*. Although various DNA fingerprint methods including microsatellite markers are well established in *P.monodon*.

OBJECTS OF THE INVENTION:

An object of this invention is to propose a Microsatellite DNA marker used for identifying disease resistant populations of *Penaeus monodon*.

Another object of this invention is to propose a method of developing Microsatellite DNA marker used for identifying disease resistant populations of *Penaeus monodon*.

Further, object of this invention is to propose a Microsatellite DNA marker, which will be useful to generate disease resistant seeds.

Still further object of this invention is to propose a Microsatellite DNA marker to help us to overcome the economic damages of the shrimp aquaculture from viral diseases.

BRIEF DESCRIPTION OF THE INVENTION:

According to this invention there is provided a microsatellite DNA marker to identify disease resistant populations of *Penaeus monodon*.

The said Microsatellite upon PCR amplification generates a unique statistically significant DNA band of 71 bp specific for disease susceptible along with a band of 317 bp, whereas disease resistant population showed only 317 bp band and absence of 71 bp band.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS:

Fig.1 Shows WSSV detection test by commercially available kit (Bangalore Genei Pvt. Ltd., India).

Fig. 2 Shows analysis of microsatellite DNA markers to identify disease resistant *P. monodon*.

Fig. 3 Shows microsatellite (KM-19/20) amplification with various concentration of genomic DNA from disease susceptible *P.monodon*.

DETAILED DESCRIPTION OF THE INVENTION:

We have collected two populations of *P.monodon* shrimp from highly virus affected shrimp farm. One of these contains shrimps that died due to virus infection; these confirmed the presence of 10-10^5 WSSV virus particles per µl of the extract from gill tissue. We called them disease susceptible populations. Another population of animals has been collected from the same disease affected shrimp farm those have no clinical signs of virus infection, survive well in the disease affected farm and also confirmed the absence of WSSV particle using commercially available kit. We named these as disease resistant populations. We have studied the genetic diversities between these two populations of shrimp using PCR based microsatellite DNA markers. Out of 42 different microsatellite one (KM-19/20) generates a unique DNA band of 71bp specific for disease susceptible along with a band of 317 bp whereas disease resistant populations showed only 317 bp band and absence of 71 bp band. Our invention suggested that as low as 14.8µg shrimp tissue is enough to identify disease resistant populations. We have also found that this marker is very specific to *P.monodon* not found in other shrimp. Since we have used naturally occurring disease resistant populations of shrimp isolated from highly disease affected farms logically one could also able to consider these populations as disease resistant while challenging with virus. Although in future we have a plan to do WSSV challenge experiment with disease resistant shrimps to monitor virus load using

real time quantitative PCR method. In conclusion, the result presented here is the preliminary work to develop disease resistant genetic marker in *P.monodon*. We need to study further to know whether this disease resistant marker is a WSSV disease resistant quantitative trait locus (QTL) region. For this reason we are in progress to clone and sequence this marker for finding disease resistant gene that will be examined the role of WSSV pathogenesis.

Detection of WSSV in disease susceptible and resistant shrimp (*P.monodon*) samples:

The viral DNA (WSSV) was isolated from 30mg gill tissue of shrimp (P. monodon) samples (disease resistant and susceptible) collected from highly disease-affected ponds. Quantification of virus load in the shrimp populations was monitored by PCR method using commercial available Kit (Bangalore Genei, India). According to the manufacturer's method, shown in the Figure 1, we observed disease susceptible individuals (those individuals having clinical symptom of virus infection and died due to disease) were infected moderately to severely with $10\text{-}10^5$ WSSV particles per µl of the above preparation (Figure 1, Lane 8-14). Where as disease resistant individuals did not exhibit any virus infection (Figure 1, Lane 1-7), no clinical symptoms of disease and survived well though they were collected from the same disease-affected ponds.

Disease resistant microsatellite DNA marker analysis:

Shrimp genomic DNA was prepared from tail tissue and was subjected for microsatellite DNA marker analysis by PCR. Out of 42 microsatellites only one (KM-19/20) showed bands specific to disease susceptible shrimps and provided reproducible fingerprints as DNA marker to identify disease resistant shrimps (*P.monodon*). The frequency of the marker in the susceptible and resistant populations is shown in Table-1. The PCR amplified products generated from KM-19/20 microsatellite using primers designed from its flanking sequence showed two DNA bands. One is 317 bp with frequency 96% in disease susceptible and 94% in disease resistant populations. Where as other one is of 71bp with frequency 80% only in disease susceptible populations and absent in disease resistant populations with 0% frequency (Table-1, Figure 2). Chi Square was calculated with this data and the value was corrected by applying Yate's correction. The probability

was done by using Fischer Exact Test. We obtained that 71bp DNA band was highly significant ($p<0.01$) genetic marker to distinguish between susceptible and resistant population.

Table-1: Microsatellite DNA marker to identify disease resistant *P.monodon*:

Micro atellite name	Repeat motifs	Primer sequences 5'→3'	Opti mum anne aling temp (°C)	Band size (bp)	Microsatellite DNA marker frequency (%) in Disease		Chi Square	Signific ance
					Suscepti ble (n=50)	Resistant (n=50)		
KM-19/20	$(ATC)_4$... ...$(TC)_3$ ...$(TA)_4$	Forward: GATCATCATCATCG GCTG	50	317	96	94	0.2105	Not Signific ant
		Reverse: TATGTACGTTAGTC CAAG		71	80	0	66.667*	Highly Signific ant i.e. $p<0.01$*

* Chi square value was corrected by applying Yates' Correction, Probability was done by using Fischer Exact Test.

The use of viral resistant shrimp lines is an important preventive measure in aquaculture management strategies aimed to reduce the economic damage due to viral diseases. At this stage we have developed a microsatellite DNA marker for the first time to identify disease resistant P.monodon based on absence of 71bp DNA band but presence of only 317bp band (Table-1, Figure-2). For this study we have used naturally occurring disease resistant population of shrimp having no clinical signs of disease, absence of virus and survival in highly virus affected shrimp aquaculture farms. However, one should also be able to study these resistant populations in the presence of virus challenge under

controlled conditions in a laboratory. We plan to perform such a WSSV challenge experiment with disease resistant shrimps to monitor virus load using real time quantitative PCT. The disease resistant microsatellite DNA marker developed in this report will be very useful for marker assisted breeding program in *P.monodon* to generate disease free brood stock in shrimp aquaculture industry.

In conclusion, the result presented here is the groundwork for further characterization of the disease resistant genetic marker in *P.monodon*. It is not known if this disease resistant marker is indicative of a WSSV disease resistant quantitative trait locus (QTL) region or if this may be due to founder effect. For this reason currently we are in progress to clone and sequence this marker for finding disease resistant gene that will be examined the role of WSSV pathogenesis.

EXAMPLE:

Collection of shrimp (P.monodon) samples:

We have collected two populations (50 individuals for each group) of shrimp (*P.monodon*) samples from highly disease-affected ponds. One population called disease susceptible contains those individuals having clinical symptoms of viral disease (most died due to viral disease before collection) and confirmed the presence of WSSV in the gill tissues by PCR method using commercially available kit (Bangalore Genei Pvt. Ltd, India). Another population called disease resistant contains those individuals survive well in the same disease-affected ponds, having no clinical symptoms of disease and also confirmed the absence of all kinds of virus (WSSV, WSBP, YHV, HPV, IHHNV and MBV) in their gills by PCR method.

Preparation of genomic DNA from shrimp:

Genomic DNA was isolated from 100 mg tail muscle tissue of shrimp (both resistant and susceptible population) using Wizard ®Genomic DNA purification Kit protocol (Promega Biotech, USA). Each DNA sample was quantitated by spectrophotometer

(Shimadzu UV160U, Shimadzu Corp., Japan). DNA samples were electrophoresed in a 1% agarose gel to determine the quality before use in subsequent PCRs.

Development of microsatellite DNA marker to identify disease resistant shrimp (*P.monodon*) population:

Forty-two different microsatellite loci from the genome of *P.monodon* were used for searching disease resistant DNA marker. The primer sequences were designed from the flanking region of their respective microsatellite locus for PCR amplification. For disease resistant microsatellite DNA marker analyses, the PCR reactions were carried out in a 25-100µl volume containing 200-500ng DNA template, 10-15 pmole of each oligonucleotide primer (forward and reverse), 0.15-0.2mM dNTPs (Roche, Germany), 1.5-2.0mM $MgCl_2$ (Bangalore Genei, India), 1x buffer (10-20mM Tris-HCl, pH 8-9 @25^{0}C, 40-50mM KCl and 0.01-0.1% gelatin (Bangalore Genei, India) and 0.6-1U Taq polymerase (Bangalore Genei, India). Each reaction was placed in a thermocycler (Perkin Elmer 2400, USA) for PCR. The thermal profile was 94°C for 3-5 min; 25-30 cycles of the following-94°C for 1min, experimental annealing temperature (48-50°C) for 30 second and 72°C for 1min; at the end final extension 72°C for 5 min. The amplified DNA was electrophoresed in 6% polyacrylamide gel at 80-100V for 1-5h and DNA was subsequently visualized with 1µg/mL ethidium bromide and photographed (Gel Doc 100, Bio-Rad Laboratories. Hercules, California).

WE CLAIM:

1. A microsatellite DNA marker to identify disease resistant populations of *Penaeus monodon* comprising a highly statistically significant DNA band of 71 bp and 317 bp bands.
2. The microsatellite DNA marker as claimed in claim 1, wherein said 71bp DNA band is present only in disease susceptible population and absent in disease resistant populations.
3. The microsatellite DNA marker as claimed in claim 1, wherein said 317bp band is present in both disease susceptible and resistant populations.
4. The method as claimed in claim 1, wherein the development of DNA marker is done from 42 microsatellite loci obtained from shrimp genomic DNA.
5. The microsatellite as claimed in claim 1, wherein said microsatellite DNA marker has the following repeat motif: $(ATC)_4$ ---$(TC)_3$---$(TA)_4$---.
6. The method as claimed in claim 1, wherein both forward and reverse primers are designed as follows: 5'-GAT CAT CAT CAT CGG CTG-3' and 5'-TAT GTA CGT TAG TCC AAG –3'.
7. The method as claimed in claim 1, wherein either the amount of 8.5-17µg tissues or 200-400ng genomic DNA required for said DNA marker.
8. The method as claimed in claim 1, wherein said disease resistant microsatellite DNA marker is analysed by PCR reactions which are carried out in a 25-100µl volume containing 200-500ng DNA template, 10-15 Pmole of each (forwards and reverse) oligonucleotide primer, 0.15-0.2mM dNTPs, 1.5-2mM $MgCl_2$, buffer containing 10-20mM Tris-Cl, 40-50mM KCl, 0.01-0.1% gelatin, pH 8-9 @25^0C and 0.6-1.0 U Taq polymerase.
9. The method as claimed in claim 1, wherein the amplified DNA was electrophoresed in 5-8% polyacrylamide gel at 80-100V for 1-5 hrs.