*World Journal of Microbiology & Biotechnology* **17**: 529–533, 2001.



# Inter-simple-sequence repeat (ISSR)-PCR for the identification of saprophytic strains of *Leptospira*

Jyoti Shukla, Urmil Tuteja, Milind B. Ratnaparkhe and Harsh V. Batra*
*Division of Microbiology, Defence R&D Establishment, Jhansi road, Gwalior 474002, India*
**Author for correspondence: Tel.: +751-341550, Fax: +751-341148, E-mail: mb@drde.8m.com*

Received 15 February 2001; accepted 2 July 2001

*Keywords:* ISSR-PCR, *Leptospira* differentiation, multiplex PCR

## Summary

The inter-simple-sequence repeat (ISSR) primers that anneal to a simple repeat of various length and at non-repetitive motifs at 3′ and 5′ end were attempted for PCR amplification of *Leptospira* genome. Of the six ISSR primers tested, namely, $(AG)_8T$, $(AG)_8C$, $(AG)_8G$, $(CA)_8A$, $(TG)_8C$ and $(TG)_8G$, only primer $(AG)_8T$ produced amplification of 1000 bp in the two non-pathogenic *Leptospira* species tested, viz; *Leptospira biflexa* serovar *patoc* and *L. meyeri* serovar *ranarum*, with no amplification in any of the 16 standard pathogenic serovars tested. The remaining five ISSR primers did not exhibit any amplification of the *Leptospira* genome in either pathogenic or non-pathogenic species. From among 35 *Leptospira* isolates recovered from hospitalized patients with pyrexia of unknown origin and/or febrile jaundice (12 in number) and from different environmental water sources (23 in number), $(AG)_8T$ ISSR-PCR correctly identified all the 22 isolates from water sources that were confirmed to be non-pathogenic by conventional tests. The results therefore, confirmed the ability of a primer, based on simple-sequence repeat motif, to produce a fragment that is useful as a group genetic marker in *Leptospira* species. The single nucleotide anchor, T′, at the 3′ end of the primer appeared to play an important role in differentiation of pathogenic and non-pathogenic species of *Leptospira*. Multiplex PCR, using ISSR primer, $(AG)_8T$ and the reported 16S rRNA gene primers, specific for pathogenic *Leptospira* species, or the 23S rRNA *Leptospira* genus specific primers, provided clear identification of serovars and isolates into pathogenic or non-pathogenic groups.

## Introduction

Leptospirosis is one of the world's most important zoonotic diseases. It is primarily an infectious disease of ruminants like cattle, sheep and shrews etc. Man usually contracts the disease by coming into contact with infected animals or contaminated animal products. *Leptospira* organisms can be found virtually in all tropical and temperate areas of the world and a large number of serovars distinguishable into broad categories of pathogenic and non-pathogenic groups have been reported. These are indistinguishable under dark field microscopy and by culture characteristics. Inhibitory or support growth in 8-azaguanine-containing media (Johnson & Rogers 1964), egg-yolk reaction test (Fuzi & Csoka 1961a), *p*-phenylenediamine (PPD) dye test (Fuzi & Csoka 1961b) and growth at 13 °C (Johnson & Harris 1967) are the conventional methods of differentiation into pathogenic and non-pathogenic groups and may take up to 2–4 weeks to obtain the results. PCR amplification of the specific 16S rRNA region (Hookey 1992) and of the 23S rRNA region (Woo *et al.* 1997a) have been recently reported for differentiation.

Inter-simple-sequence repeats (ISSRs) are DNA markers that involve the use of microsatellite sequences directly in the PCR for the DNA amplification (Zietkiewicz *et al.* 1994). Microsatellite or simple-sequence repeat (SSR) are short tandem repetitive DNA sequences with a repeat length of 1–5 bp (Litt & Luty 1989). Multilocus fingerprinting methods have been developed using oligonucleotides based on SSRs as primers in PCR amplification (Gupta *et al.* 1994; Sanchez *et al.* 1996). These methods enable amplification of genomic DNA and provide information about many loci simultaneously. These markers have been used for genetic diversity, gene tagging and genome mapping in plant species (Tsumura *et al.* 1996; Ratnaparkhe *et al.* 1998a, b). Microsatellites have been characterized in number of microorganisms (Van Belkum *et al.* 1998), however, ISSR have not been used for the study of microorganisms of clinical importance. We report here an ISSR-PCR that appears to differentiate pathogenic and non-pathogenic *Leptospira* serovars.

## Materials and methods

### Leptospira *serovars*

Pathogenic serovars of *Leptospira* species, viz., *Leptospira interrogans* serovars *bataviae*, *australis*, *canicola*, *copenhageni*, *hebdomadis*, *icterohaemorrhagiae* and *djasmin*; *Leptospira kirschneri* serovars *cynopteri* and *grippotyphosa*; *Leptospira santarosai* serovars *shermani* and *celledoni*; *Leptospira weilii* serovar *sarmin* and *Leptospira borgpetersenii* serovars *ballum*, *javanica* and *tarassovi* and the saprophytic *Leptospira* of species *Leptospira biflexa* serovar *patoc* and *Leptospira meyeri* serovar *ranarum* were obtained from WHO Collaborating Center, Netherlands. These serovars were maintained in EMJH media. Twelve isolates of *Leptospira* recovered from hospitalized patients of pyrexia of unknown origin (PUO) and/or from cases of febrile jaundice and also 23 isolates obtained from different water sources of sewage, stagnant water and tap water of Gwalior city were utilized in the present study.

### *DNA extraction*

For extraction of DNA, exponentially growing leptospire cultures were centrifuged at 12,000 × *g* for 30 min at 4 °C. The pellet was suspended in glucose–tris–EDTA buffer (50 mM Glucose, 1 M Tris–HCl, 0.5 M EDTA, pH 8.0) and then resuspended in lysis buffer (10% SDS and 10 N NaOH). This was then subjected to phenol–chloroform extraction twice and precipitated with ethanol. After two washes in 70% ethanol, the DNA preparation thus obtained was air dried and redissolved in ultrapure water (Brendle *et al*. 1974).

### *PCR primers*

The set of ISSR primers were obtained from University of British Columbia, Vancouver, Canada. The primers utilized were of 17 nucleotides in length with two nucleotides repeating eight times and having one nucleotide at 3′ end as anchor. The list of ISSR and rRNA gene specific primers used in present study is shown in Tables 1 and 2.

### *PCR amplifications*

The PCR amplification using ISSR primers was performed with following conditions: Denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, for 35 cycles, followed by 10 min extension at 72 °C. Each 25 $\mu$l PCR reaction contained 2.5 mM $MgCl_2$, 200 $\mu$M dNTPs, 50 mM KCl, 10 mM Tris–HCl, 1% Triton X-100, 1 unit of Taq DNA polymerase, 5 pmol of primers, and 30 ng of genomic DNA. For multiplex PCR the annealing temperature was 52 °C when ISSR $(AG)_8T$ repeat primer was used with pathogenic specific 16S rRNA gene primers and 55 °C when ISSR and primers of 23S rRNA genes were utilized.

*Table 1.* ISSR primers.

| | |
|---|---|
| $(AG)_8T$ | AGAGAGAGAGAGAGAGT |
| $(AG)_8C$ | AGAGAGAGAGAGAGAGC |
| $(AG)_8G$ | AGAGAGAGAGAGAGAGG |
| $(CA)_8A$ | CACACACACACACACAA |
| $(TG)_8C$ | TGTGTGTGTGTGTGTGC |
| $(TG)_8G$ | TGTGTGTGTGTGTGTGG |

*Table 2.* Primer sequences of 23S rRNA and 16S rRNA gene.

| |
|---|
| 23S rRNA genus specific primers |
| Forward primer 5′ GACCCGAAGCCTGTCGAG 3′ |
| Reverse primer 3′ CATTAGCCCTGATTCGTACCG 5′ |
| 16S rRNA pathogenic serovar specific primers |
| Forward primer 5′ CGCTGGCGGCGCGTCTTAAA 3′ |
| Reverse primer 3′ AAGGTCCACATCGCCACTT 5′ |

## Results

### *Marker $(AG)_8T$-1000 is specific to the non-pathogenic species of* Leptospira

DNA of standard non-pathogenic serovars, namely, *patoc* and *ranarum* belonging to *Leptospira* species *L. biflexa* and *L. meyeri* and of pathogenic serovars belonging to species, *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. weilii* and *L. borgpetersenii* were tested with ISSR-PCRs. Amplification of DNA could be observed only with primer $(AG)_8T$, wherein a region of 1000 bp was amplified in non-pathogenic species of *Leptospira*. Figure 1 shows the PCR-amplified product using primer $(AG)_8T$. The marker $(AG)_8T$-1000 was absent in the standard pathogenic serovars of the *Leptospira* species *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. weilii*, and *L. borgpetersenii*. The ISSR-PCR using primer $(AG)_8T$ was repeated a number of times to confirm the reproducibility of the amplified product. The primer $(AG)_8T$ amplified the marker region in non-pathogenic species whereas other primers based on (AG) repeats with different anchors such as $(AG)_8G$ and $(AG)_8C$ did not produce amplification. This indicated that a single nucleotide anchor at 3′ end of the primer played an important role for specificity of PCR reaction in non-pathogenic *Leptospira* species.

### *Screening of* Leptospira *isolates from the patients and from the environmental samples*

For evaluating the ISSR marker $(AG)_8T$-1000, 12 isolates of *Leptospira* recovered from hospitalized patients of PUO and/or from cases of febrile jaundice and 23 isolates obtained from different water sources were used. DNA was extracted from these 35 isolates and used for PCR analysis. PCR was performed using 23S rRNA genus specific primers which amplified 482 bp fragment from all the isolates, further confirming that these isolates belong to *Leptospira* species. All the isolates were then characterized with the conventional proce-

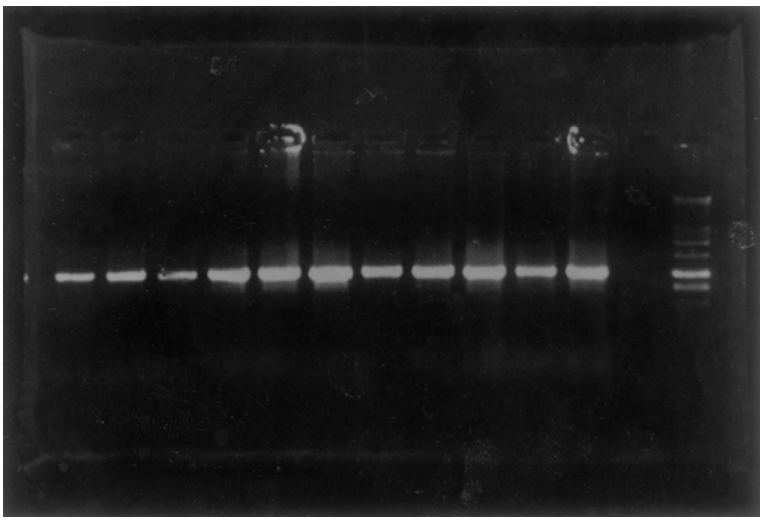

*Figure 1*. PCR-amplified ISSR pattern on 1.2% agarose gel. Lanes 1–9: non-pathogenic *Leptospira* isolates. Lanes 10–11: standard non-pathogenic *Leptospira* serovars, namely, *patoc* and *ranarum*. Lane 12: standard pathogenic *Leptospira* serovar *australis*. Lane 13: 200 bp ladder.

dures of differentiation following growth in 8-azaguanine and at 13 °C, egg-yolk reaction and PPD dye tests. In the 8-azaguanine test, the growth of all the 12 isolates from human patients and one from a tap water source of the same hospital ward was inhibited after 3 weeks of incubation at 28 °C, while the remaining 22 isolates from different water sources had confluent growth. Growth of these isolates at 13 °C after 3 weeks of incubation had identical results to that of growth in 8-azaguanine. In the egg-yolk reaction test, the same group of 13 isolates (12 from patients, one from tap water) did not produce pellicle or turbidity in the overlayed media but the other group of 22 isolates from water sources decomposed egg-yolk and produced turbidity and a pellicle after 4 weeks incubation at 28 °C. Similarly, in the PPD dye test, 2-weeks-old cultures of the same 13 isolates produced a reddish brown coloration and the remaining 22 producing blackish brown coloration. Based upon these observations, 12 isolates from hospitalized patients and one from tap water source were identified as pathogenic and the rest 22 as non-pathogenic. The data indicated that 13 isolates belong to pathogenic species while 22 isolates were saprophytic. These isolates were then screened using primer $(AG)_8T$ ISSR primer in PCR. The band of 1000 bp was observed in the non-pathogenic isolates while it was absent in all the pathogenic isolates. Figure 2 shows the amplification of $(AG)_8T$-1000 in non-pathogenic isolates of *Leptospira*. Table 3 presents results of the conventional tests and the PCRs on 35 isloates.

Multiplex PCR was performed using reported primers of 23S rRNA or 16S rRNA genes in combination with $(AG)_8T$ ISSR primer on standard serovars of *Leptospira* as well as isolates. In combination of 23S rRNA and ISSR primers, amplification of the 482 bp fragment

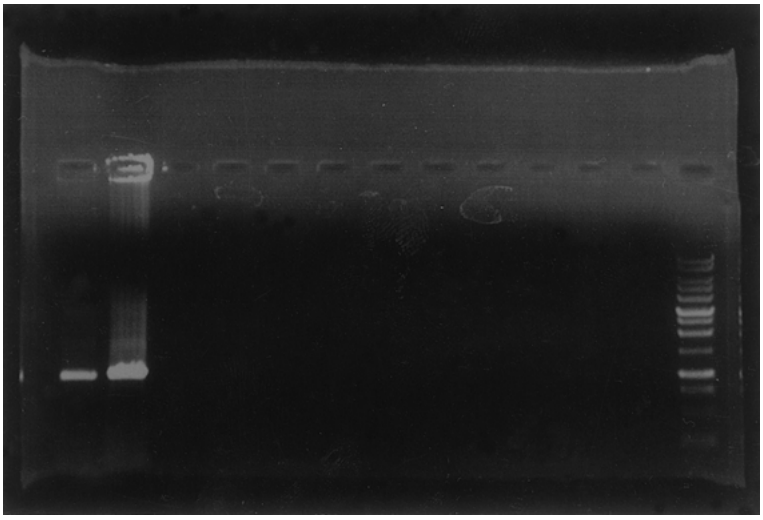

*Figure 2*. PCR-amplified ISSR pattern on 1.2% agarose gel. Lanes 1–2: standard non-pathogenic *Leptospira* serovars *patoc* and *ranarum*. Lanes 3–8: pathogenic *Leptospira* isolates. Lanes 9–12: standard pathogenic *Leptospira* serovars, namely, *australis*, *autumnalis*, *ballum*, and *bataviae*. Lane 13: 200 bp ladder.

*Table 3.* Characterization of *Leptospira* isolates into pathogenic and non-pathogenic groups.

| Source | Number tested | Isolates recovered | Conventional methods | | | | rRNA PCR | | ISSR-PCR |
|---|---|---|---|---|---|---|---|---|---|
| | | | 8-Azaguanine | 13 °C | Egg-yolk | PPD | 16 S | 23 S | $(AG)_8T$ |
| Ward patient | 55 | 12 | NG | NG | ND | RB | + | + | – |
| Tap water | 9 | 1 | NG | NG | ND | RB | + | + | – |
| Sewage | 28 | 15 | G | G | D | BB | – | – | + |
| Stagnant water | 23 | 7 | G | G | D | BB | – | – | + |

NG – no growth; ND – no decomposition; RB – reddish brown; G – growth; D – decomposition; BB – blackish brown.

appeared in all the standard serovars and isolates, whereas amplification of the 1 kb fragment was observed only in non-pathogenic leptospires. Figure 3 shows the representative picture of multiplex PCR using $(AG)_8T$ primer and 23S rRNA genus specific primers. In multiplex PCR of 16S rRNA gene primers and ISSR combination, a 631 bp fragment was obtained in pathogenic serovars and isolates, while the 1 kb fragment was amplified only in non-pathogenic leptospires. Figure 4 shows the representative picture of multiplex PCR using $(AG)_8T$ primer and 16S rRNA pathogenic specific primers. These results indicated that multiplex PCR can easily distinguish pathogenic and non-pathogenic leptospires when $(AG)_8T$ primers are utilized.

## Discussion

SSRs have been characterized in microorganisms like *Mycobacteria*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Neisseria meningitidis* and *N. gonorrhoeae*. Successful use of PCR-mediated SSR amplifications to track the spread of microbial pathogen has been reported for *H. influenzae* and *Candida albicans* (van Belkum *et al.* 1998). However, it is difficult to identify the repeat sequences and then design locus specific PCR primers. Identification of repetitive sequences involves genomic library construction, screening and DNA sequencing. ISSR-PCR with arbitrarily predesigned primers can provide a faster way for detection of SSRs in the genome. Of the six predesigned primers for repeat sequences utilized in this study, primer $(AG)_8T$ amplified a single band of 1000 bp which is helpful for differentiation of pathogenic and non-pathogenic *Leptospira* serovars and isolates. The differentiation of pathogenic species, *L. interrogans* from saprophytic species, *L. biflexa* was reported earlier using 23S rRNA gene specific primers (Woo *et al.* 1997a). Pathogenic leptospires were also detectable by amplification of 631 bp using specific primer of 16S rRNA gene sequences (Hookey 1992) and by specific hybridization of a 23S rRNA region employing a fluorogenic probe (Woo *et al.* 1997b). The ISSR $(AG)_8T$ repeat sequence region identified in this study is a new region that differentiates the pathogenic and non-pathogenic *Leptospira* serovars. The significance of this 1 kb region, present exclusively in saprophytic serovars, is not known but could be investigated in terms of attenuation or loss of virulence in these microorganisms. At present no information is available about the genes involved in the virulence of *Leptospira*. Simple sequence repeats have been associated with the pathogenicity, virulence, evolution, gene expression and other important characteristics of microbes. Variability of SSR with clear

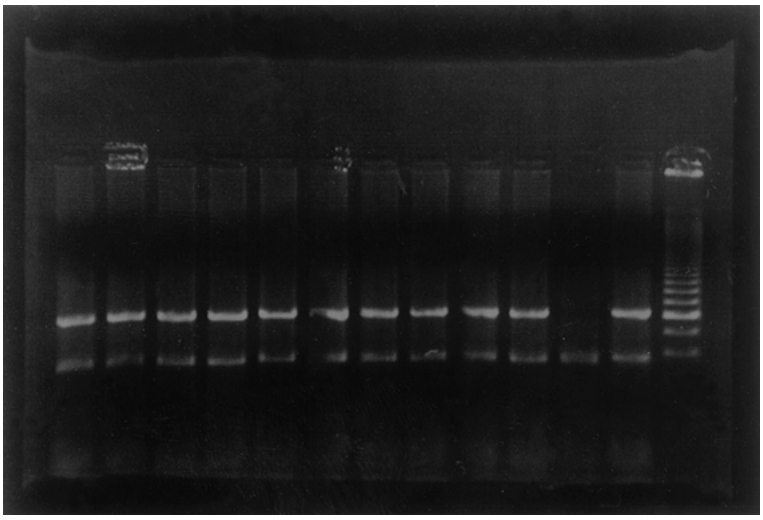

*Figure 3.* Multiplex PCR using $(AG)_8T$ ISSR primer and 23S rRNA genus specific primers. Amplification pattern on 1.2% agarose gel. Lanes 1–10: non-pathogenic *Leptospira* isolates. Lane 11: standard pathogenic serovar *canicola*. Lane 12: standard non-pathogenic *Leptospira* serovar *patoc*. Lane 13: 200 bp ladder.

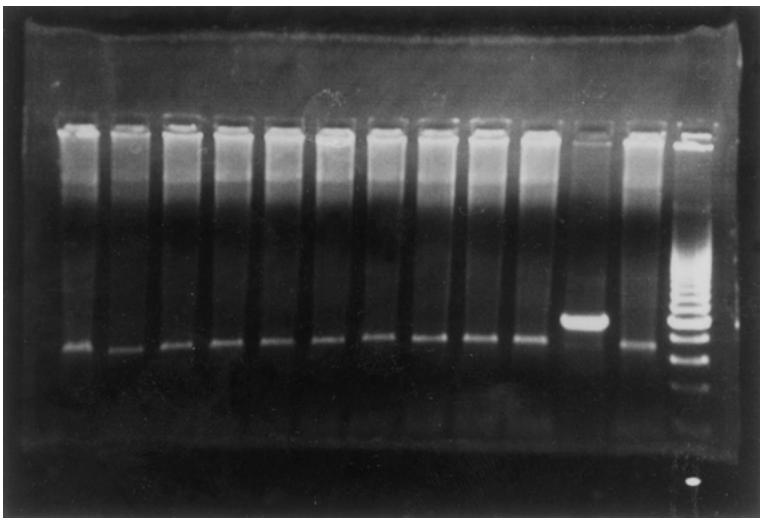

*Figure 4*. Multiplex PCR using $(AG)_8T$ ISSR primer and 16S rRNA pathogenic specific primers. Amplification pattern on 1.2% agarose gel. Lanes 1–6: standard pathogenic *Leptospira* serovars, namely, *autumnalis*, *ballum*, *cynopteri*, *djasmin*, *grippotyphosa*, and *javanica*. Lanes 7–10: pathogenic *Leptospira* isolates. Lane 11: standard non-pathogenic *Leptospira* serovar, namely, *ranarum*. Lane12: standard pathogenic *Leptospira* serovar *sarmin*. Lane 13: 200 bp ladder.

implications for virulence has been reported in certain organisms (Van Belkum *et al*. 1998).

The ISSR marker $(AG)_8T$-1000 possess a number of advantages. The procedure is simple, quick, reliable and can be used to process many samples simultaneously. The amplification conditions with the ISSR-PCR are very stringent and this results in a high reproducibility. Multiplex PCR of ISSR with 16S rRNA primers amplifying the 1 kb fragment in non-pathogenic serovars and the 631 bp fragment in pathogenic serovars and similarly with 23S rRNA genus specific primers presenting two bands in non-pathogenic leptospires (1 kb and 482 bp) and one band in pathogenic leptospires (482 bp) adds to clarity in differentiation.

## Acknowledgements

The authors are grateful to Dr R. V. Swamy, Director D. R. D. E., Gwalior for the encouragement to conduct the study. We are also thankful to Commandant, Military Hospital, Gwalior for helping in collection of clinical samples.

## References

Brendle, J.J., Rogul, M. & Alexander, A.D. 1974 Deoxyribonucleic acid hybridization among selected leptospiral serotypes. *International Journal of Systematic Bacteriology* **24**, 205–214.

Fuzi, M. & Csoka, R. 1961a An egg-yolk reaction test for the differentiation of leptospirae. *Journal of Pathology and Bacteriology* **82**, 208–211.

Fuzi, M. & Csoka, R. 1961b Rapid method for the differentiation of parasitic and saprophytic leptospirae. *Journal of Bacteriology* **81**, 1008.

Gupta, M., Chyi, Y.-S., Romero-Severson, J. & Owen, J.L. 1994 Amplification of DNA markers from evolutionary diverse genomes using single primers of simple sequence repeats. *Theoretical and Applied Genetics* **89**, 998–1006.

Hookey, J.V. 1992 Detection of Leptospiraceae by amplification of 16S ribosomal DNA. *FEMS Microbiology Letters* **90**, 267–274.

Johnson, R.C. & Harris, V.G. 1967 Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. *Journal of Bacteriology* **94**, 27–31.

Johnson, R.C. & Rogers, P. 1964 Differentiation of pathogenic and saprophytic leptospires with 8-azaguanine. *Journal of Bacteriology* **88**, 1618–1623.

Litt, M. & Luty J.A. 1989 A hypervariable microsatellite revealed *in vitro* amplification of dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics* **44**, 397–401.

Ratnaparkhe, M.B., Santra D.K., Tullu A. & Muehlbauer, F.J. 1998a Inheritance of inter-simple-sequence-repeat polymorphisms and linkage with a fusarium wilt resistance gene in chickpea. *Theoretical and Applied Genetics* **96**, 348–353.

Ratnaparkhe, M.B., Tekeoglu, M. & Muehlbauer, F.J. 1998b Inter-simple-sequence repeat are useful for finding markers associated with disease resistance gene clusters. *Theoretical and Applied Genetics* **97**, 515–519.

Sanchez, la Hoz, M.P., Davila, J.A., Loarce, Y. & Ferrer, E. 1996 Simple sequence repeat primers used in polymerase chain reaction amplification to study diversity in barely. *Genome* **39**, 112–117.

Tsumura, Y., Ohba, K. & Strauss, S.H. 1996 Diversity and inheritance of inter-simple-sequence-repeat polymorphism in Douglas-fir (*Pseudotsuga menziesii*) and Sugi (*Cryptomeria Japonica*). *Theoretical and Applied Genetics* **92**, 40–45.

van Belkum, A., Scherer, S., van Alphen, L. & Verbrugh, H. 1998 Short-sequence DNA repeats in prokaroytic genomes. *Microbiology and Molecular Biology Reviews* **62**, 275–293.

Woo, T.H.S., Smythe, L.D., Symonds, M.L., Norris, M.A., Dohnt, M.F. & Patel, B.K.C. 1997a Rapid distinction between *Leptospira interrogans* and *Leptospira biflexa* by PCR amplification of 23S ribosomal DNA. *FEMS Microbiology Letters* **150**, 9–18.

Woo, T.H.S., Patel, B.K.C., Smythe, L.D., Symonds, M.L., Norris, M.A. & Dohnt, M.F. 1997b Identification of pathogenic *Leptospira* genospecies by continuous monitoring of fluorogenic hybridization probes during rapid-cycle PCR. *Journal of Clinical Microbiology* **54**, 3140–3146.

Zietkiewicz, E, Rafalaski, A. & Ladunda, D. 1994 Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplifications. *Genomics* **20**, 176–183.