Chapter 3

Development of species-specific primers for five species of the Botryosphaeriaceae

Abstract

Certain species of the Botryosphaeriaceae have been reported to cause destructive canker and dieback diseases of trees such as *Eucalyptus* in South Africa and other parts of the world. These fungi can also live latently inside healthy *Eucalyptus* tissue. Species of Botryosphaeriaceae are morphologically difficult to distinguish making it necessary to develop DNA based identification techniques. Such techniques are also potentially useful in determining the presence of these fungi directly from plant material. The aim of this study was to design species-specific primers for rapid and reliable *in-vivo* identification of *Neofusicoccum parvum*, *N. eucalypti* prov. nom., *N. ursorum* prov. nom., *N. crypto-australe* prov. nom. and *B. dothidea*, which were reported from *Eucalyptus* in Pretoria in a recent study. Five species-specific primers were designed by exploiting differences in the translation elongation factor 1α (EF-1α) gene region. The general primer (EF-Br) was used in all cases as the complimentary primer in the amplification. Primer specificity was tested on DNA templates extracted from isolates of all the five species, in known samples and a blind test. The sensitivity was determined by serial dilutions with the purified DNA in sterilized distilled water and plant DNA solution from *Eucalyptus* leaves. All primers detected as little as 10 pg of fungal DNA when serially diluted with water and with plant DNA. In a preliminary *in vivo* experiment *N. parvum* was identified directly from the plant material. The sensitivity of these primers will allow reliable and rapid detection of the five species *in vivo*. The strategy used to develop these primers could be extended to detect other endophytes and latent pathogens.

Introduction

Species of the Botryosphaeriaceae are among the most destructive pathogens of forest trees worldwide, including *Eucalyptus*. Most *Eucalyptus* spp. originate from Australia (Poynton 1979) and many species are grown commercially in many countries around the world. Introduction of *Eucalyptus* spp. in the form of seed and cuttings around the world is thought to have resulted in the concomitant introduction of their fungal pathogens (Burgess *et al*. 2002*a*; 2002*b*; Slippers *et al*. 2004). Among these pathogens are species of Botryosphaeriaceae. These fungi are endophytes and / or latent pathogens on woody hosts, and they often remain undetected until the host suffers from some sort of environmental stress (Schoeneweiss 1981; Old *et al*. 1990; Smith *et al*. 1996; Slippers and Wingfield 2007).

The endophytic occurrence of the Botryosphaeriaceae on leaves have been reported on different hosts around the world (e.g. Johnston 1998; Swart *et al*. 2000; Denman *et al*. 2003; Pavlic *et al*. 2007). In South Africa, Botryosphaeriaceae were first reported on *Eucalyptus* foliage in the 1980's (Crous *et al*. 1989). Smith (1995) demonstrated penetration of *Eucalyptus* leafs through the stomatal openings by germinating conidia of *Botryosphaeria dothidea*, and also found Botryosphaeriaceae on leaves of *E. smithii*, *E. camaldulensis*, *E. grandis* and *E. nitens*. *Botryosphaeria dothidea* was also thought to be the cause of canker and dieback of *Eucalyptus* in South Africa (Smith *et al*. 1996). In the recent study it was shown that *N. parvum* was the most common pathogen causing canker and dieback of *Eucalyptus* in South Africa, while *B. dothidea* was rarely isolated (Slippers *et al*. 2004).

Identification of species of Botryosphaeriaceae is often based on anamorph morphology, because the sexual structures (teleomorph) are rarely encountered in nature and are not easily induced in culture. During the last decade DNA based tools have provided more robust methods to identify the members of the Botryosphaeriaceae (e.g. Denman *et al*. 2000; Crous *et al*. 2006; Slippers and Wingfield 2007). These studies have resulted in a much clearer view of the diversity of Botryosphaeriaceae that infect the particular hosts in certain areas. For example, a recent study revealed the presence of five species (*Neofusicoccum* and *Fusicoccum*) in a small *Eucalyptus* arboretum in Pretoria (Chapter 2).

The standard method of detecting species of Botryosphaeriaceae from asymptomatic tissue has been by primary isolation from leaves and twigs on agar media. This method can be time consuming and slow growing species may be undetected or under represented (Zhang *et al*. 2006). The aim of this study is to design species-specific primers for rapid detection of five species of Botryosphaeriaceae isolated from *Eucalyptus* in Pretoria, South Africa (see Chapter 2). For this purpose, variations among the translation elongation factor (EF-1α) gene region sequences of the five species were targeted. The second aim was to test the ability of these primers to detect these species *in-vivo* in *Eucalyptus* leaves.

Materials and Methods

Isolates and DNA extraction

Twenty-two isolates representing six species of the Botryosphaeriaceae were used in this study (Table 1). These isolates were obtained from a recent study of Botryosphaeriaceae on *Eucalyptus* spp. in Pretoria, South Africa (Chapter 2) and from culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

. DNA template was obtained from fungal cultures by using the modified version of phenol:chloroform DNA extraction method described in Pavlic *et al*. (2007). The concentration of the extracted DNA was determined by using the nanodrop (ND-1000 v3.3.0; Inqaba biotech, South Africa) and accompanied software.

DNA was also extracted from *Eucalyptus* leaves. Visually healthy leaves were randomly sampled from *Eucalyptus* trees. All leaves were surface sterilized by placing them sequentially for 1 min in 96 % ethanol, undiluted bleach (3.5–5 % available chlorine) and 70 % ethanol, and rinsed in sterile distilled water. The leaves were freeze dried with liquid nitrogen and crushed with a mortar and pestle. The DNA was extracted by using a CTAB extraction method (Doyle and Doyle 1987).

PCR with general primers

The translation elongation factor 1α (EF-1α) gene region of all isolates was amplified with the general primers EF-AF (5' CAT CGA GAA GTT CGA GAA GG 3')

and EF-BR (5' CRA TGG TGA TAC CRC GCT C 3') (Sakalidis 2004). A PCR reaction mixture contained 1 µL of template DNA (60-100 ng/µL), 10mM of each primer, 0.2 mM of dNTPs, PCR buffer with $MgCl_2$ (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 2.5 units of *Taq* polymerase (Roche Molecular Biochemicals, Almeda California), and the final reaction mixture volume was 25 µL. PCR conditions were as follows: denaturation at 95 ^{o}C for 2 min, followed by 35 cycles of denaturation (95 ^{o}C for 20s), annealing (55 ^{o}C for 45s) and elongation (72 ^{o}C for 1½ min), followed by a final elongation step at 72 ^{o}C for 7 min. PCR amplicons were viewed on a 1.5 % agarose gel stained with ethidium bromide under UV-light.

DNA sequencing and primer design

After purifying PCR products using the High Pure PCR Product Purification kit (Roche Diagnostics, Mannheim, Germany) amplicons were sequenced in both directions using the same primers that were used in PCR reactions. The ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Warrington, U.K.) was used for sequencing reactions as specified by the manufacturer and reactions were run on an ABI PRISM 3100^{TM} automated DNA sequencer (Perkin-Elmer, Warrington, U.K.). The nucleotide sequences were aligned with MAFFT software version 5.667 (http://timpani.genome.ad.jp/~mafft/ server/) (Katoh *et al*. 2002).

The variations between the EF-1α gene region sequence data of five species of Botryosphaeriaceae were used for designing forward primers. The species-specific primers were designed by using the software Primer 3 (v. 0.4.0) (http://frodo.wi.mit.edu/) (Rozen and Skaletsky 2000). Online software Netprimer (www.PremierBiosoft.com) was used to further check for potential primer dimer and hairpin formation, and presence of repeated sequences on all selected putative primer sequences. All putative primer sequences were compared using BLAST (http://www.ncbi.nlm.nih.gov) against known species of Botryosphaeriaceae from GenBank in order to confirm their compatibility to relevant species. The primer EF-BR was used as complement to all the designed species-specific primers.

Specificity and sensitivity tests

All five primers were optimized by adjusting temperature to the relevant annealing temperatures as well as the amount of $MgCl_2$ added for each species and tested for specificity on isolates representing five species of Botryosphaeriaceae of known identity (Table 1). DNA extracted from representatives of five species of the Botryosphaeriaceae was diluted to a starting concentration of ~100 ng/µL with sterile distilled water. DNA concentration was verified by using nanodrop (ND-1000 v3.3.0) and accompanied software. The DNA was further diluted to 50, 25, 12.5, 4, 0.01, 0.001, 0.0001 ng/µL and was used in the PCR amplification with the species-specific primers designed in this study to determine the detection limits of the technique using these primers. PCR conditions were as described above, except that annealing temperatures were adjusted (Table 2). Both sterilized distilled water and DNA extracted from the youngest *Eucalyptus* leaves (expected to be endophyte free) were used as dilutents. DNA extracted from the youngest *Eucalyptus* leaves was checked for the presence of species of Botryosphaeriaceae by amplifying with EF-1α primers EF-AF and EF-BR and the designed species-specific primers (Table 2).

DNA from fifteen isolates representing six species of Botryosphaeriaceae of known identity were used for performing a blind test. Prior to this analysis all isolates were renamed and a DNA sample (1 µL) with a concentration between 60-100 ng/µL was used for each in PCR reactions. PCR amplification of DNA template with all five (BdeF, NueF, NceF, NeeF, NpeF) species-specific primer pairs was performed separately. DNA template of *N. ribis*, a close relative of *N. parvum*, was also included in the blind test to check the specificity of the *N. parvum* primer (NpeF). The PCR conditions for this test were the same as those described for PCR with the general EF-1α primers.

Detection of species of Botryosphaeriaceae from naturally infected Eucalyptus *leaves*

Sections of about 2 x 2 cm in size were cut from visually healthy leaves that were randomly sampled from *Eucalyptus* trees in a Pretoria arboretum. All leaves were cut into 8 segments and labelled following surface sterilization by sequential emersion for 1 min in 96 % ethanol, undiluted bleach (3.5–5 % available chlorine) and 70 % ethanol, and rinsed in sterile distilled water. Samples were then freeze dried with liquid nitrogen and crushed

with a mortar and pestle. DNA extraction was done from each segment using the CTAB extraction method for *Eucalyptus* leaves (Doyle and Doyle 1987). DNA amplification from each section was performed by using all the species-specific primers pairs separately; using PCR conditions described above and appropriate annealing temperatures (Table 2). The DNA from known species of Botryosphaeriaceae was used as positive controls.

Results

PCR with general primers

Amplification of DNA templates with the EF-1α general primers showed the expected amplicons of about 330 bp in size for all isolates used (Fig. 1). Comparison of the EF-1α DNA sequences of five species of Botryosphaeriaceae revealed conserved sequences when compared to the ITS sequence data.

DNA sequencing and primer design

Analysis of the DNA sequences of the EF-1α gene region revealed conserved regions within species which differed between the five species of Botryosphaeriaceae analysed. Forward primers were designed based on the differences between the species (Table 2 and 3). The BLAST results from the analysis of the putative primer designed for *Botryosphaeria dothidea* (BdeF) and *Neofusicoccum parvum* (NpeF), showed that the sequence corresponded to these species. However, the *N. eucalypti* prov. nom. primer (NeeF) is identical to a corresponding locus in *N. vitifusiforme*. The latter species does not occur on *Eucalyptus* and this primer should still be useful for detecting *N. eucalypti*. *N. ursorum* (NueF) and *N. crypto-australe* (NceF) primer sequences did not result in any identical matches within any of the known Botryosphaeriaceae from the GenBank. This is expected as these two species have been recently characterized (Chapter 2) and their sequences have been submitted. However, *N. crypto-australe* (NceF) primer sequence was expected to amplify its close relatives *N. australe* and *N. luteum*.

Specificity and sensitivity test

The species-specific primers only amplified DNA fragments from the species for which they were designed for (Fig. 2). An amplicon of about 330 bp was observed for

each species under different annealing temperatures (Table 2). The sensitivity test performed with serial dilutions of the genomic DNA of all species of Botryosphaeriaceae revealed that 10 pg of the fungal genomic DNA could be detected when diluted with both sterile distilled water and a plant DNA solution (Fig. 3). No species of Botryosphaeriaceae were detected from the DNA isolated from surface sterilized *Eucalyptus* leaves that were used as a dilutent in performing serial dilutions. The identity of all isolates that were randomly selected and used for the blind test were correctly determined using the species-specific primers (Fig. 4).

Detection of species of Botryosphaeriaceae from naturally infected Eucalyptus *leaves*

Neofusicoccum parvum was the only species that was detected from six of the eight leaf segments tested using the species-specific primers NpeF and EF-BR (Fig. 5). The PCR amplicons of the positive control was similar in size (330 bp) to that of the tested samples. No other species were detected when using the rest of the designed primers (Table 2).

Discussion

In this study, five species-specific primers were developed for the identification of *Neofusicoccum parvum*, *N. ursorum*, *N. eucalypti*, *N. crypto-australe* and *Botryosphaeria dothidea* based on the interspecies sequence variations of the translation elongation factor 1α gene fragment. Three species tested in this study have been previously reported as endophytes and latent pathogens on *Eucalyptus* in different parts of the world (Smith *et al*. 1996; Slippers *et al*. 2004; Burgess *et al*. 2006). *Neofusicoccum ursorum* and *N. crypto-australe* have only been previously reported from *Eucalyptus* in Pretoria (Chapter 2). The development of these primers provides a promising method for the detection of these species from healthy leaves.

The EF-1α gene region was targeted in this study for designing species-specific primers. Previously, ITS rDNA sequences have been used to design species-specific primers for identification of the Botryosphaeriaceae as well as other fungal species. For example, species-specific primers were designed from ITS rDNA sequence data for identification of *Fusicoccum* spp. from pistachio in California (Ma and Michailides 2002),

Diplodia pinea from *Pinus* in Lexington, Wisconson and South Africa (Flowers *et al*. 2003) and soil microfungi in Oregon USA (Martin and Rygiewicz 2005). The ITS rDNA sequence is, however, not always ideal for this purpose as differences amongst species of the Botryosphaeriaceae are often small. Furthermore, the most variable region of the ITS rDNA is also highly repetitive in many Botryosphaeriaceae, making primer design difficult. Therefore, exploiting variations in gene regions other than ITS might in some cases be necessary for designing primers for the identification of multiple species belonging to this group. EF-1α is useful for this purpose as it has been characterized in a fairly large number of species of Botryosphaeriaceae. It is also less repetitive and contains many indels that enhance specificity of primers as seen in (Table 3).

In this study, the primers designed could detect as little as 10 pg/µL of fungal DNA when used in a dilution series with both sterile distilled water and plant DNA as dilitents. These results are consistent with findings of Smith and Stanosz (2006) where the same detection limit was found following amplification with species-specific primers for detecting the latent Botryosphaeriaceous pathogen, *Diplodia pinea* in *Pinus*. Amplification using all the primers, even in the presence of high concentration of plant DNA, shows that these primers have the potential to be used for *in vivo* detection for the Botryosphaeriaceae.

In the preliminary tests on DNA isolated from sections of naturally infected *Eucalyptus* leaves from Pretoria arboretum, only *N. parvum* was detected using the species-specific primer NpeF and general primer EF-BR. This finding was expected since *N. parvum* was found to be the most abundant species on *Eucalyptus* in the arboretum (Chapter 2). The small sample size and rare occurrence of other *Neofusicoccum* species in the *Eucalyptus* arboretum could explain the failure to detect them from the leaves sampled in this study.

Neofusicoccum ribis was also included amongst the isolates selected for the blind test. All species were correctly identified using the species-specific primers with the exception of *N. ribis* where an amplicon was produced in the reaction containing the NpeF primer designed for identification of *N. parvum*. This was not unexpected as these two species are closely related to each other (Slippers 2003; Slippers *et al*. 2004). *Neofusicoccum australe* and *N. luteum* also share the DNA sequence which was used for

designing the species-specific primer for *N. crypto-australe* (NceF), and DNA from the former species would be expected to result in an amplification product using this primer. However, *N. australe* and *N. luteum* are not known from *Eucalyptus* in South Africa. The primer (NeeF) specific for *N. eucalypti* would also possibly amplify a locus in *N. vitifusiforme* as it has significant similarity to the sequence of this gene region in that species. The latter species, however, has not been identified from *Eucalyptus*. In cases such as this, the use of molecular tools like PCR-RFLP fingerprinting, as well as phylogenetic analysis can be used to further verify the identity of pathogens where necessary (Slippers *et al*. 2004; Alves *et al*. 2005; Pavlic *et al*. 2007).

With the species-specific primers developed in this study identification of endophytic Botryosphaeriaceae directly from the plant material can be achieved relatively easily. The species-specific primers apparently allows for the detection of small amounts of fungal cells. This technique can thus provide an easy way for future studies in trying to understand the relative abundance and distribution of species of the Botryosphaeriaceae that colonize *Eucalyptus* leaves.

References

Alves A, Phillips AJL, Henriques I, Correia A (2005) Evaluation of amplified ribosomal DNA restriction analysis as a method for the identification of *Botryosphaeria* species. *FEMS Microbiology Letters* **245**, 221–229.

Burgess T, Wingfield MJ (2002*a*) Impact of fungal pathogens in natural forest ecosystems; a focus on *Eucalyptus*. In 'Microorganisms in plant conservation and biodiversity.' (Eds K Sivasithamparam, KW Dixon, RL Barrett) pp. 285–306. (Kluwer Academic Publishers: Dordrecht)

Burgess T, Wingfield MJ (2002*b*) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the Southern Hemisphere. *International Forestry Review* **4**, 56–65.

Burgess TI, Sakalidis ML, Hardy GEStJ (2006) Gene flow of the canker pathogen *Botryosphaeria australis* between *Eucalyptus globulus* plantations and native eucalypt forests in Western Australia. *Austral Ecology* **31**, 559–566.

Crous PW, Knox-Davies PS, Wingfield MJ (1989) Newly-recorded foliage fungi of *Eucalyptus* spp. in South Africa. *Phytophylactica* **21**, 85–88.

Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Phillips AJL, Alves A, Burgess T, Barber P, Groenewald JZ (2006) Phylogenetic lineages in the Botryosphaeriaceae. *Studies in Mycology* **55**, 235–253.

Denman S, Crous PW, Taylor JE, Kang J-C, Pascoe I, Wingfield MJ (2000). An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Studies in Mycology* **45**: 129–140.

Denman S, Crous PW, Groenewald JZ, Slippers B, Wingfield BD, Wingfield MJ (2003) Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequence data. *Mycologia* **95**, 294–307.

Doyle JJ, Doyle JL (1987) A rapid isolation procedure to small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11–57.

Flowers J, Hartman J, Vaillancourt L (2003) Detection of latent *Sphaeropsis sapinea* infections in Austrian Pine Tissues using Nested–polymerase chain reaction. *Phytopathology* **93**, 1471–1477.

Johnston PR (1998) Leaf endophytes of manuka (*Leptospermum scoparium*). *Mycological Research* **102**, 1009–1016.

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**, 3059–3066.

Ma Z, Michailides TJ (2002) A PCR-based technique for identification of *Fusicoccum* sp. from Pistachio and various other hosts in California. *Plant Disease* **86**, 515–520.

Martin KJ, Rygiewicz PT (2005) Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BioMed Central Microbiology* **5**, 28.

Old KM, Gibbs R, Craig I, Myers BJ, Yuan ZQ (1990) Effect of drought and defoliation on the susceptibility of Eucalypts to cankers caused by *Endothia gyrosa* and *Botryosphaeria ribis*. *Australian Journal of Botany* **38**, 571–581.

Pavlic D, Slippers B, Coutinho TA, Wingfield MJ (2007) Botryosphaeriaceae occurring on native *Syzygium cordatum* in South Africa and their potential threat to *Eucalyptus*. *Plant Pathology* **56**, 624–636.

Poynton RJ (1979) Tree planting in Southern Africa, Vol. 2. The *Eucalyptus*. Department of Forestry. South Africa.

Rozen S, Skaletsky HJ (2000) Primer 3 on the www for general users and for biologist programmers. In 'Bioinformatics Methods and Protocols; Methods in Molecular Biology.' (Eds S Krawetz, S Misener) pp. 365–386. (Humana Press: Totowa, NJ)

Sakalidis M (2004) Resolving the *Botryosphaeria ribis-B. parva* species complex; a molecular and phenotypic investigation. B. Sc. Thesis. School of Biological Sciences and Biotechnology, Murdoch University, Western Australia.

Schoeneweiss DF (1981) The role of environmental stress in diseases of woody plants. *Plant Disease* **65**, 308–314.

Slippers B, Fourie G, Crous PW, Coutinho TA, Wingfield BD, Carnegie AJ, Wingfield MJ (2004) Speciation and distribution of *Botryosphaeria* spp. on native and introduced *Eucalyptus* trees in Australia and South Africa. *Studies in Mycology* **50**, 343–358.

Slippers B, Wingfield MJ (2007) Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology Reviews* **21**, 1–17.

Smith H (1995) *Botryosphaeria dothidea* as a pathogen and endophyte of *Eucalyptus* in South Africa. M.Sc. Thesis, Department of Microbiology and Biochemistry, University of the Orange Free State, South Africa.

Smith H, Wingfield MJ, Crous PW, Coutinho TA (1996) *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany* **62**, 86–88.

Smith DR, Stanosz GR (2006) A species-specific PCR assay for detection of *Diplodia pinea* and *D. scrobiculata* in dead red and jack pines with collar rots symptoms. *Plant Disease* **90**, 307–313.

Swart L, Crous PW, Petrini O, Taylor JE (2000) Fungal endophytes of Proteaceae, with particular emphasis on *Botryosphaeria proteae*. *Mycoscience* **41**, 123–127.

Zhang HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. *Natural Product Report* **23**, 753–771.

Fig. 1. An agarose gel (1.5 %) showing amplification of a fragment of the elongation factor 1-α gene region in species of Botryosphaeriaceae, using primers EF-AF and EF-BR. PCR products of each species are indicated in the gel. Lane 15 is negative control (PCR without DNA template); M = 100 bp marker.

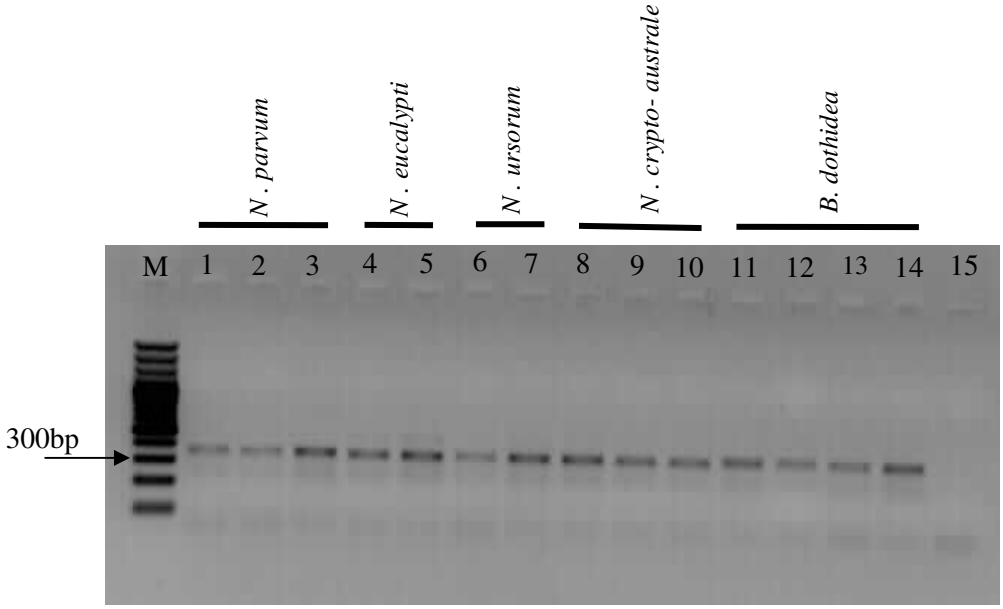
N . parvum
N . eucalypti
N . ursorum
N . crypto- australe
B. dothidea
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
300bp

Fig. 2. An agarose gel (1.5 %) showing the specificity of the different primers. For each set of lanes, two lanes contain PCR product from DNA of the intended species, while the next four contain PCR product where DNA from the other four species were included. Thus, the gels contain *N. parvum* (*A*, lane 1 and 2), *B. dothidea* (*A*, lane 7 and 8), *N. crypto-australe* (*B*, lane 13 and 14), *N. ursorum* (*B*, lane 19 and 20), *N. eucalypti* (*C*, lane 25 and 26). M = 100 bp marker.

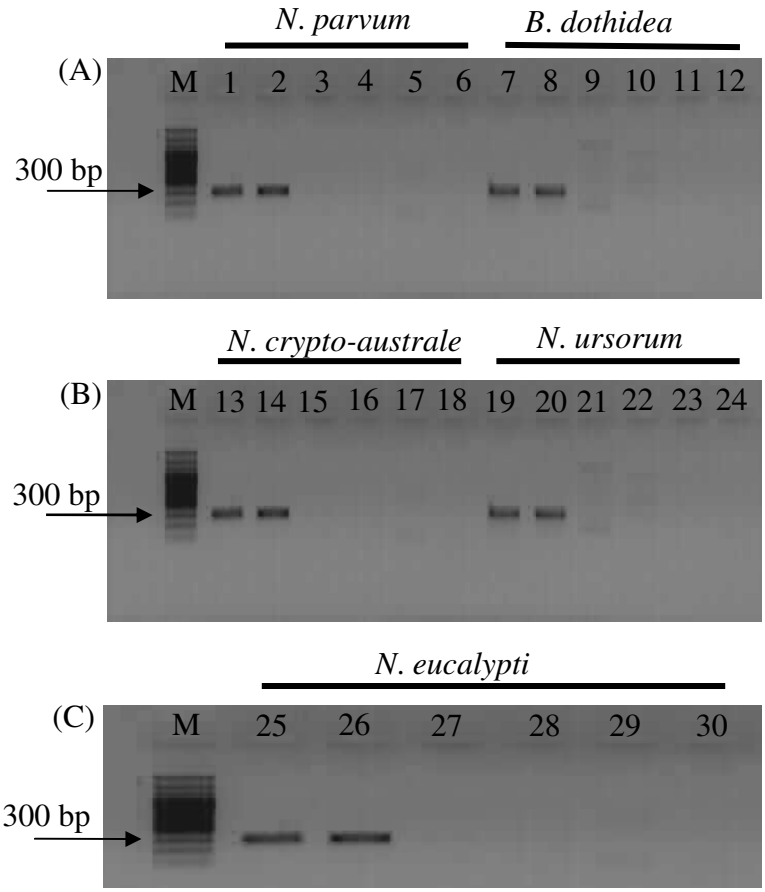
N. parvum
B. dothidea
(A)
M 1 2 3 4 5 6 7 8 9 10 11 12
300 bp
N. crypto-australe
N. ursorum
(B)
M 13 14 15 16 17 18 19 20 21 22 23 24
300 bp
N. eucalypti
(C)
M 25 26 27 28 29 30
300 bp

Fig. 3. An agarose gel (1.5 %) showing the smallest concentration of DNA for which an amplicon could be detected. Lanes 1-8 (*A*) shows the amplicons produced using EF-1α primers (EF-AF and EF-BR) and lanes 1-8 (*B*) shows using the forward BdeF primer and EF-Br reverse primer, as an example. Lanes 1-8 (*A* and *B*) represents different concentrations of the DNA template (50, 25, 12.5, 4, 0.01, 0.001, 0.0001 and 0.00001 ng/µL). M (*A* and *B*) = 100 bp marker.

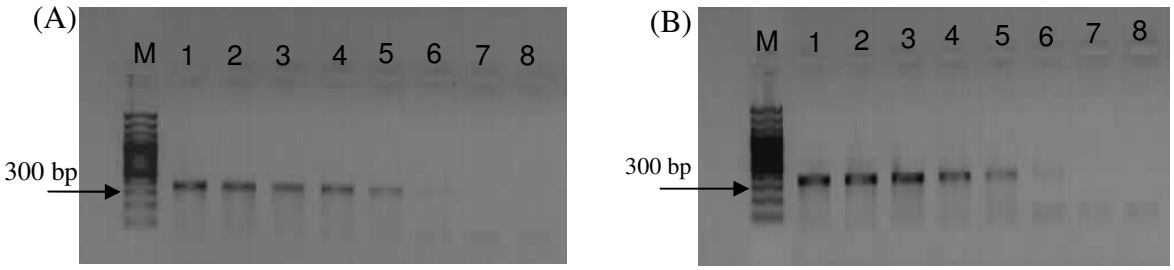
(A)
M 1 2 3 4 5 6 7 8
300 bp
(B)
M 1 2 3 4 5 6 7 8
300 bp

Fig. 4. An agarose gel (1.5 %) showing the specificity of primers for five species of Botryosphaeriaceae in the blind test. Isolates of the five species of Botryosphaeriaceae of known identity were randomly selected and relabeled. Amplification was done with each of the five species specific primers namely NpeF (*N. parvum*), NueF (*N. ursorum*), NeeF (*N. eucalypti*), NceF (*N. crypto-australe*) and BdeF (*B. dothidea*). PCR products amplified for each species are indicated in gels (*A-D*); M = 100 bp marker.

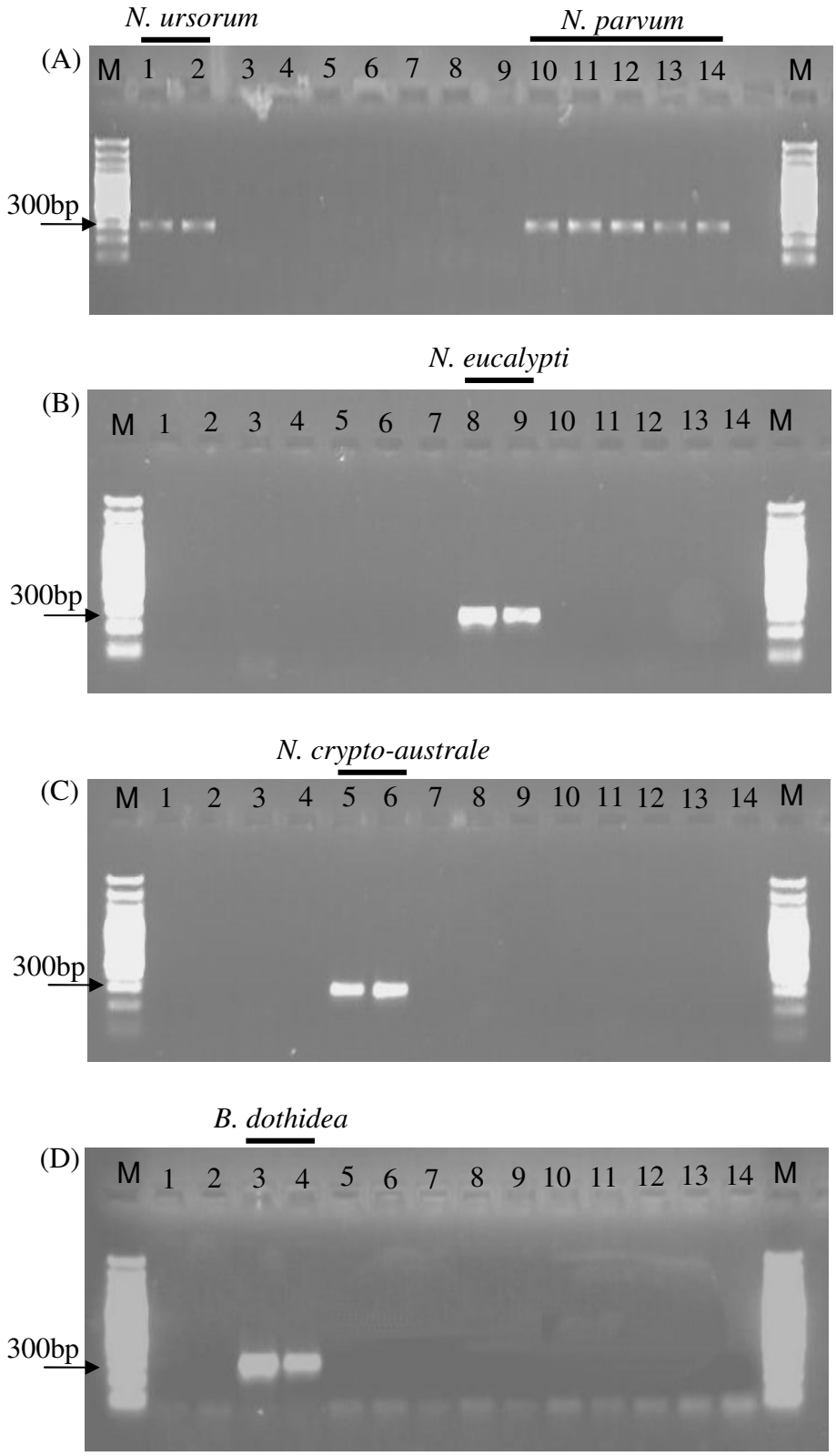
N. ursorum
N. parvum
(A)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
300bp
N. eucalypti
(B)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
300bp
N. crypto-australe
(C)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
300bp
B. dothidea
(D)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
300bp

Fig. 5. An agarose gel (1.5 %) showing detection of *N. parvum* directly from *Eucalyptus* leaf segments. Lanes 1-8 show the amplicons produced using NpeF species specific primer together with EF-1α reverse primer (EF-BR). Lane 9 is negative control (PCR product without DNA template). Lane 10 shows positive control of purified genomic DNA of *N. parvum*. M = 100 bp marker.

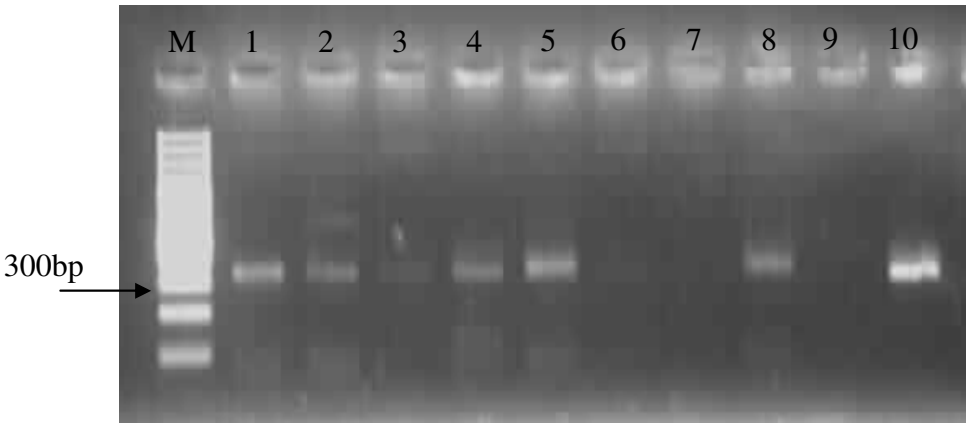
M
1
2
3
4
5
6
7
8
9
10
300bp

Table 1. Isolates representing species of Botryosphaeriaceae used in the specificity tests and primer design

Culture no.[A,B]	Other no.	Identity	Host	Location	Collector	Genbank EF
CMW7772 [A]		*Neofusicoccum ribis*	*Ribes* sp.	New York	B. Slippers & G. Hudler	AY236877
CMW7054 [A]	CBS 121.26	*N. ribis*	*R. rubrum*	New York	N. E. Stevens	AY236879
CMW14035[A, B]		*N. ribis*	*Syzygium cordatum*	Kwambonambi, S. Africa	D. Pavlic	AF283681
CMW14140 [A, B]		*N. ribis*	*S. cordatum*	Tzaneen, S. Africa	D. Pavlic	AY236885
CMW9078 [A]	ICMP 7925	*Neofusicoccum parvum*	*Actinidia deliciosa*	New Zealand	S. R. Pennycook	AY236885
CMW10122 [A]		*N. parvum*	*Eucalyptus grandis*	Mpumalanga Province, S. Africa	H. Smith	AF283681
CMW14021 [B]		*N. parvum*	*S. cordatum*	Kwambonambi, S. Africa	D. Pavlic	
CMW14022 [B]		*N. parvum*	*S. cordatum*	Kwambonambi, S. Africa	D. Pavlic	
CMW14019 [B]		*N. parvum*	*S. cordatum*	Kwambonambi, S. Africa	D. Pavlic	
CMW14018 [B]		*N. parvum*	*S. cordatum*	Kwambonambi, S. Africa	D. Pavlic	
CMW20736 [A, B]		*N. parvum*	*E. robusta*	Pretoria, S. Africa	H. M. Maleme	
CMW23792 [A, B]		*N. parvum*	*E. dorrigoensis*	Pretoria, S. Africa	H. M. Maleme	
CMW24571 [A, B]		*N. eucalypti*	*E. paniculata*	Pretoria, S. Africa	H. M. Maleme	
CMW23791 [A, B]		*N. eucalypti*	*Eucalyptus* spp.	Pretoria, S. Africa	H. M. Maleme	
CMW23790 [A, B]		*Neofusicoccum ursorum*	*Eucalyptus* spp.	Pretoria, S. Africa	H. M. Maleme	
CMW24480 [A, B]		*N. ursorum*	*Eucalyptus* spp.	Pretoria, S. Africa	H. M. Maleme	
CMW9076 [A,]	ICMP 7818	*Neofusicoccum luteum*	*Malus domestica*	New Zealand	S. R. Pennycook	AY236893
CMW10309 [A,]	CAP 002	*N. luteum*	*Vitis vinifera*	Portugal	A. J. L. Phillips	AY339266
CMW3386 [A]		*Neofusicoccum crypto-australe*	*Wollemia nobilis*	Queensland, Australia	M. Ivory	AY615157
CMW20738 [A, B]		*N. crypto-australe*	*E. citriodora*	Pretoria, S. Africa	H. M. Maleme	
CMW23787 [A, B]		*N. crypto-australe*	*Eucalyptus* spp.	Pretoria, S. Africa	H. M. Maleme	
CMW9073 [A]		*Neofusicoccum australe*	*Acacia* sp.	Melbourne, Australia	J. Roux & D. Guest	AY615269
CMW1110 [A]		*N. australe*	*Widdringtonia nodiflora*	Cape Province, S. Africa	W. J. Swart	AY615158
CMW8000 [A]		*Botryosphaeria dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236898
CMW9075 [A]	ICMP 8019	*B. dothidea*	*P. nigra*	New Zealand	G. J. Samuels	AY236899
CMW20728 [A, B]		*B. dothidea*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme	
CMW20739 [A, B]		*B. dothidea*	*E. microcorys*	Pretoria, S. Africa	H. M. Maleme	

[A]Culture collections: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria; CAP = Culture collection of A. J. L. Phillips, Lisbon, Portugal; CBS =Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand.

[A]Sequences of isolates used for designing primers.

[B]Isolates used in the blind test.

Table 2. Species-specific primers designed for five species of Botryosphaeriaceae and their PCR annealing temperatures

Identity	Primer	Primer sequences	Annealing tempetature (^{o}C)
Neofusicoccum parvum	NpeF	5' CTG GGT GCC AGG TGC 3'	60
N. eucalypti	NeeF	5' GTT TTT CCG CTG CAC GCA 3'	55
N. ursorum	NueF	5' GCA CGC TGG GTG GTG GGT 3'	60
N. crypto-australe	NceF	5' GCG ATG GTC CTG GGA TC 3'	60
Botryosphaeria dothidea	BdeF	5' GTT CCT GCG CCG AAT T 3'	54

Table 3. Multiple aligned sequences of representative isolates of Botryosphaeriaceae used for designing species specific primers. Primer sequences designed are in bold. Only the portion where primers where designed is shown. Each couloured block represent primer sequences for different species of Botryosphaeriaceae. (*Neofusicoccum ribis*), (*N. parvum*), (*N. eucalypti*), (*N. vitifusiforme*), (*N. ursorum*), (*N. luteum*), (*N. australe*), (*N. crypto-australe*) and (*Botryosphaeria dothidea*)

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CMW_7772     cga gaa ggt aag aaa gtt ttt cct tcc -gc tgc acg c-- gct ggg tgc tgg gtg ctg ggt gct ggg tgc tgg gtt ccc gca ctc aat ttg
CMW_7054     cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tcg tgg gtg ctg ggt gct ggg tgc tgg gtt ccc gca ctc aat ttg
CMW_9078     cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tcg cag gtg ctg ggt --- --- --- --- --t ccc gca ctc aat ttg
CMW_10122    cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tcg cag gtg ctg ggt --- --- --- --- --t ccc gca ctc aat ttg
CMW_23792    cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tgc cag gtg ctg ggt --- --- --- --- --t tcc gca ctc aat ttg
CMW_20736    cga gaa ggt aag aaa gtt ttt cct tcc -gc tcc acg c-- gct ggg tgc cag gtg ctg ggt --- --- --- --- --t tcc gca ctc aat ttg

CMW_15952    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_15953    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc agc cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_23791    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_24460    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_24571    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
STE_U5252    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
STE_U5050    cga gaa ggt aag aaa gtt ttt cc- --- -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gcg ctc aat ttg
CMW_23790    cga gaa ggt aag aaa gtt ttt cct tcc -gc tgc acc cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_24480    cga gaa ggt aag aaa gtt ttt cct tcc -gc tgc acg cac gct ggg tgc tgg gt- --- --- --- --- --- --- --t gcc gca ctc aat ttg

CMW_10309    cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- cga tgg tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_9076     cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- cga tgg tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_9073     cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- cga tgg tgc tgg ga- --- --- --- --- --- --- --t gct gca ctc aat ttg
CMW_1110     cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- cga tgg tgc tgg ga- --- --- --- --- --- --- --t gct gca ctc aat ttg
CMW_3386     cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- tgc tgc tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_20738    cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- tgc tgc tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_23787    cga gaa ggt gag aag att ttt cgt tcc -gc tgc ccg cg- tgc tgc tgc tgg ga- --- --- --- --- --- --- --t gcc gca ctc aat ttg
CMW_8000     cga gaa ggt aag cac aca ttt tct gtg -cc tgc ac- --- --- gtg tgc tgg gtt cct gc- --- --- --- --- --- --- --g ccg aat ttg
CMW_9075     cga gaa ggt aag cac aca ttt tct gtg -cc tgc ac- --- --- gtg tgc tgg gtt cct gc- --- --- --- --- --- --- --g ccg aat ttg
CMW_23787    cga gaa ggt aag cac aca ttt tct gtg -cc tgc ac- --- --- gtg tgc tgg gtt cct gc- --- --- --- --- --- --- --g ccg aat ttg
CMW_20739    cga gaa ggt aag cac aca ttt tct gtg -cc tgc ac- --- --- gtg tgc tgg gtt cct gc- --- --- --- --- --- --- --g ccg aat ttg
```