RESEARCH PROTOCOLS FOR CASSAVA BROWN STREAK DISEASE

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This document is an output from Project R8227 funded by the UK Department for International Development [DFID] Crop Protection Programme, for the benefit of developing countries.

Contents

Introduction

Cassava brown streak disease [CBSD] is caused by *Cassava brown streak virus* [CBSV], that has been tentatively placed in the genus *Ipomovirus* in the family *Potyviridae* [Monger *et al*., 2001a]. Although CBSD was first described in the 1930s [Storey, 1936] there was renewed interest in the disease in the 1990s, when surveys funded by the UK Department for International Development, Crop Protection Programme [DFID-CPP] showed it to be a major threat to food security in the coastal regions of Tanzania [Legg and Raya, 1997; Hillocks *et al*.,1996] and northern Mozambique [Hillocks *et al*.,2002] and also in the coastal strip of Lake Malawi from Tanzania [Legg and Raya, 1997] into southern Malawi [Gondwe *et al*., 2003].

In the absence of comparative data from earlier surveys, it is not possible to assess whether or not CBSD has increased in importance in recent years. A widely held view among smallholders in Mozambique, is that CBSD became a major problem after highly sensitive cultivars such as cv. Calamidade, were distributed following destruction of much of the cassava crop by a hurricane during the late 1980s. However, the disease was known to be prevalent in Tanzania at altitudes below 1000m during the 1940s [Nichols, 1950]. Resistance to both CBSD and cassava mosaic disease [CMD] were major selection criteria in the cassava breeding programme at Amani Research Station which, until 1952, was the headquarters of the East African Agriculture & Forestry Research Organisation. Since then, some research has continued sporadically, on the biology, distribution and control of CBSD. Research on the disease conducted up until 2001 is reviewed by Hillocks and Jennings (2003).

By the end of the 1990s, it was recognised that CBSD was diminishing the role of cassava as a food security crop in coastal areas of eastern and southern Africa. This recognition led to the incorporation of CBSD control measures into food security programmes run by several NGOs in Mozambique, that were funded by DFID and US AID. By 2001, resistance to CBSD had become a priority in the cassava improvement programmes of IITA, for varieties intended for the East African coast. From 2002, a number of breeding, research and other CBSD management projects have been funded by the Rockefeller Foundation, US AID, IFAD and DFID. In October 2002 a workshop was held in Mombasa, attended by representatives of each of these projects [See Legg and Hillocks, 2003]. At the workshop it was agreed that a set of research protocols should be developed to serve as a guide to appropriate methodology and to standardise the methods used by the various projects working on CBSD.

The Natural Resources Institute [NRI] has developed methods for research on CBSD in projects funded by DFID in the period 1996 - 2002, and DFID-CPP agreed to provide funding for NRI to publish these methods as a set of research protocols. It is hoped that this manual will provide a practical guide for researchers working to decrease the impact of CBSD, and in so doing, to enhance food security which is the basis of sustainable livelihoods.

Disease identification

Leaf symptoms

CBSD is often unrecognised in the field because of the inconspicuous leaf symptoms compared to those of CMD, and they may be disguised by symptoms of CMD and cassava green mite [CGM]. Symptoms of CBSD on the leaf can take different forms depending on cassava variety, the age of the plant and the conditions under which it is growing. Leaf symptoms may disappear altogether when new growth appears after a period of drought-induced abscission. The typical symptoms on green leaves are a feathery chlorosis around the secondary veins [Fig.1.]. Unlike CMD there is no leaf distortion and the plant may look otherwise healthy and vigorous. Fig. 2 shows some of the common variations in leaf symptoms of CBSD. CBSD symptoms tend to be more pronounced on the lower leaves, while for CMD they can be seen on the upper leaves. Fig. 3 shows symptoms of CMD and Fig. 4 those of cassava green mite [CGM] for comparison.

Fig.1. Typical leaf symptoms of CBSD showing 'feathery' chlorosis on the lower canopy of young green cassava plants.

Fig.2. Two variations of CBSD leaf symptoms

Fig.3. Leaf symptoms of cassava mosaic disease [CMD]

Fig. 4. Symptoms caused on cassava leaves by green mite

Stem symptoms

Stem symptoms may not always occur and their severity may be a varietal characteristic. Purple/brown markings appear on the green bark [Fig. 5] and on older bark, the discolouration extends into the underlying cortex . Plants with root necrosis may exhibit shoot die-back .

Fig.5. Symptoms of CBSD on the green stem

Root symptoms

Root symptoms appear sometime after the leaf symptoms. If an infected cutting is planted, the period from sprouting to the first appearance of root symptoms seems to be a varietal characteristic. In the varieties that are most sensitive to root necrosis, early signs of necrosis can be found from 6 months after planting. In the more tolerant varieties symptoms may not appear until 12 - 18 months after planting. The development of root necrosis symptoms seems to be associated with the physiological age of the plant, and necrotic areas in the starch-bearing tissues appear around the time of maximum root-bulking. Symptoms begin with small yellowish/brown, corky specks that increase in size and number, until in sensitive varieties, the whole root consists of yellow/corky material which is inedible [Fig. 6]. While the necrosis is confined to patches, the root may still be used for cooking but the diseased areas have to be removed before cooking or drying for storage. In the advanced stages of root necrosis, secondary organisms can cause soft rot, leading to compete root decay [Fig. 7], especially in wet soil.

Fig.6. Tuberous roots showing patches of yellowish/brown necrosis associated with CBSD.

Fig.7. Soft rot can sometimes result from CBSD root necrosis

Fig.8. Scoring system used to assess the severity of CBSD root necrosis.

Survey methods

Disease incidence survey [leaf symptoms]
Disease incidence surveys should be based on leaf symptoms and conducted when most of the crop is 4 -5 months old and before the onset of leaf senescence. Leaf damage by cassava green mite[CGM] progresses as the crop matures and this too, can obscure CBSD symptoms. If many fields are be surveyed, then disease incidence estimates may be based on a sample of the first 30 plants encountered on a diagonal transect through the field. If more accurate estimates are required, the number of plants in the sample should be increased. Farmers often grow a number of cassava varieties in the same field that may vary in sensitivity to CBSD, so it is helpful to note the cultivar name for each of the 30 plants in the sample. Assessment of the severity of above-ground symptoms of CBSD is of doubtful value, as they vary with cultivar, growing conditions and age of the plant.

Disease severity survey [root necrosis]
The main damage to the cassava crop by CBSD is caused by the root symptoms. These appear as constrictions and as yellow, corky areas of necrosis in the starch-bearing tissues. Disease severity is therefore usually based on an assessment of the severity of root necrosis. Surveys of root necrosis must be carried out on crops that are at least 8 months old, and preferably older than this, as the severity of root necrosis increases with increasing age of the plants. The main period for the development of root necrosis seems to be after the main period of root bulking. The dry season is not a good time for surveying leaf symptoms, due to the often poor conditions of the foliage, induced by drought and green mites. Ideally, 30 plants should be assessed per field, but this is usually reduced to 10 per field because of time limitations and the unwillingness of the farmer to see so many plants sampled destructively. As this is a severity score, there is no point in including non-infected plants [i.e. those without above-ground symptoms], so the first step is to select 10 plants with clear CBSD symptoms - this may have to be based on stem symptoms if the foliage is in poor condition. [NB. If results of the root necrosis assessment are to

be used for crop loss assessment, a disease incidence [leaf symptoms] record will be required for each field]. The plants are then carefully uprooted and examined for CBSD symptoms. Each root has to be cut transversely and examined for necrotic patches. If only mild symptoms of necrosis are present, it may be necessary to make several cuts along the root to find the symptoms. The severity of any root necrosis is then scored using a 1 - 5 scale [see Fig.8]. A mean score for each plant can be derived from the individual root scores, or, alternatively, if time is limited, the highest score from each plant may be recorded.

Crop loss assessment

A full assessment of economic loss to CBSD is difficult to conduct, as there are several ways in which the disease contributes to loss of income and decreased food security.

Direct yield loss
Loss of root weight as a result of retarded development either within the root or loss of photo-assimilate due to reduced growth of the above ground parts of the plant.

This is the most straight-forward to assess, by comparing the root weight from plants with foliar symptoms and root necrosis with similar nearby plants showing no symptoms. However, often there may be little effect on root weight, only a loss in useable root. Furthermore, the large variation that occurs in root yield between neighbouring cassava plants may exceed any variation due to CBSD. After harvest when the roots are peeled and made into chips for drying, the usual practice is to cut out and discard the necrotic areas. In order to obtain an accurate measure of total loss in root weight, the necrotic areas must be removed and weighed. This weight is then added to any loss in total weight relative to symptomless controls. An attempt was made to short-cut this process by Hillocks *et al*. [2001] by removing all roots with a root necrosis score of 3 or above and taking this weight as the additional loss. However this tends to over-estimate yield loss, as women [who are responsible for preparing cassava chips] will have managed to save some tissue from roots with patches of necrosis.

When calculating crop loss in an area, the proportion of yield lost is multiplied by the mean disease incidence in the area or for a given variety. For example 40% mean disease incidence and 30% loss in root yield [based on excision of necrotic areas] = 40 x 30/100 = 12%

Loss of marketable yield
Hillocks *et al*. [2001] assumed that all roots with a root necrosis score of 3 or above were unmarketable and these were removed from the harvest before calculating marketable root yield. The validity of this has been questioned, as it is generally considered that any evidence of root necrosis makes the roots unsuitable for market. This seems to be confirmed at markets in areas with a high incidence of CBSD, where cassava roots sold on the stalls have been brought from outside the immediate area. However, as stated above, when there is a food shortage, women will try to cut-out the necrotic patches.[If symptoms are severe but food scarce the complete diseased root may still be consumed. Roots infected by even a small amount of CBSD necrosis do not dry properly and produce poor flour with a tainted flavour].

Loss of useable yield
It is often the practice in areas where CBSD is endemic, for women to cut out the necrotic areas of cassava roots after harvest when the roots are peeled and made into chips for drying. Experimentally, the most accurate way to assess loss in useable yield would be to cut out the necrotic areas and weigh the discarded tissue. However, this would be very time consuming, and it is probably more practical to estimate yield loss by cutting roots and giving them a score for root necrosis severity. The roots can be cut transversely for a quick assessment, but longitudinal cuts avoid missing small

areas of necrosis. The cut roots for each plant can be laid out and a mean score estimated. If this is a replicated experiment, a mean plot score can be derived from the total score of all roots harvested in that plot. Tissue loss can then be estimated by converting scores to percentage tissue loss using the following guide:

SCORE	**APPROX TISSUE LOSS [%]**
Score 1	0
Score 2	1 - 10
Score 3	11 - 25
Score 4	26 - 50
Score 5	>50

[e.g Mean root necrosis severity score = 2.8 therefore, approx tissue loss would be around 22%].

The disadvantage of this is that it does not allow accurate calculation of tissue loss which can only be done by removing the diseased tissue and reweighing the root sample.

Loss of yield as a consequence of early harvesting

The severity of CBSD root necrosis increases with plant age, but also root necrosis seems to develop most during, or, immediately after, the main period of root-bulking. It is well known in areas where CBSD has long been present, that farmers harvest early *i.e*. before the crop reaches its full yield potential, in order to avoid root necrosis. This means that early-bulking varieties are favoured and storage in the field becomes less common. This decreases the value of cassava as food security crop, because it can no longer be left in the field to provide a reserve of food during periods when there is little other food available. These effects are very difficult to quantify. The impact of early harvesting could be assessed for individual varieties by comparing plots harvested at 7-9 months with those harvested at 12 - 18 months. Alternatively, farmers could be surveyed and after being shown the symptoms, asked about their harvesting practices for the different varieties they grow and to estimate the yield loss due to early harvesting.

Screening for resistance

Theoretically, there may be several different types of resistance:

Type 1. The plant readily becomes infected with the virus and shows typical leaf symptoms, but root necrosis is less severe, or its onset is delayed until after the main period of root-bulking. [This is refereed to as 'tolerance' and is the type of resistance most often found in local cultivars].[tolerance]

Type 2. The plant readily becomes infected with the virus but only mild symptoms are expressed in leaves and roots. In this case the virus should be detectable in the plant by PCR-diagnostics. [tolerance]

Type 3. Plants will show leaf symptoms when inoculum pressure is high but fewer plants become infected compared to fully susceptible controls. However, once infected, severe root necrosis may develop. [resistance to infection]

Type 4. Under high inoculum pressure, the plant shows no symptoms and the virus cannot be detected in any part of the plant. This may be due either to failure of the vector to transmit to these varieties or the virus is transmitted but does not multiply beyond the site of infection. [hypersensitivity or immunity]

Further research is required to determine if all these types of reaction occur in practice, among different cassava genotypes. However, the method of disease assessment employed in screening for resistance will influence the type of 'resistance' obtained.

The practice at Amani [Jennings] in screening for resistance to CBSD under EAAFRO cassava improvement programmes, was to base selection on the severity of root symptoms. However, this requires a record of the presence or absence of leaf symptoms during the growing season to avoid selection of disease 'escapes'. Placing emphasis on root symptoms leads to selection for type I or type 3 resistance or tolerance.

If the severity of above ground symptoms [leaf and stem] are taken into account in making selections [with reference to standard controls], there is more chance of selecting for type 3 and type 4 resistance [if it exists]. This would be the preferred resistance as widespread distribution of varieties with this type of resistance would lead to a reduction in inoculum pressure allowing limited cultivation of more susceptible varieties that may be favoured by communities for particular characteristics.

Assessment of resistance

There are two ways to assess the resistance of a cassava variety to CBSD and ideally, both should be used:

a) *With 'clean' planting material*. Take planting material from symptomless mother plants [this might need to be done in a non-CBSD area because apparently symptomless plants can give rise to plants that show CBSD symptoms at or soon after sprouting]. The material to be screened must then be planted in an area of high inoculum pressure. This requires that the virus is present in the form of infected plants - usually provided by infector rows, and that transmission is also taking place. This second condition can be difficult to ensure as the amount of transmission taking place varies between seasons and can sometimes be very little. [The absence of knowledge of the vector makes it even more difficult to predict periods of high inoculum pressure]. So, the disadvantage of this method is that if little or no spread occurs, no information can be obtained on the reaction of the cultivars to CBSD.

b) *With infected planting material*. For varieties that show leaf symptoms, cuttings can be taken from mother plants showing symptoms. These can be planted in screening trials and the severity of root necrosis assessed at harvest. Clearly, this will assess only type 1 and possibly, type 2 resistance [tolerance]. The advantage of this

method however, is that the sensitivity of the cultivar to root necrosis can be assessed, even when there is no transmission taking place.

It is important that standard controls [see below] are included in screening trials, as the presence of a known susceptible control [planted from healthy cuttings] is the only way to check that transmission of CBSD took place.

Disease scoring

In screening trials disease assessment is more simple to carry out than when yield loss is being calculated. Two things are vital – that the initial disease status of the material is known and that this is confirmed by a first score for the incidence leaf symptoms as early as possible, after sprouting. After that new symptoms may be due to insect transmission.

Disease incidence [foliar symptoms] - can be recorded monthly, but the key data is the maximum incidence for each cultivar.

Disease severity [root symptoms] – recorded at harvest. Ideally all roots have to be examined, but a single severity score [see above] can be given to each plant and a plot mean calculated. I would suggest also recording the highest score in the plot as this indicates the propensity of the cultivar to develop root necrosis.

If selections are being made, then only those free of necrosis should go forward. NB. This is where the initial disease status of the planting material is important. For if it was CBSD-free at planting and escaped infection during the season, then the absence of root necrosis will NOT indicate resistance.

Importance of high inoculum pressure

Plants grown from symptomless mother plants that remain free of CBSD leaf and root symptoms cannot be said to be resistant, unless it is known that they were exposed to high inoculum pressure *i.e* in situations where vector transmission tales place and there are many plants nearby, showing leaf symptoms of CBSD. This requires the inclusion of healthy plants of a susceptible control in the trial or selection plots. It is useful to use a plot to record whitefly populations each week [top two fully expanded leaves] as this will show if the season experienced low or high populations.

Standard controls

It was agreed at the Mombassa workshop that a set of standard controls should be adopted by all projects working on CBSD. The agreed standards were:

Highly susceptible :	TMS 42025
Less susceptible:	TMS 30001

A source of these lines that is free of CBSD will be required.

One or more local standards [i.e. a commonly grown cultivar in the locality] may also be included, but they should be virus-free at planting.

If the international standards are not available, then the same national ones should be used each year and at every location. [A set of standards should be chosen to represent, high medium and low susceptibility].

Production and distribution of CBSD-resistant planting material

The methods used depend to some extent on which approach is being adopted:

a) *Distibution of CBSD-tolerant cvs. which may or may not be infected*. - Where CBSD is widespread and losses represent a serious threat to food security, the decision may be taken to multiply and distribute local cultivars that have been identified as having some tolerance to CBSD. This means in practice that they are cultivars that become infected with the virus but are less prone to develop root necrosis as a result.

If this is the option chosen, then much time can be saved as there is no need to find a source of disease-free cassava and multiplication can be done, even in areas of high inoculum pressure.

b) *Distribution of CBSD-tolerant cvs. that are virus-free*. Where CBSD is less widespread and some farmers may have cassava fields that are free of the disease, distributing virus-infected planting material is inappropriate. The process of obtaining virus-free cuttings is more time consuming, and may not be worthwhile if the risk is high that some of the crop may become infected before harvest.

The method described below covers the whole process from identification of resistant material to distribution.

1. Identification of CBSD-resistant [tolerant?] cassava cultivars.

2. Identification of a source of the chosen cultivar that is free of CBSD [and free of CMD]. This may be difficult in some areas.

[If a completely virus-free source cannot be found, it may be necessary to try to obtain a clean stock by roguing after the collection has been made.

3. Collected material should be planted in isolation from other cassava by at least 200m.

4. Inspection should be done twice a week from early sprouting stage and any plants with symptoms of CBSD or CMD are removed. If the chosen option is to distribute CBSD-tolerant varieties that are not necessarily free of CBSD, only plants showing CMD symptoms should be rogued.

5. Even if the aim is not to produce virus-free planting material, it may still be desirable to remove any infected plants if numbers are small [say up to 5%]. The distribution of infected planting material should not be of great concern provided the variety does not develop more than mild root necrosis within the first growing year.

5. Plan for delivery of planting material - Vehicles, land prepared by farmers etc.

6. Harvest and deliver as soon as possible. Only cut stems that will be delivered that day.

7. Leave plants as a ratoon crop for the next season. It is probably best to replant after the first ratoon crop.

The use of 'rapid multiplication' techniques

Cassava can be multiplied rapidly in a small area by two-node cuttings grown at high density in a propagation bed and then keeping the soil moist by irrigation. Many plants can be obtained in this way in a short time from small amounts of starting material. However, the resulting plants are not ideal for distribution to farmers as they are very vulnerable in transit and to moisture stress on transplanting.

Propagation by seedlings

Cassava is propagated routinely by vegetative cuttings but seeds may be used in breeding programmes. The plant is normally cross-pollinated and seed is a source of variability. Self-sown seedlings in the field may sometimes be retained by farmers and represent a source of new land races. Freshly collected seed may be slow to germinate and germination can be facilitated by nicking the seed coat with a file.

Propagation *'in vitro'*

Cassava plantlets can be generated from meristem tips or from nodes in tissue culture medium in glass tubes. This requires facilities for sterile culturing and growth room where light and temperature can be regulated. Tissue-cultures plantlets can be used to obtain pest and disease-free plants for transporting across international boundaries. Heat therapy with meristem culture can be used to obtain virus-free explants from virus-infected material.

Wash trimmed explants in tap water, then immerse in 70% ethanol for 3 -5 s. Shake explants in sterilisation solution [180 ml SDW + 20 ml domestic bleach + one drop

Tween 20] for 10 - 20 min. Rinse 4 times in SDW. Place explants into tubes containing growth medium under asceptic conditions and replace the caps.

Growth medium for cassava node-bud cuttings

Murashige & Skoog basal medium	2.2g/l
Sucrose	20 g/l
Phytagel	2 g/l

Growth medium for cassava meristem culture

MS basal medium	4.4 g/l
BAP	0.1 mg/l
NAA	0.15 mg/l
GA3	0.003 mg.l
Sucrose	20 g/l
Phytagel	2 g/l

Diagnosis of CBSD by RT-PCR

Leaf symptoms of CBSD are usually clear on vigorous green plants during the first 4-5 months of growth. However, leaf symptoms may vary according to the variety and growing conditions. It is common for leaf symptoms to be absent in leaves that grow under very hot conditions or that sprout after a period of drought-induced defoliation. There may be other reasons why it may be necessary to detect the virus in the absence of leaves e.g. quarantine tests on cuttings. For these reasons a PCR-based diagnostic test was developed at Bristol University with funding from DFID-CPP. This method has been used routinely at NRI for virus indexing of tissue-cultures plants and for testing for the presence of the virus in transmission experiments.

Collection of diseased leaf samples for RT-PCR
Remove leaves exhibiting clear CBSD symptoms [usually the oldest leaves in cassava] from the plant using previously autoclaved forceps. Transfer the leaves into self-sealable plastic bags to avoid desiccation and return them to the laboratory for analysis. Analyse the samples either fresh or after storage at 80°C. Note: All experiments should be conducted wearing plastic gloves to avoid contamination with RNase enzyme, which will degrade virus particles.

RNA extraction
Extract total RNA from cassava and leaves using the RNesay Plant Mini Kit (Qiagen) according to the manufacturer's instructions as follows. (A handbook supplied with the kit will explain the following protocol in greater detail).

- Grind 100 mg of leaf sample under liquid nitrogen to a fine powder using a mortar and pestle and transfer the contents to 1.5 ml sterile eppendorf tube.

(Or, the sample can also be ground using a thick-gauged plastic bag and a roller-grinder in the absence of liquid nitrogen).

- Prepare buffer RLT by adding 10 µl of β-mercaptaethanol per 1 ml of RLT (supplied with the kit). Add 450 µl of RLT to the sample and vortex vigorously.
- Apply sample to the QIA shredder spin column (lilac) sitting in a 2 ml collection tube and centrifuge for 2 min at maximum speed. Transfer the flow-though to a new 1.5 ml sterile eppendorf tube without disturbing the cell-debris pellet in the collection tube.
- Add 0.5 ml volume (usually 225 µl) of absolute ethanol to the sample and mix well by pipetting.
- Apply sample (usually 675 µl) onto an RNeasy mini spin column (pink) sitting in a 2 ml collection tube. Centrifuge for 15 sec at 10,000 rpm. Discard the flow-through.
- Pipette 700 µl of buffer RW1 onto the RNeasy column and centrifuge for 15 sec at 10,000 rpm to wash. Discard the flow-through.
- Prepare RPE buffer by adding 4 volumes of absolute ethanol to RPE. Transfer RNeasy column into a new 2 ml collection tube. Add 500 µl buffer RPE onto the RNeasy column and centrifuge for 15 sec at 10,000 rpm. Discard the flow-through and reuse the collection tube.
- Add 500 µl buffer RPE onto the RNeasy column and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Discard the flow-through.
- Transfer RNeasy column in a new 1.5 ml collection tube and add 30 µl of RNase free water directly onto the RNeasy membrane. Centrifuge for 1 min at 10,000 rpm to elute the CBSV RNA particles. Flow-through contains CBSV RNA particles.
- Store RNA at -20°C.

Set up of RT-PCR

Subject extracted RNA to reverse transcriptase polymerase chain reaction (RT-PCR) using the One Step RT-PCR Kit (Qiagen) for virus detection. Primers CBSV 10 (5'-ATCAGAATAGTGTGACTGCTGG-3') and CBSV 11 (5'-CCACATTATTATCGTCACCAGG-3'), which amplify ~200 bp length nucleotides. The RT-PCR reaction contained the following reagents:

Reagent	**Volume per sample (µl)**
2 X Reaction buffer	12.5
20 µm primer CBSV 10	1.5
20 µm primer CBSV 11	1.5
RT *Taq* polymerase mix	0.5
RNase free water	7.0
Sample	2.0
Total	25.0

Run in a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, UK) under the following amplification cycles:

Function	Temperature (°C)	Time (min)	Cycle(s)
Conversion of RNA into cDNA	50	30	1
Denaturation of cDNA	94	1	1
Denaturation of DNA	94	1	
Annealing of primer	52	1	40
DNA synthesis	72	1	
Further extension of DNA	72	10	1

Gel electrophoresis

Load the amplified products of the reaction onto a 1.5% (w/v) agarose gel in 0.5x Tris Boric acid EDTA (TBE) electrophoresis buffer. Mix 4 µl of orange G loading dye (15% (w/v) Ficoll Type 400, 0.25% (w/v) orange G, 40 mM EDTA, pH 8.0) with 15 µl of the sample product prior to loading the gel, and run at 5.0 V/cm. Visualise the products by staining the gel in 1 µg/ml ethidium bromide for 30 minutes and photographed under an ultraviolet transilluminator. The DNA size marker "KB ladder" (Gibco BRL) can be used for estimating the size of the DNA strands.

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