Castanea (Sweet chestnut) & *Castanopsis* (Chinquapin)

Post-Entry Quarantine Testing Manual

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Ministry of Agriculture and Forestry
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Castanea & *Castanopsis* Post-Entry Quarantine Testing Manual

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1. SCOPE

The scope of this manual is limited to nursery stock (whole plants, dormant and non-dormant cuttings, and plants in tissue culture), seed for sowing and pollen of *Castanea* and *Castanopsis* species permitted entry into New Zealand as listed in the Ministry of Agriculture and Forestry's (MAF) Plants Biosecurity Index (see: http://www1.maf.govt.nz/cgi-bin/bioindex/bioindex.pl). At the date of publication of this manual, these species were as follows:

Castanea crenata
Castanea denata
Castanea henryi
Castanea mollissima
Castanea sativa

Castanopsis carlesii
Castanopsis cuspidata
Castanopsis delavayi
Castanopsis orthacantha
Castanopsis tribuloides

This manual describes the testing protocols specified in the import health standards for these commodities. The manual also provides an introduction to the crop and guidance on the establishment and maintenance of healthy plants in quarantine.

2. INTRODUCTION

Chestnuts (*Castanea* spp.) belong to the Fagaceae family and are a close relative of oaks and beech. The related genus, *Castonopsis* (chinquapin, chinkapin), belongs to the same family and consists of about 120, mainly evergreen tree species which are native to North America, and tropical and subtropical Asia. Most species are used for timber and some species (e.g. *Castanopsis chrysophylla*) produce edible nuts.

The chestnut tree is a large, relatively fast-growing deciduous hardwood, similar in appearance to oak. Male and female flowers are found on the same tree but cross-pollination is essential for good fruit set. There are many species but the most common and commercially important are *Castanea sativa* (European or Sweet chestnut), *C. crenata* (Japanese chestnut), *C. mollissima* (Chinese chestnut), and *C. dentata* (American chestnut). There are at least a further 16 species and many species hybridise freely. The nuts produced by the genus *Castanea* should not be confused with those produced by horse chestnuts (*Aesculus* spp.) or the tubers formed by water chestnuts (*Trapa* spp.).

Chestnuts are a temperate zone crop grown worldwide, mostly in the northern hemisphere (Europe, Asia and North America) and to a lesser extent in New Zealand, Australia, Chile, and southern Africa. Global annual production exceeds 400,000 tonnes of which New Zealand produces about 300 tonnes. The main producers (in descending order) are China, Turkey, Korea, Italy, Japan, Spain, Portugal and France (Breisch, 1995). Most of the world production is either *C. sativa*, mainly in Europe, *C. crenata* in Japan and *C. mollissima* in China. *Castanea dentata* used to be a widespread timber and nut tree in North America, but was largely wiped out by chestnut blight (*Cryphonectria parasitica*) over the last century, and most commercial production in America is now from the blight-resistant *C. mollissima*.

Chestnuts are a major food crop in Asia (especially China, Japan and Korea) and Europe (especially France, Italy, Portugal, Turkey and Spain), and are an important source of high quality, durable, hardwood timber and commercially extracted tannins. The nuts themselves are nutritious and gluten-free and are eaten fresh or processed in a variety of forms such as paste, puree, crumb, confectionery, and flour.

It is thought that all four economically important *Castanea* species have been imported into New Zealand in the past. Although *C. sativa* and *C. crenata* can be found throughout the country, no specimens of *C. dentata* or *C. mollissima* seem to have survived, and they do not seem well-suited to the New Zealand climate.

In New Zealand, chestnuts flower from November-January and produce up to 50-100 kg of nuts per tree (2-3 tonnes per hectare) between March and May. All commercial trees are grafted or budded and care must be taken to avoid rootstock incompatibility.

Most commercial chestnut cultivars in New Zealand are selections of trees imported many years ago and many are *C. crenata* × *sativa* hybrids. Three cultivars, '1002', '1005', and '1015', are planted in the vast majority of the estimated 200-300 commercial plantings. Most orchards are in the Auckland, Waikato, and Bay of Plenty but plantings can be found from Northland to Central Otago. Named overseas cultivars have been imported from Asia, Europe and North America, but have generally not performed well under New Zealand conditions.

The priorities for importing *Castanea* germplasm for the New Zealand industry include the importation of cultivars that have nuts which are easier to peel, named cultivars of *C. mollissima*, *C. crenata* and *C. dentata*, for which demand exceeds supply overseas, and cultivars with resistance to fungal root and storage rots.

Further information about chestnuts can be found on the websites of the New Zealand Chestnut Council (www.nzcc.org.nz), Connecticut Agricultural Experiment Station (http://www.caes.state.ct.us/FactSheetFiles/IndexHeadingFiles/FSnut.htm) and the University of Tennessee (www.utc.edu/~jcraddoc/chestnutlinks.html).

3. IMPORT REQUIREMENTS

The import requirements for nursery stock (whole plants, dormant and non-dormant cuttings, and plants in tissue culture) and pollen of *Castanea* and *Castanopsis* are set out in MAF's import health standard "Importation of Nursery Stock" (see: http://www.biosecurity.govt.nz/files/ihs/155-02-06.pdf). Imported nursery stock must meet the general requirements (sections 1-3) and the additional specific requirements detailed in the "*Castanea*" schedule. In summary, an import permit is required and a phytosanitary certificate must accompany all consignments certifying that the nursery stock has been inspected and found to be free of any visually detectable regulated pests, and has been treated for regulated insects and mites (whole plants and cuttings only). Specific additional declarations are also required for *Ceratocystis fagacearum*, *Cryphonectria parasitica*, *Phytophthora ramorum*, and *Xylella fastidiosa*. On arrival in New Zealand, nursery stock must be grown for a minimum period of 3 months in a Level 3 post-entry quarantine facility where it will be inspected, treated and/or tested for regulated pests.

The import requirements for *Castanea* and *Castanopsis* seed for sowing are set out in MAF's import health standard "Importation of Seed for Sowing" (see: http://www.biosecurity.govt.nz/files/ihs/155-02-05.pdf). Imported seed must meet the general requirements (sections 1-2) and the specific requirements detailed in the "*Castanea*" schedule in section 3. In summary, an import permit is required and a phytosanitary certificate must accompany all consignments certifying that the seeds have been inspected and found free of any visually detectable regulated pests. On arrival in New Zealand, the seed must be grown for a minimum period of 3 months in a Level 3 post-entry quarantine facility where it will be inspected, treated and/or tested for regulated pests.

The import requirements for pollen are stated in section 2.2.3 in MAF's import health standard "Importation of Nursery Stock" (see: http://www.biosecurity.govt.nz/files/ihs/155-02-06.pdf) and further details can be found in section 5.3 of this manual.

4. PESTS

The following section lists regulated pests of *Castanea* and *Castanopsis* nursery stock and seed for sowing that require generic or specific measures.

4.1 Regulated pests for which generic measures are required

Insects:

Conotrachelus carinifer	[Nursery stock only]	
Conogethes punctiferalis	[Seed for sowing only]	
Curculio spp.	[Nursery stock & seed for sowing]	Figure 1.1
Cydia spp.	[Seed for sowing only]	
Cyrtepistomus castaneus	[Seed for sowing only]	
Dryocosmus kuriphilus	[Nursery stock only]	Figure 1.2

Fungi:

Amphiporthe castanea	[Nursery stock & seed for sowing]	
Ceratocystis fagacearum	[Nursery stock & seed for sowing]	
Cryphonectria parasitica	[Nursery stock & seed for sowing]	Figures 1.3, 1.4
Helicobasidium mompa	[Nursery stock only]	
Phytophthora ramorum	[Nursery stock only]	Figure 1.5
Sclerotinia pseudotuberosa	[Seed for sowing only]	

Virus:

Chestnut mosaic virus	[Nursery stock only]	Figures 1.6, 1.7, 1.8

Diseases of unknown aetiology:

Chestnut little leaf	[Nursery stock only]
Chestnut yellows	[Nursery stock only]

4.2 Regulated pests for which specific tests are required

Bacterium:

Xylella fastidiosa	[Nursery stock only]

4.3 Regulated pests for which specific tests are optional

Fungus:

Cryphonectria parasitica	[Nursery stock & seed for sowing]	Figures 1.3, 1.4

Phytoplasma:

Chestnut witches' broom ("*Candidatus* Phytoplasma castaneae")	[Nursery stock only]

5. PROPAGATION, CARE AND MAINTENANCE IN POST-ENTRY QUARANTINE

Plants must be maintained in a healthy, vigorous state. *Castanea* and *Castanopsis* plants in quarantine may exhibit symptoms associated with abiotic stresses such as water and nutrient deficiencies. Plants grown in containers can become deficient in magnesium, calcium, manganese and zinc and other trace elements. Symptoms associated with magnesium deficiency are illustrated in Appendix 2. Potting up the plants to allow large root volumes and foliar feeding can help avoid these problems, as can regular re-potting into fresh media.

5.1 Nursery stock

Castanea and *Castanopsis* nursery stock may be imported as whole plants, dormant and non-dormant cuttings, and plants in tissue culture.

5.1.1 Whole plants

To date, whole plants have never been imported into New Zealand as the risk of importing pests and diseases has been judged to be too high. As New Zealand's pest and disease status is considered relatively clean, whole trees of some New Zealand cultivars have been exported bare-rooted and packed in Sphagnum moss to various overseas destinations.

5.1.2 Dormant and non-dormant cuttings

Imported cuttings must be free of fruit, leaves, roots, and soil to minimise the risk of importing pests and diseases. It is best to import *Castanea* and *Castanopsis* cuttings between December to February (from northern hemisphere), and June to July (from southern hemisphere).

Chestnuts cannot easily be rooted from cuttings and this technique of growing chestnut plants has never been practiced in New Zealand. Likewise, this technique is rarely practiced overseas and is usually only used for growing specialist chestnut timber, or for growing rootstock selections that are not well suited to New Zealand conditions.

Chestnuts will usually bud or graft easily. A number of techniques have been successfully used for grafting and budding including, whip-and-tongue grafting, T-budding, and chip-budding. The success rate of budding or grafting is markedly increased by wrapping the scion with plastic food wrap, or similar, to prevent desiccation. However, care must be taken to genetically match the scion with the rootstock to avoid rootstock incompatibility. Taking the scion and the rootstock from the same cultivar reduces the risk of incompatibility but does not eliminate the risk totally. The risk of incompatibility is especially high if the scion originates from a different species of *Castanea* than the rootstock, e.g. using combinations of *C. sativa, C. crenata,* and *C. mollissima.*

Rootstock incompatibility poses a major problem for the importation of chestnut material through quarantine. Since it is unlikely that imported scion wood can be successfully budded or grafted onto a New Zealand rootstock, it may be necessary to first import seed to use as a rootstock, and then import the scionwood 1-2 years later for budding or grafting. Note that rootstocks grown directly from seed either in New Zealand or from imported seed rarely, if ever, produce worthwhile chestnuts.

5.1.3 Plants in tissue culture

Tissue culture plants should ideally be imported in winter (June to July in the southern hemisphere) to allow them to become established prior to summer.

Plantlets to be tested are carefully excised from the tissue culture vessel and washed to remove any remaining agar and planted into pots of planting media containing 50:50 (v/v) pasturised peat:perlite or 50:50 (v/v) peat:vermiculite. The plantlets must be protected from evaporation for approximately three weeks by covering initially with a vented plastic tub or bag. Alternatively, the plants can be misted regularly to keep the planting media moist, and to maintain a high relative humidity. Pots should be placed in bright light, but not direct sunlight during the three weeks. After this period, any coverings should be removed and the plants moved to higher light intensity.

Tissue culture propagation of chestnuts is technically difficult, very expensive, and requires specialised handling. Tissue cultured plants are available in Europe, but only a limited range of cultivars are commercially available.

Tissue culture propagation of chestnut has never been carried out in New Zealand, and to date, none of the commercially available cultivars from overseas have been imported into New Zealand.

5.2 Seed for sowing

At present, the simplest way to produce rootstocks for grafting and budding is to grow seedlings. Seed (i.e. the nuts) should be grown in a loamy growing media in deep pots to allow good taproot growth. Chestnuts are best grown at a pH of around 5 to 6.5. Seedlings grow best in well-drained soil, but require adequate moisture to achieve optimum growth and yields. Chestnut seedlings can tolerate nutrient-poor conditions, but will respond well to an application of a complete balanced fertiliser with nitrogen, potassium and magnesium. Fertiliser should be applied twice, once in early spring and once in mid summer (Lyle, 2006).

Fresh chestnut seed in good condition will germinate readily. Seeds from some cultivars have a much higher germination percentage than others. Also, some cultivars produce a high percentage of multi-embryonic seeds, which can complicate nursery management.

Unprotected seeds are highly perishable and readily loose moisture and viability if they dry out. Viability also reduces as seeds age; most chestnut seed will not retain viability for more than 6-8 months, even under optimum storage conditions. Chestnuts should be stored in a moist environment e.g. in a plastic bag with little ventilation, and under refrigeration at around 0ºC, but do <u>not</u> freeze. Even at 0ºC gradual deterioration can occur over time, especially due to fungal rots.

Chestnut seeds are prone to a number of pests and diseases which can pose a problem for their importation into New Zealand. Imported seed can harbour chestnut blight within the pellicle, which is a thin skin covering the kernel, but no symptoms can be seen externally. Likewise, the seeds can harbour insect pests such as chestnut weevil larvae without visible external damage.

Immediately prior to sowing, nuts should be carefully peeled using a sharp knife to remove the outer shell but without damaging the embryonic shoot tip which lies just beneath the tip of the nut. Once the outer shell is removed, the kernel can be examined for insect damage. To check the pellicle for chestnut blight, shelled nuts should be sown with the pellicles on; this avoids damaging the embryonic shoot tip. Once the seeds have germinated and the seedling has expanded its first true leaves, the remains of the kernel can be removed and plated onto bacterial isolation agar medium. Removing the kernel can have a detrimental effect on the new seedling's growth as its

food supply is being removed. Most seedlings will survive and continue to grow but growth will be slower than if the kernel was still present. An additional season may be required to get the seedlings large enough to act as rootstocks for grafting/budding using imported scionwood.

5.3 Pollen

Anthers can be collected from mature but unopened flowers and dried in warm, light conditions. Following this drying period, pollen should be collected into a centrifuge vial or into gel capsules and stored at 4°C in a sealed container in the presence of a strong desiccant such as calcium chloride.

There are no regulated pests affecting pollen of *Castanea* or *Castanopsis* spp., and pollen may be imported without specific testing or the need for post-entry quarantine.

6. INSPECTION

The inspection requirements for the operator of the facility are set out in the "MAF Biosecurity Authority Standard PBC-NZ-TRA-PQCON" (see: http://www.biosecurity.govt.nz/border/transitional-facilities/plants/pbc-nz-tra-pqcon.htm).

Rootstock incompatibility may be seen quite soon after grafting/budding, or it may take several years to become obvious. The first symptom of delayed incompatibility is a swelling and/or cracking around the graft union. The trees will generally show poor health and slow growth, which sometimes leads to wilting and even tree death. Incompatibility symptoms can be similar to *Phytophthora* root-rot infection, but differ in that the rootstock will produce a lot of suckering below the graft union, and the trees will generally have a healthy root system. In severe cases of incompatibility, the whole top of the tree may simply break off at the graft union.

High temperatures can cause the leaves to partially "roll up" in some cultivars but generally the trees will recover from this naturally. Some relatively new introduced species, especially *C. mollissima* and *C. dentata*, can show unusual "curly leaf" symptoms and stunting and/or dieback of new shoots during spring in some years, especially on the first leaves to emerge. The cause of this is thought to be attributed to cold temperatures and not to pests or diseases.

A more serious disorder reported mostly in Australia and Chile is "bubbly bark" where early spring growth may show a severe and rapid onset of twig/branch die-back associated with characteristic "pimples" or "bubbly bark" on the remaining live wood. "Bubbly bark" can lead to tree death. However, root systems can remain healthy on trees which have had their entire tops killed, but usually extensive and vigorous sucker growth is observed from the rootstock. Young grafted and fast growing apparently healthy trees are the most prone to this problem. Some cultivars are much more susceptible than others. As with "curly leaf" no pest or disease has been proven as the cause and it is suspected that the disorder is related to cold conditions. In a controlled glasshouse environment, it is unlikely that either "curly leaf" or bubbly bark" disorders would be observed.

Inspection of imported chestnut seed is especially difficult because chestnuts may look healthy from the outside yet can harbour insect larvae, internal fungal rots (e.g. chestnut blight) and bacteria. Any soft nuts should be immediately discarded and careful shelling and peeling of the nut will reveal most internal rots (usually visible on the kernel surface, just beneath the pellicle). Infestations of insect larvae can often only be observed if the kernel is cut in half. However, as larvae mature they create an exit hole, at which stage their presence is obvious.

7. TESTING

Each of the specific tests required in the import health standard (as described in section 4 and summarised in Table 1) must be done irrespective of whether plants exhibit symptoms. This testing is required to detect latent infections.

Xylella fastidiosa must be tested for by PCR at the end of the summer using the bud growth from propagated plants. If phytoplasma testing is done, this should also be tested for by PCR at the end of the summer using the bud growth from propagated plants. Detection of both bacteria and phytoplasma requires testing mid-veins from at least two fully expanded leaves. The leaves should be sampled from each of two different branches of the main stem, one a younger leaf and one an older leaf. The sampled leaves from each plant must be bulked together and tested as soon as possible after removal from the host.

If leaf samples have to be stored before testing, the plant material must be kept whole, all surface water removed, and the material stored in a plastic bag at $4^{o}C$ for no more than 7 days. Samples that become partially decayed or mouldy should not be tested, and further samples must be collected.

Chestnut seed can be screened for chestnut blight by sowing shelled nuts with their pellicles on. Once the seeds have germinated and the seedling has expanded its first true leaves, the remains of the kernel can be removed and plated onto bacterial isolation agar medium as described in section .17.2.1.

Table 1: Summary of the regulated pests for *Castanea* indicating the specific tests that are required (■) or optional (★)

Organism type	PCR	Selective media
Fungus		
Cryphonectria parasitica[1]		★
Bacterium		
Xylella fastidiosa	■	
Phytoplasma		
Chestnut witches' broom phytoplasma	★	

[1]Seed for sowing only

7.1 Specific tests for nursery stock

Each plant must be tested separately with the following exceptions, samples from up to 5 plants may be bulked for testing provided that either:

(a) the plants are derived from a single imported dormant cutting which was split into separate cuttings upon arrival in New Zealand, in accordance with the requirements of the permit to import; or

(b) in the case of tissue culture where clonal plants are derived from the same mother plant, and this is confirmed by evidence from the national plant protection organisation in the exporting country.

7.1.1 Molecular assays

PCR **MUST** be carried out for the following bacterium:

- *Xylella fastidiosa*

PCR is **OPTIONAL** for the following phytoplasma:

- Chestnut witches' broom phytoplasma

7.1.1.1 Polymerase chain reaction (PCR)

The following section describes the molecular tests for regulated pests listed on the import health standard for *Castanea*. The recommended published PCR primers for these tests are listed in Table 2 along with plant internal control primers for conventional and real-time PCR. The Gd1/Berg54 internal control primers amplify the 16S rRNA gene from chloroplasts, whereas the COX primers amplify the cytochrome oxidase gene from mitochondria. The inclusion of an internal control assay is recommended to eliminate the possibility of PCR false negatives due to extraction failure, nucleic acid degradation or the presence of PCR inhibitors.

DNA extracted from chestnut contains many PCR inhibitors and it is recommended to purify DNA prior to PCR. An effective method to purify the DNA is to use a MicroSpin™ S-300 HR column following the manufacturer's instructions (GE Healthcare Cat. No. 27-5130-01). Bovine serum albumin (BSA) is included in the PCR reactions which also helps overcome inhibitors.

The PCR reagents listed for the methods described in this section have been tested by the MAF Investigation and Diagnostic Centre – Tamaki. Alternative reagents may give similar results but will require validation.

Table 2: PCR primers used for the detection of regulated pests of *Castanea* and plant internal controls

Target organism	Primer name	Sequence (5´-3´)	Tm (°C)	Expected size (bp)	Reference
Phytoplasmas					
Universal phytoplasma	P1	AAGAGTTTGATCCTGGCTCAGGATT	53	1,800	Deng & Hiruki, 1991
	P7	CGTCCTTCATCGGCTCTT			Schneider *et al.*, 1995
Universal phytoplasma	R16F2	ACGACTGCTAAGACTGG	50	1,248	Lee *et al.*, 1995
	R16R2	TGACGGGCGGTGTGTACAAACCCCG			
Bacterium					
Xylella fastidiosa	RST31	GCGTTAATTTTCGAAGTGATTCGA	55	733	Minsavage *et al.*, 1994
	RST33	CACCATTCGTATCCCGGTG			
	XF-F	CACGGCTGGTAACGGAAGA	62	70	Harper *et al.*, 2010
	XF-R	GGGTTGCGTGGTGAAATCAAG			
	XF-P[1]	FAM-TCGCATCCCGTGGCTCAGCC-NFQ[2]			
Internal control					
Plant DNA control	Gd1	ACGGAGAGTTTGATCCTG	50-62	1,500	Andersen *et al.*, 1998
	Berg54	AAAGGAGGTGATCCAGCCGCACCTTC			
Plant DNA control	COX-F	CGTCGCATTCCAGATTATCCA	60	74	Weller *et al.*, 2000
	COX-R	CAACTACGGATATATAAGAGCCAAAACTG			
	COX-P[1]	FAM-TGCTTACGCTGGATGGAATGCCCT-NFQ[2]			

[1]Real-time probe.
[2]NFQ = Non-fluorescent quencher.

7.1.1.1.1 Phytoplasma PCR

Recommended method

1. Extract total DNA from the petioles and/or mid-veins of leaves sampled from imported plants. Successful PCR amplification can be achieved using the following DNA extraction procedures:
   (a) DNeasy® Plant Mini Kit (Qiagen Cat. No. 69104); or
   (b) a phytoplasma enrichment procedure as described by Kirkpatrick *et al*. (1987) and modified by Ahrens & Seemüller (1992).
   See Appendix 3 for details of these extraction methods. Alternative methods may also be used after validation.
2. Optional: Perform a PCR with the Gd1/Berg54 internal control primers (Table 2) using the components and concentrations listed in Table 3 and cycle under the conditions listed in Table 4.
3. Perform a nested PCR on the DNA using the universal phytoplasma primer pair, P1/P7 (Table 2), for first-stage PCR followed by the R16F2/R16R2 primer pair for the second-stage PCR (Table 2).
4. Set up the first-stage and second-stage PCR reactions using the components and concentrations listed in Table 3 and cycle under the conditions listed in Table 4. The first-stage PCR products, including the controls, are diluted 1:25 (v/v) in water, and 2 µl of the diluted products are used as the template in the second-stage PCR. The following controls must be included for each set of PCR reactions:
   (a) positive control: DNA from any phytoplasma extracted from any host tissue. A cloned fragment of the phytoplasma may also be used. If the internal control primers are not used, then the phytoplasma DNA must be mixed with healthy *Castanea* or *Castanopsis* DNA to rule out the presence of PCR inhibitors; and
   (b) no template control: water is added instead of DNA template. An additional no template control is included in the second-stage PCR.
   When setting up the test initially, it is advised that a negative control (DNA extracted from healthy *Castanea* or *Castanopsis* leaf tissue) is included.
5. Analyse the PCR products (second-stage PCR products only) by agarose gel electrophoresis.

Table 3: Generic conventional PCR reaction components using GoTaq® Green Master Mix

Reagent	**Volume per reaction (µl)**
Sterile H_2O	5.0
GoTaq® Green Master Mix (Promega M7122)	10.0
10 µg/µl Bovine Serum Albumin (BSA) (Sigma A7888)	1.0
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
DNA template	2.0
Total	**20.0**

Table 4: Generic conventional PCR cycling conditions

Step	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	40
Annealing	See Table 2	30 sec	
Elongation	72°C	1 min	
Final elongation	72°C	10 min	1

Interpretation of results
The pathogen-specific PCR test will only be considered valid if:

(a) the positive control produces the correct size product as indicated in Table 2; and
(b) no bands are produced in the negative control (if used) and the no template control.

If the Gd1/Berg54 internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce a 1,500 bp band. Failure of the samples to amplify with the control primers suggests that the DNA extraction has failed, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

Phytoplasma positive controls for PCR
Phytoplasma positive controls (in the form of DNA) may be obtained from the MAF Investigation and Diagnostic Centre – Tamaki (see the Contact Point, section 8). A charge may be imposed to recover costs.

7.1.1.1.1.1 Chestnut witches' broom phytoplasma

It is optional to test plants for Chestnut witches' broom phytoplasma by nested-PCR using the universal primers listed in Table 2. See section 7.1.1.1 for details of test methods and interpretation of results.

7.1.1.1.2 Bacteria PCR

Recommended method: Conventional PCR

1. Extract total DNA from the petioles and/or mid-veins of leaves sampled from imported plants. Successful PCR amplification can be achieved using the DNeasy® Plant Mini Kit (Qiagen Cat. No. 69104). An alternative method may also be used after validation.
2. Optional: Perform a PCR with the Gd1/Berg54 internal control primers (Table 2) using the components and concentrations listed in Table 3 and cycle under the conditions listed in Table 4.
3. Perform a PCR with bacteria-specific primers on the DNA using the components and concentrations listed in Table 3 and cycle under the conditions listed in Table 4. The following controls must be included for each set of PCR reactions:
   (a) positive control: DNA extracted from any host infected with *X. fastidiosa* or from a pure culture of *X. fastidiosa*. If the internal control primers are not used, then the *X. fastidiosa* DNA must be mixed with healthy *Castanea* or *Castanopsis* DNA to rule out the presence of PCR inhibitors; and
   (b) no template control: water is added instead of DNA template.

   When setting up the test initially, it is advised that a negative control (DNA extracted from healthy *Castanea* or *Castanopsis* tissue) is included.
4. Analyse the PCR products by agarose gel electrophoresis.

Interpretation of results for conventional PCR
See section 7.1.1.1.1 for details of interpretation of results.

Recommended method: Real-time PCR

1. Extract total DNA from the petioles and/or mid-veins of leaves sampled from imported plants according to a standard protocol (as described above).
2. Optional: Perform a PCR on the nucleic acid with the COX internal control primers (Table 2) using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6.
3. Set-up the real-time PCR using pathogen-specific primers (Table 2) and the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6. The reaction

and cycling conditions can be changed depending on the real-time reagents and machine used, but this would require validation.

4. The following controls must be included for each set of reactions:
   (a) positive control: DNA extracted from any host infected with *X. fastidiosa* or from a pure culture of *X. fastidiosa*. If the internal control primers are not used, then the *X. fastidiosa* DNA must be mixed with healthy *Castanea* or *Castanopsis* DNA to rule out the presence of PCR inhibitors; and
   (b) no template control: water is added instead of DNA template.

   When setting up the test initially, it is advised that a negative control (DNA extracted from healthy *Castanea* or *Castanopsis* tissue) is included.
5. Analyse real-time amplification data according to the real-time thermocycler manufacturer's instructions.

Table 5: Generic real-time PCR reaction components for DNA templates using Invitrogen Platinum® qPCR SuperMix-UDG

Reagent	**Volume per reaction (µl)**
Sterile H_2O	4.2
2 × Reaction Mix (Invitrogen 11730-017)	10.0
10 µg/µl Bovine Serum Albumin (BSA) (Sigma A7888)	0.6
50 mM $MgCl_2$	0.4
5 µM Forward primer	1.2
5 µM Reverse primer	1.2
5 µM Dual-labelled fluorogenic probe	0.4
DNA	2.0
Total	**20.0**

Table 6: Generic real-time PCR cycling conditions

Step	**Temperature**	**Time**	**No. of cycles**
UDG incubation hold	50ºC	2 min	1
Initial denaturation	95ºC	2 min	1
Denaturation	95ºC	10 sec	40
Annealing and extension	See Table 2	40 sec	

Interpretation of results for real-time PCR

The real-time PCR test will only be considered valid if:

(a) the positive control produces an amplification curve with the pathogen-specific primers; and
(b) no amplification curve is seen (i.e. cycle threshold [C_T] value is 40) with the negative control (if used) and the no template control.

If the COX internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification plot with the internal control primers suggests that the DNA extraction has failed or compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

Bacteria positive controls for PCR

X. fastidiosa positive control (in the form of DNA) may be obtained from the MAF Investigation and Diagnostic Centre – Tamaki (see the Contact Point, section 8). A charge may be imposed to recover costs.

7.1.1.1.2.1 *Xylella fastidiosa*

Plants must be tested for *Xylella fastidiosa* by conventional or real-time PCR using the primers and probe (for real-time PCR) listed in Table 2. See section 7.1.1.1.2 for details of test methods and interpretation of results.

7.2 Specific tests for seed for sowing

Tests are to be carried out on plants germinated from imported seeds.

An optional test is available for the following pathogen:

- *Cryphonectria parasitica*

See Table 1 for the optional test for this pathogen and section .17.2.1 for details of the test methods and interpretation of results.

7.2.1 Fungi isolation on media

It is optional to test for *Cryphonectria parasitica* from *Castanea* and *Castanopsis* seed by isolation of the fungus on agar media. Plants should be tested separately. Aseptic techniques should be used throughout the test procedure.

7.2.1.1 *Cryphonectria parasitica*

The pellicle of *Castanea* seed can harbour *Cryphonectria parasitica*. Shelled nuts should be sown with the pellicles on as described in section 5.2. Once the seeds have germinated and the seedling has expanded its first true leaves, the remains of the kernel can be removed and plated onto agar media.

Recommended method
Surface sterilise the kernel for up to 2 min in a solution of sodium hypochlorite (2-6% active chlorine) and rinse thoroughly in sterile distilled water. Aseptically transfer the kernel onto potato dextrose agar (PDA) and incubate at room temperature for 3-5 days. Alternatively, the kernel can be plated onto 1.5% water agar with 100 µg/ mL^{-1} streptomycin sulphate; sparse mycelium growing from the kernel can be sub-cultured onto PDA. Cultures can be exposed to an alternating cycle of 12 h fluorescent light and 12 h darkness to induce colony pigmentation and pycnidia formation (OEPP/EPPO, 2005).

Interpretation of results
Mycelial growth can be up to 5 mm per day. Slower growth rates are observed for hypovirulent isolates. Mycelium is white when young but changes to yellow, then orange-yellow and finally red-orange to violet after several months. Hypovirulent isolates remain white. Intermediate isolates vary from white to yellow to orange and the pigment remains in the centre on the colony only.

Around 5 days after sub-culturing, conidiomata are produced in diurnal concentric rings which form a dense globose mat over the media. Hypovirulent isolates are characterised by a decreased ability to produce conidiomata and these will be irregularly distributed over the surface of the culture (OEPP/EPPO 2005).

8. CONTACT POINT

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Appendix 1: Symptoms of significant regulated pests of *Castanea*

1.1 *Curculio elephas*

Larva of *Curculio elephas* emerging from a nut of *Castanea sativa.* (Courtesy J. A. Payne; USDA Agricultural Research Service, reproduced from www.forestryimages.org).

1.2 *Dryocosmus kuriphilus*

Developing gall caused by larva of *Dryocosmus kuriphilus* during spring growth. (Courtesy J. A. Payne; USDA Agricultural Research Service, reproduced from www.forestryimages.org).

1.3 *Cryphonectria parasitica*

Bark necrosis on *Castanea sativa* caused by *Cryphonectria parasitica*. (Courtesy A. Kunca; National Forest Centre – Slovakia, reproduced from www.forestryimages.org).

1.4 *Cryphonectria parasitica*

Canker with bark discoloration and orange stroma caused by *Cryphonectria parasitica*. (Courtesy R. L. Anderson; USDA Forest Research Service, www.forestryimages.org).

1.5 *Phytophthora ramorum*

Foliage necrosis on epicormic shoots caused by *Phytophthora ramorum* arising from a mature *Castanea sativa* tree trunk. (Courtesy S. Denman; Forest Research, UK).

1.6 *Chestnut mosaic virus*

Deformed foliage of *Castanea sp*. caused by *Chestnut mosaic virus*. (Courtesy H. Breisch; CTIFL, France).

1.7 *Chestnut mosaic virus*

Apical necrosis of shoots of *Castanea sp*. caused by *Chestnut mosaic virus*. (Courtesy H. Breisch; CTIFL, France).

1.8 *Chestnut mosaic virus*

Vein clearing and deformed foliage on *Castanea crenata* × *sativa* 'Maraval" caused by *Chestnut mosaic virus*. (Courtesy H. Breisch; CTIFL, France

Appendix 2: Symptoms of nutrient deficiencies in *Castanea*

2.1 Magnesium deficiency

Chestnut leaves with interveinal chlorosis and necrotic patches caused by magnesium deficiency.
(Both images courtesy of R. Knowles, New Zealand Chestnut Council).

Appendix 3: Protocols referenced in the manual

3.1 Phytoplasma enrichment extraction method (Kirkpatrick *et al*., 1987 and modified by Ahrens & Seemüller, 1992)

1. Grind 0.3 g leaf petioles and midveins or buds and bark scrapings (1/10; w/v) in ice-cold isolation buffer (0.1 M Na_2HPO_4, 0.03 M NaH_2PO_4, 10% [w/v] sucrose, 2% [w/v] PVP-40, 10 mM EDTA, pH 7.6 to which 0.15% [w/v] bovine serum albumin and 1 mM ascorbic acid were added just before use).
2. Transfer ground sample to a cold 2 ml microcentrifuge tube and centrifuge at 4ºC for 5 min at 4,500 rpm.
3. Transfer supernatant into a new 2 ml microcentrifuge tube and centrifuge at 4ºC for 15 min at 13,000 rpm.
4. Discard the supernatant.
5. Resuspend the pellet in 750 µl hot (55ºC) CTAB buffer (2% [w/v] CTAB, 100 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 1.4 M NaCl, 1% [w/v] PVP-40). It is easier to resuspend the pellet by first adding a small volume of CTAB buffer (e.g. 100 µl) then the remaining volume of CTAB buffer is added once the pellet has been resuspended.
6. Incubate at 55ºC for 30 min with intermittent shaking then cool on ice for 30 seconds.
7. Add 750 µl chloroform:octanol (24:1 v/v), vortex thoroughly and centrifuge at 4ºC or at room temperature for 4 min at 13,000 rpm.
8. Carefully remove upper aqueous layer into a new 1.5 ml microcentrifuge tube.
9. Add 1 volume ice-cold isopropanol, vortex thoroughly and incubate on ice for 4 min. Centrifuge at 4ºC or at room temperature for 10 min at 13,000 rpm. Discard supernatant.
10. Wash DNA pellet with 500 µl ice-cold 70% (v/v) ethanol, centrifuge at 4^oC or at room temperature for 10 min at 13,000 rpm.
11. Dry DNA pellet in a DNA concentrator or air-dry.
12. Resuspend in 20 µl sterile distilled water. Incubating the tubes at 55^oC for 10 min can aid DNA resuspension.
13. Store DNA at -20ºC for short term storage or -80ºC for long term storage.