

National Diagnostic Protocol

Endocronartium harknessii

The cause of pine gall rust



NDP 32 V1

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

<https://www.ippc.int/core-activities/standards-setting/ispms>

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at

<http://plantbiosecuritydiagnostics.net.au/sphd/sphd-reference-standards/>

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for *Endocronartium harknessii* is current as at the date contained in the version control box below.

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Further information

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1 INTRODUCTION

1.1 The Pathogen

The rust fungus *Endocronartium harknessii* is considered the most destructive disease of pine in Canada and the USA (Cannon, 2007). It causes witches' brooms, galls and cankers that are susceptible to secondary wood rotting pathogens. Galls formed on the main stem damage wood quality and lead to stem breakage. Infections on lateral branches are a source of inoculum, but have little effect on wood quality. Only one host is needed for *E. harknessii* to complete its life cycle (autoecious), which consists of only one spore type (microcyclic) (Ramsfield *et al.* 2007, Ramsfield and Vogler 2010, Cannon 2007). The spores are referred to as aeciospores, or aecioid-teliospores, as they have been reported to produce basidia.

1.2 Host range

Endocronartium harknessii occurs on many *Pinus* species in the subgenus *Pinus*, including *P. radiata*, *P. elliotii*, *P. pinaster* and *P. caribaea*, which make up the majority of over one million hectares of Australia's softwood plantations (Gavran and Parsons, 2010). Other known hosts are *P. attenuata*, *P. balfouriana*, *P. banksiana*, *P. canariensis*, *P. contorta*, *P. coulteri*, *P. densiflora*, *P. engelmannii*, *P. halepensis*, *P. insignis*, *P. jeffreyi*, *P. mugo*, *P. muricata*, *P. nigra*, *P. pinaster*, *P. ponderosa*, *P. radiata*, *P. sabiniana*, *P. sylvestris*, *P. taeda* and *P. thunbergiana* (Cannon, 2007).

1.3 Available protocols

Morphology is adequate to identify the genera *Cronartium* and *Endocronartium* when aeciospores are present on galled infections. A molecular test developed by Ramsfield and Vogler (2010) exists for the detection of *E. harknessii* from non-sporulating galls. This test, based on the Intergenic Spacer (IGS) of ribosomal DNA (rDNA), is included in the diagnostic protocol and supersedes another molecular test based on the Internal Transcribed Spacer (ITS) region of rDNA (Ramsfield and Vogler 2004).

2 TAXONOMIC INFORMATION

- Kingdom: Fungi
- Phylum: Basidiomycota
- Class: Pucciniomycetes
- Order: Pucciniales
- Family: Cronartiaceae *sensu* Cummins and Hiratsuka (2003)
- Genus: *Endocronartium*
- Species: *harknessii* (J.P Moore) Y. Hirats.
- Synonyms: *Cronartium harknessii* E. Meinecke
Peridermium cerebroides E. Meinecke
Peridermium harknessii J.P. Moore
- Common names: Western gall rust, pine gall rust

3 DETECTION

3.1 Plant parts affected

Galls caused by *E. harknessii* form on branches and young stems of *Pinus* species. Leaves are not infected.

3.2 Symptom description

Endocronartium harknessii forms well-delimited, spherical to oblong woody galls on branches and stems. Small galls may girdle and kill branches over several years. Galls may grow for up to 4 years and reach 10 cm in diam. before producing powdery yellow-orange spores (aecioid teliospores) from blister-like structures on the gall surface. Witches' brooms are sometimes associated with the infection (Figure 1–3).



Figure 1. Spherical to oblong galls formed on the trunk of *Pinus contorta* var. *latifolia*. Aecioid teliospores are not present on the galls. Image courtesy of Tod Ramsfield, Canada.



Figure 2. Galls formed on *Pinus* sp. producing orange aecioid teliospores, USA. Image courtesy of Andrew Khitsun, USA.



Figure 3. Galls formed on *Pinus* sp. producing orange aecioid teliospores, USA. Image courtesy of Andrew Khitsun, USA.

3.3 Diseases causing similar symptoms

Many gall-forming pine rusts from a closely related genus *Cronartium* have a similar appearance to damage caused by *E. harknessii*. These rusts are heteroecious (require more than one host to complete their life cycle) and form their aecial stage on the stems and cones of *Pinus* spp. The potential spread of *Cronartium* may be limited by the absence of their alternate host, but aecial cankers of species such as *C. ribicola*, *C. quercuum* and *C. coleosporioides* sporulate for several years. *Cronartium quercuum* is morphologically similar to *E. harknessii* in its aecial state and is difficult to distinguish by spore morphology. The uredinial and telial stages of *C. quercuum* infect *Quercus*, which is not present in Australia. The molecular test by Ramsfield and Vogler (2010) differentiates these closely related species.

Rusts in the genus *Peridermium* and other species of *Endocronartium*, such as *E. pini* and *E. sahoanum*, are autoecious pathogens of *Pinus* and differ to *E. harknessii* in their narrower host range and distribution. The spores of *E. pini* are coarsely verrucose and lack smooth areas, whereas spores of *E. harknessii* have smooth regions (Peterson, 1967). *Endocronartium sahoanum* occurs on *P. pumila* in the subgenus *Strobus*, whereas *E. harknessii* occurs on hosts in the subgenus *Pinus*. Rusts in the genus *Coleosporium* also infect *Pinus*, however, they are non-gall forming and occur on pine leaves.

4 IDENTIFICATION

Morphological identification of *E. harknessii* is based on the host plant and size and ornamentation of the aecioid teliospores, ie. spores that look like aeciospores but function as teliospores. If spores are not present on suspect galls, the molecular test developed by Ramsfield and Vogler (2010) can detect *E. harknessii* from infected plant tissue. This molecular test can also distinguish *E. harknessii* from other rusts that infect *Pinus*.

4.1 Morphological methods

The shape and size of the gall cannot be used to differentiate *E. harknessii* from other *Endocronartium* or *Cronartium* species. A morphological inspection of the aecioid teliospores will distinguish *E. harknessii* from other pine rusts, with the exception of *Cronartium quercuum*.

4.1.1 Microscopic identification

Galls on infected *Pinus* species should be inspected for a powdery, yellow-orange spore coating. The aecioid teliospores can be removed from the gall with a needle, scalpel or forceps and mounted on a microscope slide in water or lactic acid. The slide should be heated and then examined with a 100× magnification oil immersion objective to visualise surface ornamentation and spore size.

Aecioid teliospores are 23–35 × 14–24 µm, subglobose to obovoid or ellipsoidal, hyaline, with coarsely verrucose walls and a lateral smooth spot (Figure 4) (from EPPO/CABI, 1996). The aecioid teliospores germinate to produce basidia, which is the main morphological character to distinguish *Endocronartium* from *Cronartium*.

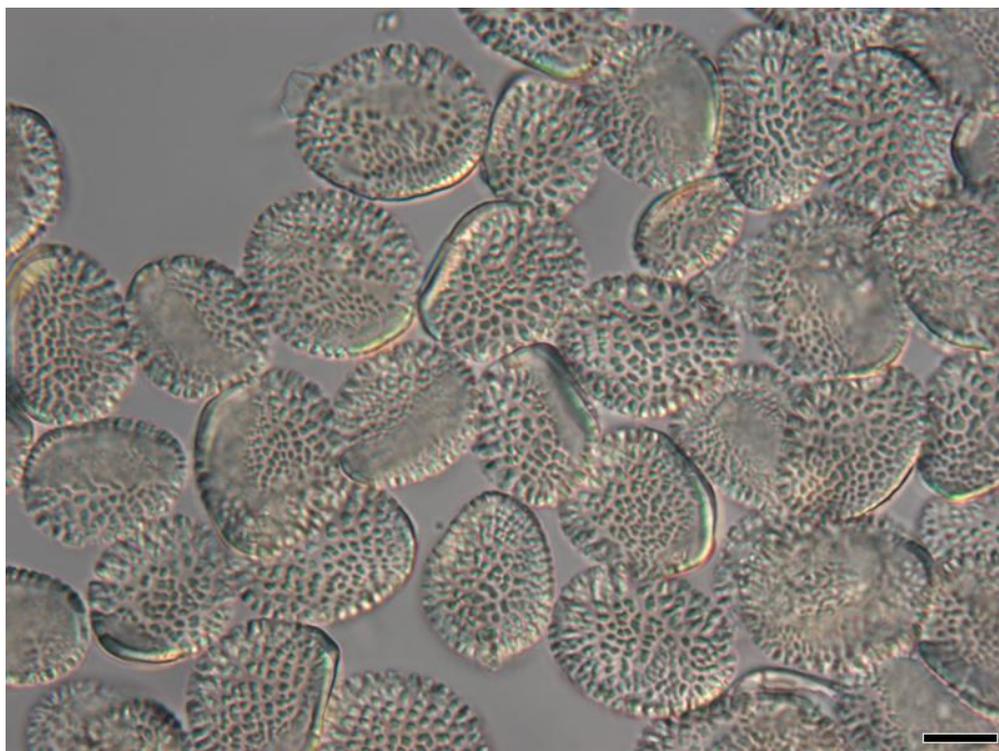


Figure 4. Aecioid teliospores of *Endocronartium harknessii* (ADW-523a). Scale = 10 µm.

4.2 Molecular methods

Two molecular based identification protocols for *E. harknessii* are described. Either approach may be used for successful identification. All PCR product from the diagnostic protocol must be sequenced. The molecular barcode for rust fungi is included as an option for diagnosticians who may have ready access to these primers.

4.2.1 Equipment and reagents

- Thermocycler
- Taq polymerase and PCR components
- Micropipettes and aerosol resistant tips
- Disposable gloves (powder free)
- Gel electrophoresis apparatus

4.2.2 DNA Extraction

Any standard fungal DNA extraction protocol can be used for rust fungi. A recommended protocol for DNA extraction from rust fungi is the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA).

For gall forming rusts, if aeciospores are present on the surface of the gall, spores are located and scraped off using a sterile scalpel. If no sporulation is evident, the outer bark of the rust gall is removed and small pieces of inner tissue are excised. The kit protocol is then followed to completion and DNA is stored at -20°C.

4.2.3 *Endocronartium harknessii* PCR assay

The reaction developed by Ramsfield and Vogler (2010) is based on the Intergenic Spacer (IGS) region of rDNA. It employs primers specific to *E. harknessii* (Phar IGS-1F and Phar IGS-1R), but will also amplify *Cronartium quercuum* f. sp. *banksianae*, a very closely related species not present in Australia. A nested reaction may be performed to increase the sensitivity of the assay and should be used in cases when the suspect material is non-sporulating.

Primers

First reaction

H: '5-CCTCGATGTCGGCTCTTC-3' (Ramsfield and Vogler, 2010)

5B: '5-AGGATTCCC GCGTGGTCCCC-3' (Ramsfield and Vogler, 2010)

Nested reaction

Phar IGS-1F '5-TCATGTGGTCACCCTATC-3' (Ramsfield and Vogler, 2010)

Phar IGS-1R '5-TGATAGGTCCCCACCTC-3' (Ramsfield and Vogler, 2010)

DNA amplification protocol

Two reactions are conducted to increase the specificity of the assay. The first reaction uses the primer pair *H* and *5B* to amplify ~1500 base pairs of the IGS. The second reaction uses *E. harknessii* specific primers to amplify ~200 base pairs from the first reaction.

1. Prepare PCR cocktail on ice in a sterile microcentrifuge tube. This reaction can be performed with any polymerase enzyme according to the manufacturer conditions.

For each sample the cocktail will contain:

PCR buffer (10x)	2.50 μ L (final concentration 1x)
MgCl ₂ (50 Mm)	2.00 μ L (final concentration 2.0mM)
dNTPs (10 mM)	0.50 μ L (final concentration 200 μ M)
Forward primer (10 μ M)	1.25 μ L (final concentration 0.5 μ M)
Reverse primer (10 μ M)	1.25 μ L (final concentration 0.5 μ M)
Taq	0.20 μ L (final concentration 1%)
H ₂ O	16.30 μ L

To prepare the cocktail, multiply the above volumes by the number of samples and add to a single tube. 24 μ L aliquots are then made into 0.2 mL tubes.

2. Add 1 μ L of DNA template to 24 μ L of PCR cocktail
3. Run PCR

Cycle conditions

First reaction with primers *H* and *5B*

Denaturation	94°C for 5 min	x 1 cycle
Denaturation	94°C for 1 min	} x 35 cycles
Annealing	55°C for 1 min	
Extension	72°C for 3 min	
Final extension	72°C for 8 min	x 1 cycle

4. Run a 5 μ L aliquot of this reaction on a 1–1.5% agarose gel to confirm successful amplification. A ~1500 base pair product should be expected for the reaction with *H* and *5B*. If a product is observed proceed to step 6. If weak or no product is observed continue with the following reaction:

Dilute 1 μ L of PCR product in 99 μ L of sterile H₂O. 1 μ L of the dilution is then used as template for the next reaction at step 2.

Nested reaction with *Phar IGS-1F* and *Phar IGS-1R*

Denaturation	94°C for 2 min	x 1 cycle
Denaturation	94°C for 30 sec	} x 30 cycles
Annealing	53°C for 30 sec	
Extension	72°C for 1 min	
Final extension	72°C for 7 min	x 1 cycle

5. Run a 5 μ L aliquot of nested reactions on a 1-1.5 % agarose gel to confirm successful amplification. A ~200 base pair product should be expected for the nested reaction with *Phar IGS-1F* and *Phar IGS-1R*. If there is no amplification from this reaction host DNA should be

amplified with plant specific primers to confirm the result is a true negative and not PCR inhibition.

6. Successful PCR product should be sequenced. An example of a third party sequencing company is Macrogen, Korea. The directions for sample submission of the third party should be followed.
7. Sequences should be analysed using chromatograms from both primers. A comparison of the consensus sequence should be made with sequences of *E. harknessii* on GenBank using a nucleotide BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A 99–100 % sequence identity to *E. harknessii* IGS sequences such as EF579743, EF579744, EF579745 and EF579746, indicates a positive sample.

4.2.4 Molecular barcoding of *Endocronartium harknessii*

Amplified copies of the Large Subunit (LSU) region of rDNA can be sequenced and compared to known sequences on GenBank for identification of rust fungi. The LSU region is more easily sequenced than the ITS region for rust fungi, as the ITS may contain indels that inhibit direct sequencing. The ITS2-LSU region can be amplified with primers *Rust 2INV* and *LR7*. In some cases if a product is not amplified, a nested reaction can be performed using the primers *LR0R* and *LR6*.

Primers

First reaction

Rust 2INV: 5'- GATGAAGAACACAGTGAAA -3' (Aime, 2006)

LR7: 5'- TACTACCACCAAGATCT -3' (Vilgalys and Hester, 1990)

Nested reaction

LR0R: 5'- ACCCGCTGAACTTAAGC -3' (Vilgalys and Hester, 1990)

LR6: 5'- CGCCAGTTCTGCTTACC -3' (Vilgalys and Hester, 1990)

DNA amplification protocol

1. Prepare PCR cocktail on ice in a sterile microcentrifuge tube. This reaction can be performed with any polymerase enzyme according to the manufacturer conditions.

For each sample the cocktail will contain:

PCR buffer (10x)	2.50 µL (final concentration 1x)
MgCl ₂ (50 Mm)	2.00 µL (final concentration 2.0 mM)
dNTPs (10 mM)	0.50 µL (final concentration 200 µM)
Forward primer (10 µM)	1.25 µL (final concentration 0.5 µM)
Reverse primer (10 µM)	1.25 µL (final concentration 0.5 µM)
Taq	0.20 µL (final concentration 1 %)
H ₂ O	16.30 µL

To prepare the cocktail, multiply the above volumes by the number of samples and add to a single tube, 24 µL aliquots are then made into 0.2 mL tubes.

2. Add 1 µL of DNA template to 24 µL of PCR cocktail

3. Run PCR

Cycle conditionsFirst reaction with primers *Rust 2INV* and *LR7*

Denaturation	94° for 4 min	x 1 cycle
Denaturation	94° for 30 sec	} x 40 cycles
Annealing	57° for 1 min	
Extension	72° for 1.5 min	
Final extension	72° for 7 min	x 1 cycle

4. Run a 5 µL aliquot of this reaction on a 1–1.5 % agarose gel to confirm successful amplification. A ~1200 base pair product should be expected for the reaction with *Rust 2INV* and *LR7*. If a product is observed proceed to step 6. If weak or no product is observed continue with the following reaction:

Dilute 1 µl of PCR product in 99 µl of sterile H₂O. 1 µl of the dilution is then used as template for the next reaction at step 2.

Nested reaction with *LROR* and *LR6*

Denaturation	94° for 2 min	x 1 cycle
Denaturation	94° for 30 sec	} x 45 cycles
Annealing	59° for 30 sec	
Extension	72° for 1.5 min	
Final extension	72° for 7 min	x 1 cycle

5. Run a 5 µL aliquot of the nested reactions on a 1–1.5% agarose gel to confirm successful amplification. A ~1000 base pair product should be expected for the nested reaction with *LROR* and *LR6*.
6. Successful PCR product should be sequenced. An example of a third party sequencing company is Macrogen, Korea. The directions for sample submission of the third party should be followed.
7. Sequences should be determined using chromatograms from both primers. A comparison of the sequence should be made with sequences of *E. harknessii* on GenBank using a nucleotide BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A 99–100% sequence identity to *E. harknessii* LSU sequence AF522175 or AY700193 with a maximum of five base pair differences indicates a positive sample.

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