**Phlyctema vagabunda** is the main causal agent of apple bull’s eye rot in New Zealand

Kerry R. Everett<br>Shamini I.P.S. Pushparajah<br>Michelle J. Vergara<br>Luna Hasna<br>Peter N. Wood<br>Brent M. Fisher

Abstract

Bull’s eye rot is a postharvest storage disease of apples. Until now the cause of this disease in New Zealand was not clear. A survey of 6880 apples from five regions of New Zealand over two seasons was conducted. *Neofabraea* *malicorticis* and *N. perennans* were not found. One hundred and seventy-nine isolates were identified as *Phlyctema vagabunda* by specific polymerase chain reactions and/or sequencing the β-tubulin gene region followed by phylogenetic analysis. Two isolates were identified as *N. kienholzii*. Previous records of the presence of *N. malicorticis* and *N. perennans* in New Zealand were based on spore morphology and presence in pruning wound cankers. There is overlap in spore morphology for *N. malicorticis*, *N. perennans* and *P. vagabunda*, accounting for misidentifications. Based on our results it is likely that previous records were *P. vagabunda*, which can also infect pruning wounds.

Keywords *Phlyctema vagabunda* · *Neofabraea alba* · Lenticel rot · Postharvest · β-tubulin

Introduction

Bull’s eye rot is a postharvest storage rot of apples. Circular, sunken, brown lesions form on fruit, usually after prolonged cool storage (Creemers 2014). Lesions have a lighter brown or tan centre (Spotts 2014). Concentrically arranged grey or brown acervuli form in the centre of older lesions (Creemers 2014; Spotts 2014). Lesions also occur around lenticels, and around the stem or the calyx (Spotts 2014).

Grove (1990) attributed the causal organisms of apple bull’s eye rot as *Pezicula malicorticis* and *Neofabraea perennans*. Verkley (1999) recognised *Neofabraea* instead of *Pezicula* as the correct name for this group of fungi, and proposed three species causing apple fruit rots: *N. alba* syn. *Pe. alba*, anamorph *Phlyctema vagabunda*, *N. malicorticis* syn. *Pe. malicorticis*, anamorph *Cryptosporiopsis curvispora* and *N. perennans* syn. *Pe. perennans*, anamorph *Cryptosporiopsis perennans*. The fourth species (*N. krawtzewii* syn. *Pe. krawtzewii* anamorph *Cryptosporiopsis* sp.) is a saprotroph of poplars.

A later molecular investigation showed that when the β-tubulin region was used, five clades were separated: *N. perennans*, *N. malicorticis*, *N. alba*, *N. krawtzewii*, and an unnamed clade (de Jong et al. 2001). This clade was later assigned the name *Cryptosporiopsis kienholzii* (Spotts et al. 2009), and finally *Neofabraea kienholzii* (Seifert 2013; Chen et al. 2016). There was some doubt expressed as to whether there was sufficient difference in morphology and sequence between *N. perennans* and *N. malicorticis* to justify the assignment of species names, as these two species have identical ITS sequence. De Jong et al. (2001) argued that, because there were no instances of vegetative compatibility between the two species and there were no intermediate types, that separate species names should be assigned. *N. kienholzii* is separate from *N. alba* and *N. malicorticis/ perennans* clades when the ITS region was used for phylogenetic analysis (de Jong et al. 2001).

The division of the *Neofabraea* fungi into five species was accepted in subsequent literature in which polymerase chain reaction (PCR) primers based on the β-tubulin gene
were designed to discriminate these species (Gariepy et al. 2005; Soto-Alvear et al. 2013).

A revision of these and related fungi (Chen et al. 2016) based on multi-locus phylogenetic analysis using ITS, LSU, rpb2 and tub2 gene regions has split Neofabraea alba into a separate clade from these other species (N. perennans, N. malicorticis, N. kienholzii, N. krawzewii), and to a different genera, and it was renamed Phlyctema vagabunda.

In New Zealand, a storage rot of apples was first noticed in 1923, and named ‘Delicious spot’ (Cunningham 1925). The causal fungus was not known, and was later morphologically identified by Brien (1932) as N. perennans. This was followed by a report of ripe spot caused by N. malicorticis (Brien 1934). Eight years later ripe spot was reported to occur in all apple growing districts of New Zealand (Taylor and Brien 1943).

The early New Zealand descriptions of N. perennans and N. malicorticis fit the criteria for conidial morphology of Verkley (1999). Rots were caused almost equally by N. perennans and a fungus with curved conidia, but hundreds of cankers yielded a fungus with straight spores and only two a fungus with curved spores (Brook 1957). However, Brook (1957) pointed out that there was overlap in conidial morphology between N. malicorticis and another described species that also causes fruit rots on apples, N. alba (Osterwalder 1907; Wilkinson 1943; Brook 1957) suggested that the only reliable means of distinguishing these two fungi, which both produce curved conidia, in contrast to the straight conidia of N. perennans, was whether they colonise apple branches and twigs to form cankers (Kienholz 1939; Corke 1955). Based on morphology, Brook (1957) concluded that the fungus with curved spores isolated from fruit was N. alba, and goes on to suggest that previous identifications of N. malicorticis (Taylor and Brien 1943) were also probably N. alba.

Phylogenetic analysis of the sequence of the inter-transcribed spacer region (ITS) of ribosomal DNA of cultures from apples deposited in ICMP (International Collection of Microorganisms from Plants, Landcare Research, Auckland) as N. alba and N. malicorticis confirmed the identifications of N. alba, but the single N. malicorticis culture was identical to a fungus from kiwifruit, N. actinidiae (Johnston et al. 2004). Whether it was isolated from apple, or whether it was incorrectly ascribed to apple but was isolated from kiwifruit, is not known. There were no vouchedered specimens available for N. perennans isolated from apple in New Zealand. Based on these results and unpublished isolation records, Johnston et al. (2005) proposed that N. malicorticis and N. perennans may not ever have been present in New Zealand.

The present study aims to investigate the causal agent of apple bull’s eye rot in New Zealand.

Materials and methods

A sample of 1879 rotten apples from long term stored (0.5 ± 0.5 °C) industry library trays from the North (1096) and South Island (799) apple growing regions of New Zealand were transported to Mt Albert Research Centre (Auckland) and placed in a cool store. Apples were inspected for bull’s eye rots and isolations made between 26/9/2013 and 16/10/2013.

Five thousand one hundred apples harvested from three commercial orchards in the North Island were placed in the cool store (0.5 ± 0.5 °C) during the 2018/19 season. Apples were inspected and isolations from 100 apples showing symptoms of bull’s eye rot were made between 28/6/2019 and 28/10/2019.

The external surface of apples were wiped with 70% ethanol and small tissue pieces from rots were excised from the margin between rotten and healthy tissue and placed on Difco® potato dextrose agar (PDA) in Petri plates under fluorescent lights at 20 °C, 12 h light/12 h dark. After 2–3 weeks’ growth on PDA at room temperature, isolated fungi were identified as P. vagabunda by colony and spore morphology. In 2013 a selection of 98 isolates and in 2019 a further selection of 83 isolates identified by morphology as P. vagabunda were single-spore cultured onto PDA. Resultant cultures were stored as agar plugs (5 mm²) in 50% glycerol in cryotubes at -80 °C until required.

Cultures retrieved from agar plugs stored in the –80 °C were grown for 21 days on PDA in Petri plates at room temperature under lights (12:12 day/night cycle). Mycelium was scraped from the surface using a sterile bent glass rod and re-suspended in 250 µL Wizard™ SV Lysis buffer in a 2 mL safe-lock micro-centrifuge tube. Three 0.2 mm silicon beads (Qiagen©) were added to each sample, then tubes were placed evenly into the TissueLyser Adapter II (Qiagen©) set and homogenised for 1.5 min at 1800 oscillations/min after which the rest of the Promega Wizard™ Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) protocol was followed. Purified DNA concentration was determined using a NanoDrop® ND-1000 spectro-photometer (Biolab, Victoria, Australia) and stored at -20 °C.

Initial PCR screening was conducted for 98 isolates in 2013. Reactions were in a total volume of 25 µL/well, consisting of 2 µL of 10 ng DNA, 2.5 µL Taq polymerase buffer, 0.5 µL 10 mM dNTP, 0.75 µL 50mM Mg Cl₂, 0.5 µM of each forward and reverse primers (Neo alba-up and Neo_alba-loTub-439; Gariepy et al. (2003); Soto-Alvear et al. (2013)), 0.125 µL Platinum Taq polymerase (Invitrogen) and 18.875 µL Milli-Q® purified water. PCR reactions were conducted in a thermocycler (Technne®) under the following conditions: initial denaturation at 96 °C for 3 min, 35 cycles of 96 °C for 3 min., 60 °C for 1 min. and 72 °C for 2 min,
Phlyctema vagabunda is the main causal agent of apple bull’s eye rot in New Zealand and curved spores were also shown to be a characteristic of Phlyctema vagabunda (CBS 109875, CBS 304.62) with 1000 bootstrap replications and the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest likelihood is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites. This analysis involved 107 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding using the partial deletion model. There were a total of 741 positions in the final dataset.

### Results

Results showed a product of the expected size (358 bp) amplified using the Neo alba-up and Neo alba-loTub-439 primers from the DNA extracted from 89 of the 98 isolates in 2013.

Phylogenetic analysis of the β-tubulin gene region of 99 isolates showed that all except two isolates were in the same clade as *P. vagabunda*. These two isolates were in the *N. kienholzii* clade (Fig. 1; Table 1). No sequences were in the same clade as *N. malicorticis* or *N. perennans* (Fig. 1).

Three small clades were separated at >60% level of probability, but BLAST analysis did not find any named fungal species more similar to these sequences than *P. vagabunda*.

### Discussion

These results suggests that Brook (1957) was correct when he stated that the isolates with curved spores resulting from a survey of New Zealand apples were not *N. malicorticis*, but were instead *P. vagabunda*. Similarly Johnston et al. (2005) also stated that *N. malicorticis* was probably not ever present in New Zealand, and that all isolates were probably *P. vagabunda*.

Brook (1957) distinguished *N. perennans* from *N. malicorticis* on the basis of spore curvature, assuming straight spores were *N. perennans* and curved spores were *N. malicorticis*. The cankers from which *N. perennans* were reported in Brook (1957) were predominantly at pruning wounds and damaged fruit spurs, of which the former has also been shown to be a characteristic of *P. vagabunda* (Creemers 2014). *N. perennans* macro-conidia are described

### Table 1: Isolate names, country of origin, host and GenBank code used in phylogenetic analysis of the β-tubulin gene region

| Isolate name | Fungus | GenBank code | Country | Host | Reference |
|--------------|--------|--------------|---------|------|-----------|
| CBS 175.74   | Infundi-chalara microchona | KR859284 | Netherlands | Pinus sylvestris | Chen et al. 2016 |
| CBS 121403   | Neofabraea actinidiae | KR859285 | New Zealand | Actinidia delicosa | Chen et al. 2016 |
| CBS 126461   | Neofabraea kienholzii | KR859288 | USA | Malus domestica cv. Fuji | Chen et al. 2016 |
| CBS 102863   | Neofabraea malicorticis | KR859290 | Canada | Malus | Chen et al. 2016 |
| CBS 275.29   | Neofabraea perennans | KR859292 | unknown | Malus sylvestris | Chen et al. 2016 |
| CBS 123727   | Neofabraea vincetoxicci | KR859312 | Czech | Vincetoxicum hirundinarium | Chen et al. 2016 |
| CBS 109875   | Phlyctema vagabunda | KR866088 | USA | Fraxinus americana | Chen et al. 2016 |
| CBS 304.62   | Phlyctema vagabunda | KR866092 | South Africa | Malus sylvestris | Chen et al. 2016 |
| KE171        | Phlyctema vagabunda | ON009248 | New Zealand | Malus sylvestris | Present study |
| KE2794       | Phlyctema vagabunda | ON009249 | New Zealand | Malus sylvestris | Present study |
| KE890        | Neofabraea kienholzii | ON009250 | New Zealand | Malus sylvestris | Present study |
| KE915        | Neofabraea kienholzii | ON009251 | New Zealand | Malus sylvestris | Present study |

1 Type culture
by Verkley (1999) as straight or weakly curved, those of P. vagabunda by Chen et al. (2016) as mostly straight and by Verkley (1999) as weakly to strongly curved. It is possible that P. vagabunda could be confused with N. perennans if conidial morphology and isolation from pruning wounds were the major criteria for identification as there is some overlap in spore morphology from these descriptions. Therefore it is possible that N. perennans was also never present in New Zealand. During a later survey of apple fruit rots only N. malicorticis (Taylor and Brien 1943) was isolated, which was probably P. vagabunda based on the present results.

P. vagabunda was first recorded in 1847 from France (Desmazières 1847), and it was first reported from North America c. 150 years later (Gariepy et al. 2005), suggesting that its origin was Europe. Indeed, the most common bull’s eye rot pathogen found in Continental Europe is Phlyctema vagabunda, but it is a minor pathogen in North America.
Phlyctema vagabunda is the main causal agent of apple bull’s eye rot in New Zealand

(Spadaro et al. 2020). *P. vagabunda* is also found in Australia (Cunnington 2004), Chile (Henriquez 2005; Soto-Alvear et al. 2013), Japan (Sato et al. 2021), New Zealand (Johnston et al. 2005) and South Africa (Den Breeyden et al. 2020).

Since the discovery of a new species of *Neofabraea* from apples in the Pacific North West USA (de Jong et al. 2001; Spotts et al. 2009), *N. kienholzii* has been reported from apples in Australia (Cunnington 2004), Canada (de Jong et al. 2001), the Czech Republic (Pesciova et al. 2017), Ecuador (Valdez Tenezaca 2020), Italy (Neri et al. 2018), the Netherlands (Weneker et al. 2017, 2018), Poland (Michalecka et al. 2016), Portugal (Lin et al. 2018), Taiwan (Zhang et al. 2019) and the United Kingdom (Kingsnorth et al. 2017). *N. kienholzii* has also been reported from olives in California (Trouillas et al. 2019), and from grapes in Hungary (Lengyel et al. 2020).

Based on re-examination of the literature, isolations, PCR tests, sequencing the β-tubulin gene region, and phylogenetic comparisons with known strains, there is no evidence that any species other than *N. kienholzii* and *P. vagabunda* have ever been present in New Zealand causing bull’s eye rot on apples, and that *P. vagabunda* is most common.

Acknowledgements The authors thank the Apple Futures II Partnership Programme (PNZEA1401) funded by the Ministry for Business, Innovation and Employment (MBIE) and New Zealand Apples and Pears Incorporated (NZAPI) for funding, and apple growers for allowing access and providing orchards and fruit. Thanks to Mike Manning for assisting with morphological identifications.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions

Declarations

Conflict of interest The authors declare no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Brien RM (1932) “Delicious spot” on apples due to *Gloeosporium perennans*. NZ J Agric 45:215–218

Brien RM (1934) The fungi causing rots of stored apples in New Zealand. NZ J Agric 48:143–149

Brook PJ (1957) Ripe spot of apples in New Zealand. NZ J Sci and Tech A38:735–741

Chen C, Verkley GJM, Sun GY, Groenewald JZ, Crous PW (2016) Redefining common endophytes and plant pathogens in *Neofabraea*, *Pezicula*, and related genera. Fungal Biol 120:1291–1322

Corke ATK (1955) Bitter rot of apples: I. Branch inoculations with *Gloeosporium perennans* and *G. album*. In: Ann Rep Long Ashton Agric Hort Res Sta 1954. pp 164–168

Creemers P (2014) Anthracnose canker and Perennal canker. In: Compendium of apple and pear diseases and pests. Second edition. APS Press, St. Paul, Minnesota, pp 51–53

Cunningham GH (1925) Fungous diseases of fruit-trees in New Zealand and their remedial treatment. The Brett Printing and Publishing Company, Ltd., Auckland, New Zealand

Cunningham JH (2004) Three *Neofabraea* species on pome fruit in Australia. Australas Plant Pathol 33:453–454

de Jong SN, Levesque CA, Verkley GJM, Abeln ECA, Rahe JE, Braun PG (2001) Phylogenetic relationships among *Neofabraea* species causing tree cankers and bull’s-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the beta-tubulin gene. Mycol Res 105:658–669

Den Breeyden A, Rochofert J, Russouw A, Meitz-Hopkins J, Lennox CL (2020) Preharvest Detection and Postharvest Incidence of *Phlyctema vagabunda* on ‘Cripps Pink’ Apples in South Africa. Plant Dis 104:841–846

Desmazières BJBH (1847) Quatorzième notice sur les plantes cryptogames récemment découvertes en France [Notice #14]. Ann Sci Nat Bot 3e:9–37

Gariepy TD, Levesque CA, de Jong SN, Rahe JE (2003) Species specific identification of the *Neofabraea* pathogen complex associated with pome fruits using PCR and multiplex DNA amplification. Mycol Res 107:528–536

Gariepy TD, Rahe JE, Levesque CA, Spotts RA, Sugar DL, Henriquez JL (2005) *Neofabraea* species associated with bull’s-eye rot and cankers of apple and pear in the Pacific Northwest. Can J Plant Pathol 27:118–124

Henriquez JL (2005) First report of apple rot caused by *Neofabraea alba* in Chile. Plant Dis 89:1360

Johnston PR, Manning MA, Meier X, Park D, Fullerton RA (2004) *Cryptosporiopsis actinidae* sp. nov. Mycotax 89:131–136

Johnston PR, Pennycook SR, Manning MA (2005) Taxonomy of fruit-rotting fungal pathogens: what’s really out there? NZ Plant Prot 58:42–46

Kienholz JR (1939) Comparative study of the apple anthracnose and perennial canker fungi. J Agric Res 59:635–666

Kingsnorth J, Perrine J, Berrie A, Saville R (2017) First report of *Neofabraea kienholzii* causing bull’s eye rot of apple in the UK. New Dis Rep 36:15

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35:1547–1549

Lengyel S, Knapp DG, Karacsony Z, Geml J, Tempfli B, Kovacs GM, Vaczy KZ (2020) *Neofabraea kienholzii*, a novel causal agent of grapevine trunk diseases in Hungary. Eur J Plant Pathol 157:975–984

Lin HJ, Jiang X, Yi JP, Wang XG, Zuo RL, Jiang ZD, Wang WF, Zhou EX (2018) Molecular identification of *Neofabraea* species associated with bull’s-eye rot on apple using rolling-circle amplification of partial EF-1 alpha sequence. Can J Microbiol 64:57–68

Michalecka M, Bryk H, Poniatowska A, Pulawska J (2016) Identification of *Neofabraea* species causing bull’s eye rot of apple in Poland and their direct detection in apple fruit using multiplex PCR. Plant Pathol 65:643–654

Neri F, Cameldi I, Menghini M, Pirondi A, Nanni IM, Collina M, Mari M (2018) *Neofabraea* spp. causing apple bull’s eye rot:
identification and characterization of some Italian isolates. IOBC/WPRS Bull 138:55–58

Osterwalder A (1907) Zur Gloeosporium-Fäule des Kernobstes. Cbl Bakt 18:825–827

Pescicova K, Kolarik M, Hortova B, Novotny D (2017) Diversity and identification of Neofabraea species causing bull’s eye rot in the Czech Republic. Eur J Plant Pathol 147:683–693

Saito N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sato Y, Hirayama K, Toda T, Nara C, Furuya H (2021) Previously unreported symptom of bull’s-eye rot (Kigusare-byo) on apple fruits caused by Phlyctema vagabunda Desm. J Phytopathol 87:133–145

Seifert KA (2013) Neofabraea kienholzii (Seifert, Spotts & Levesque). Nomenclatural novelties. Index Fung 18:1

Soto-Alvear S, Lolas M, Rosales IM, Chavez ER, Latorre BA (2013) Characterization of the bull’s eye rot of apple in Chile. Plant Dis 97:485–490

Spadaro D, Torres R, Errampalli D, Everett K, Ramos L, Mari M (2020) Chap. 2. Pome fruits. In: Palou L, Smilanick JL (eds) Postharvest pathology of fresh horticultural produce. CRC Press, Boca Raton, Florida, USA, pp 55–110

Spotts RA, Seifert KA, Wallis KM, Sugar D, Xiao CL, Serdani M, Henriquez JL (2009) Description of Cryptosporiopsis kienholzii and species profiles of Neofabraea in major pome fruit growing districts in the Pacific Northwest USA. Fungal Biol 113:1301–1311

Spotts RA (2014) Postharvest diseases: Bull’s-Eye Rot. In: Sutton TB, Aldwinckle HS, Agnello AM, Walgenbach JF (eds) Compendium of Apple and Pear Diseases. 2nd edition. American Phytopathological Society, St. Paul, MN, pp 78–79

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512–526

Taylor GG, Brien RM (1943) Ripe-spot of apples (Neofabraea malicorticis). NZ J Sci Tech A25:63–72

Trouillas FP, Nouri MT, Lawrence DP, Moral J, Travadon R, Aegerter BJ, Lightle D (2019) Identification and characterization of Neofabraea kienholzii and Phlyctema vagabunda causing leaf and shoot lesions of olive in California. Plant Dis 103:3018–3030

Valdez Tenezaca AV (2020) Neofabraea kienholzii isolate apple internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. MT644347, 17 June 2020 edn., www.ncbi.nlm.nih.gov

Verkley GJM (1999) A monograph of the genus Pezicula and its anamorphs. In: Stud Mycol. p 180

Wenneker M, Pham KTK, Boekhoudt LCEa (2017) First report of Neofabraea kienholzii causing bull’s eye rot on pear (Pyrus communis) in the Netherlands. Plant Dis 101:634

Wenneker M, Pham K, Leeuwen Pv (2018) Emerging and threatening postharvest diseases in pome fruit in the Netherlands. IOBC/WPRS Bull 138:90–92

Wilkinson EH (1943) Bitter rot of apples caused by Gloeosporium album Osterw., with special reference to the variety Allington Pippin. Rep Agric Hort Res Sta Bristol 1943:81–89

Zhang H, Li X, Duan L, Xu Y, Chen X, Duan W (2019) Isolation and identification of Neofabraea kienholzii on Malus pumila imported from Taiwan Province. J Plant Prot 46:90–96

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.