Virology 499 (2016) 203–211

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Novel Endorna-like viruses, including three with two open reading frames, challenge the membership criteria and taxonomy of the *Endornaviridae*

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ARTICLE INFO

Article history:
Received 7 June 2016
Returned to author for revisions
11 August 2016
Accepted 19 August 2016

Keywords:
Ceratobasidium
Endornavirus
Indigenous virus
Orchid mycorrhizae
Mycovirus
Virus taxonomy
Wild plant virology

ABSTRACT

Viruses associated with wild orchids and their mycorrhizal fungi are poorly studied. Using a shotgun sequencing approach, we identified eight novel endornavirus-like genome sequences from isolates of *Ceratobasidium* fungi isolated from pelotons within root cortical cells of wild indigenous orchid species *Microtis media*, *Pterostylis sanguinea* and an undetermined species of *Pterostylis* in Western Australia. They represent the first endornaviruses to be described from orchid mycorrhizal fungi and from the Australian continent. Five of the novel endornaviruses were detected from one *Ceratobasidium* isolate collected from one *Pterostylis* plant. The partial and complete viral replicases shared low (9–30%) identities with one another and with endornaviruses described from elsewhere. Four had genome lengths greater than those of previously described endornaviruses, two resembled ascomycete-infecting endornaviruses, and unlike currently described endornaviruses, three had two open reading frames. The unusual features of these new viruses challenge current taxonomic criteria for membership of the family *Endornaviridae*.

1. Introduction

Members of genus *Endornavirus* (family *Endornaviridae*) are non-encapsidated viruses with double-stranded (ds) RNA genomes. The genomes of described members range from 9 kb to 17.6 kb (Fukuhara and Gibbs, 2012), and there is always only one open reading frame (ORF) encoding a replicase. Current species are distinguished on the basis of host, genome size and organization, and nucleotide sequence variations. The nucleotide identities of different endornavirus species range from 30–75% identity (Fukuhara and Gibbs, 2012). The first endornaviruses were described from broad bean (*Vicia faba*), where the occurrence of large dsRNAs was linked to cytoplasmic male sterility (Grill and Garger, 1981). Endornaviruses have since been identified from plants, e.g. rice (*Oryza sativa*) (Moriyama et al., 1995) and capsicum (*Capsicum annuum*) (Valverde et al., 1990), fungi, e.g. *Helicobasidium mompa* (Osaki et al., 2006) and *Tuber aestivum* (Stielow et al., 2011), and oomycetes – e.g. *Phytophthora* sp. (Hacker et al., 2005).

Currently, there are seven fungus-infecting, nine plant-infecting and one oomycete-infecting endornaviruses described, of which 12 have been ratified by the International Committee on Taxonomy of Viruses (ICTV) (International Committee on Taxonomy of Viruses, 2015, 2016). Endornavirus clades Alphaendornavirus (Clade I) and Betaendornavirus (Clade II) are proposed (Khalifa and Pearson, 2014) but not ratified by the ICTV. This classification reflects relationships of active domains within the ORF (Khalifa and Pearson, 2014), which can include two or more of the following: helicase (Hel), methyltransferase (MTR), glucosyltransferase (GT) and RdRp (Roossinck et al., 2011; Fukuhara and Gibbs, 2012). The number and combination of domains differ between species, with only the RdRp common to all endornaviruses (Roossinck et al., 2011; Fukuhara and Gibbs, 2012). With the exception of Persea americana endornavirus 1 (Villanueva et al., 2012), all endornaviruses also encode a helicase domain. Current members of Alphaendornavirus have larger genomes ($>13{,}000$ bp) and include viruses from basidiomycetes, oomycetes, and plants. Members of Betaendornavirus have smaller genomes that range from 9760 bp (TaEV) to 10,513 bp (SsEV1) that lack a GT domain, and they infect ascomycetes (Steilow et al., 2011;

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http://dx.doi.org/10.1016/j.virol.2016.08.019
0042-6822/

Khalifa and Pearson, 2014).

Members of the *Endornaviridae* persist in their hosts over multiple generations (Roossinck, 2010; Roossinck et al., 2011). Infection with the majority of endornaviruses does not appear to negatively influence the growth and development of the host (Grill and Garger, 1981; Pfeiffer, 1998; Ikeda et al., 2003; Osaki et al., 2006; Roossinck, 2015). There is no evidence to support horizontal transmission of endornaviruses to other hosts; the lack of movement protein indicates absence of ability to move from cell to cell (Roossinck et al., 2011; Fukuhara and Gibbs, 2012). In plants they rely on vertical transmission through infected pollen and ova (Valverde and Gutierrez, 2007; Okada et al., 2011, 2013). In fungal hosts, they transmit vertically via spores and horizontally via hyphal anastomosis (Ikeda et al., 2003; Tuomivirta et al., 2009). Endornaviruses occur in all cells of studied hosts at copy numbers of 20–100 genomes per cell (Fukuhara et al., 2006; Fukuhara and Gibbs, 2012). The cluster of basidiomycete-, oomycete- and plant-infecting endornaviruses within the alphaendornaviruses demonstrates that their evolution has involved horizontal transmission between host types, e.g. between fungi and plants (Gibbs et al., 2000; Roossinck et al., 2011; Khalifa and Pearson, 2014), but how this occurred is unknown.

Terrestrial orchids rely on symbiotic associations with mycorrhizal fungi to provide nutrients and other molecules required for germination and growth. The fungi form pelotons in the cortex of the root systems, which are digested by the orchids to acquire the required nutrients (Swarts and Dixon, 2009; Smith and Read, 2010). This process provides a possible route by which viruses are exchanged – either from fungus to plant or vice versa. Here, we used a shotgun sequencing approach to identify endornaviruses from orchid leaves and from fungal cultures initiated from mycorrhizal fungal pelotons isolated from orchid root cells.

2. Materials and methods

2.1. Collection sites

Leaves and underground stem or root tissue was collected from plants of the common mignonette orchid (*Microtis media*; 2 populations), an unidentified snail orchid (*Pterostylis* sp.; 3 populations), and dark banded greenhood orchid (*Pterostylis sanguinea*; 4 populations) from remnant native forest located on the Murdoch University campus, Western Australia (W.A.) (Fig. S1, Table 1). It is uncertain if the three populations of the snail orchid *Pterostylis* (*Pterostylis* sp. isolates 1, 2 and 3) represented the same genetic lineage because snail orchids exhibit variable morphology and interspecies hybridization is common (Brundrett, 2014).

2.2. Fungus isolation from root pelotons

Each underground stem or root tissue sample (Fig. 1) was surface-sterilized by immersion in 2% sodium hypochlorite solution, then in 70% ethanol for 10 s followed by washing in sterile water, before being ground in sterile water with a pestle. The resulting liquid mixture was viewed under a compound microscope to identify fungal pelotons (undifferentiated hyphae; Fig. 1). Individual pelotons were transferred onto fungal isolation medium (FIM) agar plates (0.3 g L^{-1} $NaNO_3$, 0.2 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $MgSO_4.7H_2O$, 0.1 g L^{-1} KCl, 0.1 g L^{-1} yeast extract, 2.5 g L^{-1} sucrose and 8 g L^{-1} agar; 100 mg L^{-1} filter-sterilized streptomycin sulfate) (Clements and Ellyard, 1979). Plates were left to incubate in the dark at 24 °C for 5–7 days. Growing mycelium was subcultured into liquid media (FIM minus agar) and left on a shaker in the dark at 24 °C until 80–100 mg fungal biomass was obtained..

Table 1
Orchids and mycorrhizal fungi sampled from the Murdoch University campus, Perth, Western Australia.

Orchid species	Common name	Plant sample No. (No. of pooled individuals[a])	Mycorrhizal fungus	Fungal sample no. (No. of individuals[a])	Latitude/Longitude of host plant population
Pterostylis sp. population 1	Snail orchid	P01 (5)	*Ceratobasidium* sp. isolate-1	C01 (1)	−32°3' 54.5034", 115°50' 19.968"
Pterostylis sp. population 2	Snail orchid	_[b]	*Ceratobasidium* sp. isolate-2	C02 (1)	−32°4' 14.0515", 115°50' 12.4667"
Pterostylis sp. population 3	Snail orchid	P02 (10)	*Ceratobasidium* sp. isolate-3	C03 (1)	−32°3' 55.70277", 115°50' 27.64415"
Microtis media	Common mignonette orchid	P03 (5)	*Ceratobasidium* sp. isolate-4	C04 (1)	−32°4' 2.5494", 115°50' 13.848"
Microtis media[c]	Common mignonette orchid	P04 (10)	–	–	−32°4' 2.334", 115°50' 17.736"/−32°4' 2.5494", 115°50' 13.848"
Pterostylis sanguinea[d]	Dark banded greenhood orchid	P05 (20)	*Ceratobasidium* sp.	C05 (5)	−32°4' 27.87305", 115°49' 54.22273"/−32°4' 13.64061", 115°50' 8.14155"/−32°4' 29.43237", 115°49' 53.43738"/−32° 4' 30.55127", 115° 49' 52.73462"

[a] Number of leaves or roots sampled and pooled from each population(s).
[b] Leaf material was not sampled for *Pterostylis* sp. population 2.
[c] Mixture of plant and fungal samples from two *M. microtis* populations; fungal sample was not tested separately.
[d] Leaf and root samples were pooled from four *P. sanguinea* populations.