Detection and analysis of endogenous badnaviruses in the New Zealand flora

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Abstract

Background and aims
Badnaviruses and their host-integrated DNA occur in tropical crops and a few northern temperate species. Following the discovery of a badnavirus on a subantarctic island with floristic links to New Zealand, we postulated that badnaviruses exist in the New Zealand flora. Badnavirus reverse transcriptase (RT) sequences consist of variable regions flanked by highly conserved regions. This study used RT sequences to detect and characterize badnavirus sequences in the New Zealand flora and to investigate their utility for the study of broader aspects of plant biology.

Methodology
Molecular diversity of RT sequences was analysed using polymerase chain reaction and denaturing gradient gel electrophoresis (DGGE). In a study of the genus Melicytus, internal transcribed spacer (ITS) sequences were compared with the RT data.

Principal results
No freely replicating badnaviruses were detected but more than half of the species (37/60) contained RT sequences. Phylogenetic analysis of 21 RT sequences formed monophyletic groups distinct from other species and from badnaviruses. No frameshift mutations occurred in any of the sequences translated in silico. More detailed study of the genus Melicytus indicated broader applications for our approach. Analysis of RT sequences revealed the presence of a previously unrecognized species (confirmed using ITS). Inheritance of DGGE profiles by Melicytus ramiflorus seedlings suggested that this species may undergo apomixis.

Conclusions
The presence of integrated badnavirus sequences in a wide range of taxa from this Southern Hemisphere flora indicates that these sequences may be common in many temperate regions. Potential to activate viruses from these sequences should be considered when placing these species in tissue culture or under other forms of abiotic or genomic stress. Analysis of endogenous RT sequences shows potential for the study of systematics, phylogenetics and plant reproductive biology.

Introduction
Members of the plant virus family Caulimoviridae possess a circular double-stranded DNA genome of ~7–8 kb. Caulimoviridae are pararetroviruses as they replicate through an obligatory RNA intermediate but, unlike animal retroviruses, do not need to integrate into the host genome to complete their replication cycle. The Caulimoviridae is divided into six genera: Caulimovirus, Soymovirus, Cavemovirus, Petuvirus, Badnavirus and Tungrovirus (Fauquet et al. 2005). Three of these genera

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have been shown to exist as integrated forms that can, under conditions of genomic or environmental stress, excise from the host genome and generate episomal virus infection (Harper et al. 2002; Hansen and Heslop-Harrison 2004). Such endogenous pararetroviruses (EPRVs) form a distinct class of retroelements and have been found in a variety of crop and ornamental plants. For example, banana contains, and taro and yucca most likely contain, integrated badnavirus sequences (Clover et al. 2003; Yang et al. 2003; Geering et al. 2005a, b; Gambley et al. 2008) as well as freely replicating, transmissible, badnaviruses. Eudicots such as Citrus, Theobroma, Ribes and Rubus contain badnaviruses, but it is not known whether they contain integrated badnavirus sequences. A number of eudicots do, however, contain integrated forms of other genera of Caulimoviridae (Hohn et al. 2008).

Although a few badnaviruses have been isolated from cultivated plants from northern temperate regions, badnaviruses are generally considered to be of tropical or subtropical origin. Recently a new species of badnavirus, Stilbocarpa mosaic bacilliform virus (SMBV), was reported infecting Stilbocarpa polaris on subantarctic Macquarie Island (Skotnicki et al. 2003). Although the subantarctic islands are among the most remote places on Earth, of the 21 species of dicotyledons that occur on Macquarie Island (19 are also part of the New Zealand flora (Allan 1982; Hnatiuk 1993). The unexpected discovery of SMBV indicated that the New Zealand flora may harbour unique viruses and pararetroviral sequences that have been evolving in isolation for a long period of their evolutionary history. To date no badnaviruses have been recorded in the New Zealand flora (Allan 1982; Hnatiuk 1993).

In this paper, we used specific polymerase chain reaction (PCR) primers to amplify a 530-nucleotide sequence of badnavirus reverse transcriptase (RT) to investigate the diversity and abundance of badnavirus sequences in the New Zealand flora. We found that often individual plants contained mixed populations of the target sequence. These mixed sequences were closely related and were resolved by denaturing gradient gel electrophoresis (DGGE) giving characteristic band patterns for each species examined. In one species (M. ramiflorus), DGGE band patterns were shown to be inherited.

Materials and methods

Study species

Unless otherwise stated, plants showing no visible signs of disease were collected from a range of habitats in the vicinity of Dunedin, East Otago, Lower South Island, New Zealand. Approximately 10% of samples were mechanically inoculated to subsets of the species listed in Guy et al. (1984) in an attempt to detect latent virus infection. Extracts of the species listed with a superscript in Table 1 (200 mM phosphate buffer pH 6.1, 0.5% sodium sulphite, 0.5% 2-mercaptoethanol, 2% polyethylene glycol 6000, 2% polyvinyl pyrrolidone) were clarified by centrifugation (10 000 × g/20 min). The precipitate was resuspended (10 mM phosphate buffer pH 7.4, 1.5% sodium chloride, 2% Triton X-100) overnight at 4 °C. Following centrifugation (5000 × g/20 min) the supernatant was centrifuged at 100 000 × g/2 h and the resulting pellets were resuspended in distilled water and then examined for virus particles in a transmission electron microscope.

Extraction of DNA

Total genomic DNA was extracted from fresh or frozen leaf tissue. A 50 mg sample of leaf tissue was frozen in liquid nitrogen and placed in a lysing matrix tube containing a garnet matrix and 1/4” ceramic sphere (Lysing Matrix A, Q-BIO gene, MP Biomedicals, LLC, South Australia and Tasmania. Other species of Melicytus have more restricted distributions, with Melicytus chathamicus being confined to the Chatham Island group which lies 700 km east of the South Island (Mitchell et al. 2009).
| Family         | Species                          | PCR | Number of badnavirus-positive samples/collections examined | Sequence (GenBank Accession) |
|---------------|----------------------------------|-----|-------------------------------------------------------------|-----------------------------|
| Apocynaceae   | *Parsonsia heterophylla*         | +   | 2/2                                                         | FJ900050, FJ900051          |
| Apiaceae      | *Stilbocarpa polaris*           | −   | 0/5                                                         |                             |
| Araliaceae    | *Raukaua anomalus*              | −   | 0/1                                                         |                             |
|             | *Schefflera digitata*           | −   | 0/1                                                         |                             |
| Asteraceae    | *Brachyglottis scidophila*      | −   | 0/1                                                         |                             |
|             | *Celmsia haastii*              | +   | 1/1                                                         |                             |
|             | *Celmsia prorepens*            | −   | 0/1                                                         |                             |
|             | *Celmsia viscosa*              | +   | 1/1                                                         |                             |
| Cornaceae     | *Grselinia littoralis*          | +   | 1/1                                                         |                             |
| Escalloniaceae| *Carpodetus serratus*           | −   | 0/1                                                         |                             |
| Poaceae       | *Chionochloa rubra subsp. cuprea* | + | 1/1                                                         |                             |
|             | *Chionochloa rubra subsp. rubra var. inermis* | + | 1/1                                                         | FJ900044                    |
| Icacinaceae   | *Pennantia corymbosa*           | −   | 0/2                                                         |                             |
| Malvaceae     | *Hibiscus trionum*              | +   | 1/1                                                         |                             |
|             | *Hoheria angustifolia*          | −   | 0/1                                                         |                             |
| Moraceae      | *Streblus microphylla*          | −   | 0/1                                                         |                             |
| Myrsinaceae   | *Myrsine australis*             | +   | 2/2                                                         |                             |
|             | *Myrsine chatharnica*           | +   | 2/2                                                         |                             |
|             | *Myrsine coxii*                 | +   | 1/1                                                         |                             |
|             | *Myrsine divaricata*            | +   | 4/4                                                         |                             |
|             | *Myrsine nummularifolia*        | +   | 1/1                                                         |                             |
|             | *Myrsine salicinia*             | +   | 2/2                                                         | FJ900049                    |
| Myrtaceae     | *Kunzea ericioides*             | −   | 0/1                                                         |                             |
|             | *Meterosideros diffusa*         | −   | 0/1                                                         |                             |
|             | *Neomyrtus obcordata*           | −   | 0/1                                                         |                             |
| Pittosporaceae| *Pittosporum tenuifolium*       | −   | 0/1                                                         |                             |
| Polygonaceae  | *Muehlenbeckia australis*       | +   | 1/1                                                         |                             |
| Ripogonaceae  | *Ripogonum scandens*            | −   | 0/4                                                         |                             |
| Rosaceae      | *Acaena juvenca*                | −   | 0/1                                                         |                             |
|             | *Rubus cissioides*              | −   | 0/1                                                         |                             |
| Rubiaceae     | *Coprosma ‘Beatson’s Gold’*     | +   | 1/1                                                         |                             |
|             | *Coprosma aerolata*             | +   | 1/1                                                         |                             |
|             | *Coprosma cheesemani*           | +   | 1/1                                                         | FJ900045                    |
|             | *Coprosma ciliata*              | +   | 1/1                                                         |                             |
|             | *Coprosma crassifolia*          | +   | 5/5                                                         | FJ900046                    |
|             | *Coprosma decurva*              | −   | 0/1                                                         |                             |
|             | *Coprosma elatiroides*          | +   | 1/1                                                         | FJ900047                    |
|             | *Coprosma foetidissima*         | +   | 1/1                                                         |                             |
|             | *Coprosma lucida*               | +   | 3/3                                                         | FJ900048                    |

*Continued*
Solon, OH, USA). The frozen sample was disrupted in a Retsch mixer mill Type 301 (Retsch GmbH & Co. KG, Haan, Germany) using three cycles of 30 s at 30 cycles per second. Following this step, the DNA was purified using a DNAeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

**Polymerase chain reaction amplification**

All PCR amplifications were performed in a 20 µL volume containing 1–50 ng of template, 10 pmol each primer and 17 µL of Reddy Mix PCR master mix containing 1.5 mM MgCl₂ (Abgene Limited, Epsom, UK). Polymerase chain reaction products were purified using a QIAquick PCR purification kit (Qiagen) prior to sequencing.

The badna RT fragment was amplified using the degenerate primers HafF (5′ ATG CCI TTY GGI ITI AAR AAY GCI CC 3′) and HafR (5′ CCA YTT RCA IAC ISC CCA ICC 3′) (Yang et al. 2003). For amplification for DGGE, the HafR primer was modified by including a GC clamp (5′ AGC CGC GCG GCG GGC GGG GCG CCG CCA YTT RCA IAC ISC CCA ICC CCA ICC 3′). Polymerase chain reaction parameters were as follows: one cycle at 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 52 °C for 1 min, 72 °C for 1 min followed by one cycle of extension at 72 °C for 30 min. For PCRs using the GC clamped primer, the cycle was modified by increasing the denaturing time to 1 min and the extension time to 2 min.

The internal transcribed spacer (ITS) of nuclear ribosomal DNA was amplified using the primers ITS-1 and ITS-4 (White et al. 1990). Polymerase chain reaction parameters were one cycle at 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 52 °C for 1 min, 72 °C for 1 min followed by one cycle of extension at 72 °C for 10 min.

**Table 1 Continued**

| Family          | Species                          | PCR | Number of badnavirus-positive samples/collections examined | Sequence (GenBank Accession) |
|-----------------|----------------------------------|-----|-----------------------------------------------------------|-----------------------------|
| Coprosma       | parviflora                       | −   | 0/1                                                       |                             |
| Coprosma       | propinqua                        | +   | 2/2                                                       |                             |
| Coprosma       | rhamnoides                       | +   | 1/1                                                       |                             |
| Coprosma       | robusta                          | +   | 1/1                                                       |                             |
| Coprosma       | rotundifolia                     | +   | 2/2                                                       |                             |
| Coprosma       | rugosa                           | −   | 0/1                                                       |                             |
| Coprosma       | virescens                        | +   | 1/1                                                       |                             |
| Plantaginaceae | Veronica sect. Hebe dieffenbachii| +   | 1/1                                                       |                             |
|                 | V. sect. Hebe odora              | +   | 1/1                                                       |                             |
|                 | V. sect. Hebe salicifolia        | +   | 1/1                                                       |                             |
| Solanaceae     | Solanum laciniatum               | −   | 0/1                                                       |                             |
| Violaceae      | Melicytus alpinus*               | +   | 13/18                                                     | FJ900052–FJ900061           |
|                 | Melicytus chathamicus            | +   | 2/3                                                       | FJ900062, FJ900063          |
|                 | Melicytus flexuosus*             | +   | 2/3                                                       | FJ900064                    |
|                 | Melicytus lanceolatus            | −   | 0/3                                                       |                             |
|                 | Melicytus macrophyllus           | +   | 1/1                                                       |                             |
|                 | Melicytus micranthus             | +   | 1/1                                                       | FJ900065                    |
|                 | Melicytus obovatus               | −   | 0/1                                                       |                             |
|                 | Melicytus ramiflorus*            | +   | 19/19                                                     | FJ900066–FJ900072          |
| Urticaceae      | Urtica australis                 | −   | 0/1                                                       |                             |
| Winteraceae     | Pseudowintera colorata           | +   | 1/1                                                       |                             |

*Species examined in the electron microscope for virus particles.
Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis was performed using the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Gradient gels containing 8% polyacrylamide (37.5:1 ratio of acrylamide:bis-acrylamide) were formed using a peristaltic pump (Gilson Minipuls) and a gradient mixing device (Hoefer SG30) with a denaturant gradient of 25–40%. Gels were run in 1 × Tris-acetate-EDTA buffer at 75 V, 400 mA for 16 h at 60 °C. Gels were stained for 60 min in MilliQ water containing 0.5 mg of ethidium bromide per litre and destained for 30 min in MilliQ water. Gels were placed on a UV transilluminator and the digital images were recorded with a Kodak Gel Logic Imaging System (Eastman Kodak Co., Rochester, NY, USA). Bands of interest were excised from DGGE gels and DNA eluted by soaking in 100 μL of water (MilliQ) overnight. Aliquots of the eluate were re-amplified using the HaFF and HaFR primers and purified prior to sequencing.

DNA sequencing

Samples were sequenced by the Allan Wilson Centre Genome Service, Massey University, using the Applied Biosystems BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Life Technologies Corporation, Carlsbad, CA, USA).

Analysis of badnavirus RT sequences

All available badnavirus RT fragment sequences from GenBank (http://www.ncbi.nlm.nih.gov/) were aligned with the sequences obtained in this study using ClustalW. Mismatched sequences and additional sequence representatives with >95% identity to existing sequences were removed from the alignment. The final nucleotide alignment was then derived from the corresponding translated amino acid alignment that had been adjusted manually. The aligned dataset contained 534 nucleotide characters, of which 448 were parsimony informative. A maximum parsimony phylogenetic analysis was done using PAUP* version 4.0b10 (Swofford 2003), with the sequences obtained in this study using ClustalW. The dataset contained 704 nucleotide characters and eight binary characters, representing indels coded as present (1) or absent (0). A Bayesian inference phylogenetic analysis was done using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), using a general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites. Four hundred and fifty thousand generations were run, sampling every hundredth generation. The standard deviation of split frequencies was 0.008268 after 450 000 generations. Trees and branch length samples were summarized after discarding the first 25% (1125) of the samples.

Analysis of Melicytus ITS region

Internal transcribed spacer sequences were aligned using ClustalW. The dataset contained 704 nucleotide characters and eight binary characters, representing indels coded as present (1) or absent (0). A Bayesian inference phylogenetic analysis was done using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), using a general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites. Four hundred and fifty thousand generations were run, sampling every hundredth generation. The standard deviation of split frequencies was 0.008268 after 450 000 generations. Trees and branch length samples were summarized after discarding the first 25% (1125) of the samples.

Results

New Zealand plant species tested for badnavirus sequences

A total of 60 species belonging to 28 genera and 22 families were tested (Table 1). A total of 37 species gave a positive result for the 530 bp fragment of RT with the remaining 23 being negative. No positives were found from species belonging to the families Apiaceae, Araliaceae, Escalloniaceae, Icacinaceae, Moraceae, Myrtaceae, Ripogonaceae, Rosaceae, Solanaceae and Urticaceae that were examined. Reverse transcriptase DNA sequences were detected in both endemic and other native species with wider distributions. Reverse transcriptase sequences were found in plant species from vegetation types ranging from tussock grassland communities to shrublands and forests.

The RT sequences reported here were detected in a monocot (Chionochloa rubra subsp. rubra var. inermis), a liane (Parsonisia heterophylla) and small trees and shrubs. Chionochloa rubra subsp. rubra var. inermis is a member of the widespread and ecologically important genus of snowgrasses (Pirie et al. 2008). Reverse transcriptase sequences were present in four widespread species of Myrsine and two Chatham Islands endemics Myrsine chathamica and M. coxii. Thirteen out of the 16 species of Coprosma, a large and taxonomically diverse genus of shrubs, surveyed contained RT sequences. The three species of V. sect. Hebe all contained RT sequences. Veronica salicifolia is a lowland forest species native to New Zealand and coastal Chile, V. dieffenbachii is endemic to the Chatham Islands and V. odora is a sub-alpine species.

Stilbocarpa mosaic bacilliform virus was not detected in S. polaris collected from Auckland and Campbell Island (no collections from Macquarie Island). None of
the Stilbocarpa plants showed symptoms of virus infection and no RT sequences were amplified using the Haf primers or the primers of Skotnicki et al. (2003).

*Melicytus ramiflorus* was the only species sampled that displayed virus symptoms. Extensive electron microscope examination of *M. ramiflorus* sap, pellets prepared from extracts by differential centrifugation and ultrathin sections failed to detect any badnavirus particles. Particles of an unidentified icosahedral virus were observed in healthy and symptomatic trees (D. J. Lyttle and P. L. Guy, unpubl. res.). However, there was no correlation between the presence of RT sequence and virus symptoms. The failure to find particles with characteristic badnavirus morphology suggests that virus DNA is present only as integrated sequence.

Virus particles were not detected in any of the preparations from the other species examined in the electron microscope. No mechanically transmissible viruses were detected during this study.

**Phylogenetic diversity of endogenous badnavirus sequences**

BLAST searching (Altschul et al. 1997) showed that the sequences reported here did not match any known badnavirus sequence. When the 21 RT DNA sequences from 12 species belonging to four genera (*Melicytus* 17, *Coprosma* 7, *Parsonia* 2, *Chionochloa* 1, *Mysine* 1) were aligned with known badnavirus sequences from GenBank, each species was shown to contain distinct sequences (Fig. 1).

With the exception of the *Coprosma propinqua* sequence, *Coprosma* sequences were monophyletic (clade A, Fig. 1) with good bootstrap support. Another monophyletic group combined the *M. alpinus* and *M. chathamicus* sequences. All the *M. ramiflorus* sequences, with the exception of *M. ramiflorus* Wa6, grouped together with strong bootstrap support. *Melicytus ramiflorus* and *Melicytus flexuosus* sequences were less closely related. Two *P. heterophylla* sequences were closely related.

**Conservation of amino acid sequences in endogenous badnavirus RT**

All the endogenous badnavirus sequences that were determined were translated into the corresponding amino acid sequence and aligned. After duplicate sequences were removed, this gave a total of 21 distinct amino acid sequences. No frameshift mutations occurred in any of the sequences and a conservative pattern of amino acid changes was observed.

**Analysis of badnavirus RT sequence diversity from *Melicytus***

Badnavirus RT sequences from the *Melicytus* spp. were investigated to determine the sequence diversity present within individual plants, among individuals of the same species and between different species in the genus. It was not possible to establish an unambiguous sequence directly from the PCR product for every collection investigated as a number of the PCR products contained a heterogeneous mixture of sequences. These mixtures were subjected to DGGE to resolve the oligonucleotide fragments.

The 530 bp RT fragment from *M. ramiflorus* was resolved into >25 distinct bands by DGGE (Fig. 2A). Overall, band patterns from different individuals were very similar in appearance and showed many bands in common. However, differences were apparent as some individuals contained additional bands or conversely lacked other bands. For example, the DGGE band pattern from a plant collected from Karamea (lane 4) was similar though distinct from eastern coastal Otago plants (lanes 2, 3) although fewer bands were observed overall. A plant from Rangitoto Island, Auckland (lane 6) showed three distinctive strong bands in the lower part of the gel but otherwise was similar to the remaining collections. Four plants collected from the same locality on the Otago Peninsula (lanes 7, 8, 9, 10) showed individual differences but contained many common bands. There were no consistent differences in the band patterns from plants collected from the southern part of the South Island (Dunedin), the northern part of the South Island (Karamea) and the northern part of the North Island (Auckland) that could be attributed to geographic location.

**Inheritance of badnavirus fragment patterns**

Six plants grown from seed collected from a single parent (Mel3) were compared in this analysis. As *M. ramiflorus* is a dioecious species only the female parent is known for this particular cross. Badnavirus fragment patterns for the female parent and the six progeny plants were generated using DGGE (Fig. 2B). The progeny plants contained all the badnavirus fragments present in the maternal plant or a subset of them. Three seedlings, P1, P3 and P5, showed DGGE patterns identical to that of the parent. Each of the remaining three seedlings (P2, P4 and P6) all showed a subset of the parental bands.

**Analysis of variation in the *M. alpinus* species complex using badnavirus fragment patterns**

Analysis of badnavirus fragment patterns for the *M. alpinus* collections showed considerable variation within the species (Fig. 3). Plants identified in the field
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Fig. 1. Phylogenetic tree showing the relationships of New Zealand endogenous badnavirus sequences to known badnaviruses.
as *M. alpinus* collected from six coastal sites in eastern Otago (Akatore Creek, Hooper’s Inlet, Nugget Point, Pyramids, Sandymount and Taiaroa Head) and from four inland sites (Swampy Summit, Middlemarch, Ida Range and Pisa Range) were subjected to PCR and DGGE analysis. DNA samples from the Swampy Summit plants (4/4) and Sandymount plants (2/2) did not amplify with the HafF and HafR primers. The remaining DNA samples all gave PCR products of the expected size. When the corresponding GC clamped primers were used for the amplification of the RT fragment, the plants collected from the coastal sites (Akatore Creek, Hooper’s Inlet, Nugget Point, Pyramids, Sandymount and Taiaroa Head) and one inland site (Middlemarch) showed identical DGGE band profiles (Fig. 3, type 1). Two of the inland plants, Pisa Range 1 and Ida Range, shared a second unique DGGE band profile (Fig. 3, type 2). The two remaining plants collected from the Pisa Range showed band profiles that were different from each other and from the other two groups. The two specimens of *M. chathamicus* examined gave identical DGGE patterns (results not shown).

**Congruence between host phylogeny and endogenous badnavirus profiles**

To clarify the phylogenetic relationships among the different species of *Melicytus* examined for badnavirus sequences in this study, the sequence of the ITS region was determined for 34 collections of various *Melicytus* spp. (Table 2) using the primers ITS-1 and ITS-4. Two major clades with weak support (posterior probability value 0.92) were resolved. One clade contained...
Fig. 3. Phylogenetic tree showing the relationships of various Melicytus species based on their ITS sequences and the DGGE band patterns of the RT fragments from M. alpinus. For M. alpinus, two predominant fragment patterns are distinguishable in the gel (designated type 1 (lanes 1–9) and type 2 (lanes 10–11)). 1: M. alpinus Akatore Creek 1; 2: M. alpinus Akatore Creek 2; 3: M. alpinus Hooper’s Inlet; 4: M. alpinus Middlemarch; 5: M. alpinus Nugget Point 1; 6: M. alpinus Nugget Point 2; M. alpinus Pyramids 1; 8: M. alpinus Pyramids 2; 9: M. alpinus Taiaroa Head; 10: M. alpinus Ida Range; 11: M. alpinus Pisa Range 1; 12: M. alpinus Pisa Range 2; 13: M. alpinus Pisa Range 3.
Table 2 *Melicytus* specimens used for ITS sequence and DGGE comparisons.

| Identifier  | Species             | Locality              | Otago Herbarium sheet no. | GenBank Accession | RT* | DGGE | ITS indel |
|-------------|---------------------|-----------------------|---------------------------|-------------------|-----|------|----------|
| alpAk1      | *Melicytus alpinus* | Akatore Creek         | OTA 060541                | FJ900084          | Present | alp Ty1 | indel3T |
| alpAk2      | *Melicytus alpinus* | Akatore Creek         | OTA 060542                | FJ900083          | Present | alp Ty1 | indel3T |
| alpHi       | *Melicytus alpinus* | Hooper’s Inlet, Otago Peninsula | OTA 061459                | FJ900085          | Present | alp Ty1 | indel3T |
| alpId1      | *Melicytus aff alpinus* | Ida Range          | OTA 060539                | FJ900088          | Present | alp Ty2 | ? |
| alpMm       | *Melicytus alpinus* | Crater, Middlemarch  | No voucher                | FJ900082          | Present | alp Ty1 | indel3T |
| alpNu1      | *Melicytus alpinus* | Nugget Point, South Otago | OTA 060848                | FJ900081          | Present | alp Ty1 | indel3T |
| alpNu2      | *Melicytus alpinus* | Nugget Point, South Otago | OTA 060849                | FJ900079          | Present | alp Ty1 | indel3T |
| alpPa1      | *Melicytus alpinus* | Locharburn, Pisa Ra  | OTA 061461                | FJ900089          | Present | alp Ty2 | indel 1a – 200 |
| alpPa2      | *Melicytus alpinus* | Locharburn, Pisa Ra  | OTA 061462                | FJ900090          | Present | alp Ty1 variant | No indel |
| alpPa3      | *Melicytus alpinus* | Locharburn, Pisa Ra  | OTA 061463                | FJ900094          | Present | variant | indel/hybrid |
| alpPy1      | *Melicytus alpinus* | Pyramids, Otago Peninsula | No voucher                | FJ900086          | Present | alp Ty1 | indel3T |
| alpPy2      | *Melicytus alpinus* | Pyramids, Otago Peninsula | OTA 060540                | FJ900080          | Present | alp Ty1 | indel3T |
| alpSmt1     | *Melicytus aff obovatus* | Sandymount, Otago Peninsula | OTA 061455                | FJ900102          | Absent | Null | No indel |
| alpSmt2     | *Melicytus aff obovatus* | Sandymount, Otago Peninsula | No voucher                | FJ900103          | Absent | Null | No indel |
| alpSw1      | *Melicytus aff obovatus* | Swampy Summit       | No voucher                | FJ900091          | Absent | Null | No indel |
| alpSw2      | *Melicytus aff obovatus* | Swampy Summit       | OTA 061453                | FJ900093          | Absent | Null | No indel |
| alpSw3      | *Melicytus aff obovatus* | Swampy Summit       | OTA 061454                | FJ900092          | Absent | Null | No indel |
| alpTh       | *Melicytus alpinus* | Taiaroa Head, Otago Peninsula | OTA 061460                | FJ900087          | Present | alp Ty1 | indel3T |
| chath       | *Melicytus chathamicus* | Landcare garden     | No voucher                | FJ900098          | Present | Chath |
| chathPJ     | *Melicytus chathamicus* | Landcare garden     | No voucher                | FJ900100          | Absent | Null |
| chathW      | *Melicytus chathamicus* | Waitangi, Chatham Islands | OTA 061464                | FJ900099          | Present | Chath |
| denSA       | *Melicytus dentatus* | South Australia     | No voucher                | FJ900101          | Present | Den |
| flexAk      | *Melicytus flexuosus* | Akatore Creek       | OTA 060545                | FJ900096          | Present | Flex | No indel |
| flexLc      | *Melicytus flexuosus* | Landcare garden     | No voucher                | FJ900097          | Present | ND |
| flexSs      | *Melicytus aff flexuosus* | Swampy Spur, Dunedin | OTA 061469                | FJ900095          | Absent | Null | indel3T |
| lan         | *Melicytus lanceolatus* | Landcare garden     | No voucher                | FJ900076          | Absent | Null |
| lanNC       | *Melicytus lanceolatus* | Nichols Creek, Dunedin | No voucher                | FJ900077          | Absent | Null |
| lanP        | *Melicytus lanceolatus* | Papatowai, South Otago | OTA 060858                | FJ900078          | Absent | Null |
| mic         | *Melicytus micranthus* | Motokarara Nursery  | No voucher                | FJ900075          | Present | ND |
| obo         | *Melicytus obovatus* | Matai Nursery, Waimate | No voucher                | FJ900104          | Absent | Null |
| ram NC      | *Melicytus ramiflorus* | Nichols Creek, Dunedin | No voucher                | FJ900074          | Present | ram |
| ramRa       | *Melicytus ramiflorus* | Rangitoto Island, Auckland | No voucher                | FJ900073          | Present | ram |

*RRT sequence.*
M. lanceolatus, M. ramiflorus and M. micranthus. The second comprised M. alpinus, M. flexuosus and M. novae zelandiae from New Zealand and M. chathamicus (Chatham Islands), M. latifolius (Norfolk Island) and M. dentatus (South Australia). The two most geographically separated species, M. dentatus and M. chathamicus, had the most closely related sequences.

In this analysis Melicytus obovatus (Cook’s Strait), together with five specimens of M. alpinus collected from Sandy Mount and Swampy Summit near Dunedin, formed an unresolved basal polytomy. Plants from all three locations had identical ITS sequences. Although the small-leaved specimens were morphologically similar to M. alpinus, their ITS sequence and lack of RT sequence separates them from M. alpinus sensu stricto.

Analysis of individual ITS sequences of Melicytus DNAs showed that two types of variation were present. Many DNA templates showed single-base polymorphisms (Table 2) where the sequencing chromatograms showed alternate bases at a number of positions. This type of polymorphism was consistent in terms of nucleotide ratios at each position and was scored if it was observed in both the forward and reverse sequences for a given sample. A second polymorphism identified was the presence of an indel that caused nucleotide shifts giving overlapping peaks in the sequencing chromatogram. Two different indels were initially identified as a character present in some species from the alignment of all the Melicytus ITS sequences. The 3T indel occurred in sequences from M. flexuosus, and the TGA indel occurred in sequences from the M. obovatus clade.

The 3T indel was found as a hybrid polymorphism in a group of specimens collected from mainly coastal sites. The simplest hypothesis to account for the 3T indel polymorphism is that the plants in which it occurs were originally derived from hybrids between two pre-existing species. From the results of this study it is likely that the coastal clade of M. alpinus arose as a hybrid between M. alpinus sensu stricto (alpId, alpPa3 and alpPa2) and M. flexuosus. In the same manner we consider that the specimens Sw1, Sw2 and Sw3 are likely to be of hybrid origin between an M. obovatus-like progenitor and M. flexuosus which is still growing nearby. The predominant sequence from these plants shows a TGA indel characteristic of M. obovatus, but a lesser amount of the 3T indel of M. flexuosus is also observed. In positions where the sequences of M. obovatus and M. flexuosus diverge, the predominant base is that of M. obovatus and the minor base is from M. flexuosus. The closely related plants Smt1 and Smt2 are isolated from any currently existing M. flexuosus population and do not show the introgression of M. flexuosus sequences.

Each group shows a badna RT DGGE pattern that is entirely consistent with the ITS sequence data.

Discussion

In spite of its Gondwanan origins, many of New Zealand’s plant lineages are more recent arrivals (Winkworth et al. 2002) with many species but few genera being endemic. The New Zealand genera which contained RT sequences also occur in other regions: S.E. Asia, Papua New Guinea, Australia, New Zealand, Pacific Islands, Sub Antarctic Islands and South America. Based on our results, we suggest that endogenous badnaviruses may be common in these regions. Melicytus dentatus collected in South Australia contained RT sequences.

Denaturing gradient gel electrophoresis proved a useful technique to analyse the sequence diversity present in many of the samples: cutting out individual bands and re-amplifying them led to additional sequence information. The most complex band pattern was found in M. ramiflorus. When PCR products from this species were analysed by DGGE, over 25 bands were observed in the majority of the samples. Some bands showed minor nucleotide changes but the amino acid sequence was conserved, while others showed additional nucleotide changes that resulted in five amino acid changes (in silico). Denaturing gradient gel electrophoresis has been used widely in environmental sampling to uncover the diversity of species within various ecosystems (Singh et al. 2004; Bull and Stach 2007; Tannock 2008), but this study shows that it is a useful technique to take a snapshot of the diversity within and between genomes.

The presence of endogenous badnaviruses, often as multiple copies, in so many of the species tested and at high incidences, indicates that they may confer a selective advantage. One such advantage could be protection from infection by related viruses via RNA interference (Covey and Al-Kaff 2000; Hull et al. 2000; Noreen et al. 2007). Multiple copies could have arisen by multiple integrations of badnaviruses into the host genome or by the single integration of concatemers whose component sequences then diverged. Typical of island floras, there is a high incidence of polyploidy in New Zealand species (Richards 1997), which could further increase copy number; however, recent studies with Nicotiana spp. (Gregor et al. 2004; Skalická et al. 2005) have shown selective deletion of repetitive sequences in some polyploids.

The inheritance of endogenous badnavirus fragment patterns was investigated by examining plants grown from the seed of a single female plant of M. ramiflorus. Three DGGE band patterns were identical to that of the
maternal plant but 3/6 progeny contained only a subset of the maternal bands. There is strong presumptive evidence that many genera of New Zealand native woody plants including Melicytus reproduce by facultative apomixis (B. P. J. Molloy, pers. comm., 2008) and this has been shown definitively for the genus Coprosma (Heenan et al. 2003). The conservation of endogenous badnavirus DGGE band patterns in M. ramiflorus is consistent with the existence of apomictic races that would give rise to additional variants whenever out-crossing occurs.

The M. alpinus complex includes a diverse assemblage of forms that encompasses a considerable amount of environmental and genetic variation, ranging from a low-growing spiny cushion, typical of the species, to shrubs and dwarf alpine forms growing in high-altitude screes. There are several unnamed shrubby taxa in the M. alpinus complex present in New Zealand (Molloy and Clarkson 1996; Mitchell et al. 2009). It is likely that apomixis also occurs in these small-leaved species and may be in part responsible for the existence of the bewildering array of forms found in the M. alpinus complex. The presence or absence of RT sequences and consistent differences in the RT fragment patterns were observed, and in conjunction with ITS sequences revealed that M. alpinus is composed of several distinct taxa.

Integrated badnavirus sequences are diverse (Geering et al. 2005a, b) and have undergone reassortment and given rise to infection during tissue culture (Ndawora et al. 1999). Recent work showed that the proliferation stage of micropropagation triggers the episomal replication (Dallot et al. 2001). Wounding and various abiotic changes (Lockhart et al. 2000; Richert-Pöggeler et al. 2003) are known to activate EPRVs and indeed other genomic sequences previously regarded as pseudogenes (Summerfield et al. 2008). Considering the growing use of tissue culture and other biotechnologies for plant conservation (Engelmann 1991; Harding et al. 1997; Harding 2004; Bapat et al. 2008) and the increasing prevalence of these ‘two-faced travellers in the plant genome’ (Staginnus and Richert-Pöggeler 2006), it now seems prudent to screen wild germplasm for endogenous badnaviruses and other endogenous Caulimoviridae (Geering et al. 2010) before tissue culture.

Conclusions and forward look

Although our preliminary focus was detecting badnaviruses and endogenous badnaviruses at large in the New Zealand flora, the detection and analysis of badnavirus RT sequences in combination with DGGE were useful for much wider applications in plant systematics, phylogenetics and reproduction biology.

Accession numbers

Reverse transcriptase (see Table 1 Accession Nos FJ90044–72) and ITS (see Table 2 Accession Nos FJ90073–101) sequences are deposited with GenBank. Melicytus spp. specimens have been lodged with the University of Otago Herbarium (see Table 2).

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Contributions by the authors

D.L., D.O. and P.G. conceived and designed the experiments. D.L. and D.O. did the experiments. D.L., D.O. and P.G. analysed the data and prepared the manuscript.

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Conflicts of interest statement

None declared.

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