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Characterization and detection of a novel idaeovirus infecting blackcurrant

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Abstract A novel virus was discovered in a blackcurrant accession (*Ribes nigrum* L.) at the USDA genebank in Oregon, USA. The genome consists of two positive-sense, single-stranded RNAs with the first encoding a 197 kDa multifunctional protein with methyl transferase, helicase and RNA-dependent RNA polymerase enzymatic motifs. The second molecule encodes two putative proteins; the 39 kDa movement and 30 kDa coat proteins. Both RNAs have conserved sequences and structures at the 5′ and 3′ termini. The genome organization, sequence and phylogenetic analyses indicate that the virus is a putative new member of the genus *Idaeovirus*, as it consistently groups with privet leaf blotch-associated virus and raspberry bushy dwarf virus. A duplex RT-PCR assay was developed for rapid detection of both genomic RNAs simultaneously. The work presented in this communication will assure the health status of blackcurrant plants in mother blocks, nurseries and production fields alike.

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The USDA National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, is a genebank preserving genetic diversity of several fruit and nut crop species from around the globe (Postman et al. 2006). A survey was initiated to study the virus status of the berry crops at NCGR and determine whether unusual symptoms might be associated with the presence of viruses. One of the NCGR berry crops, blackcurrant (*Ribes nigrum* L.), is popular because of its piquant berries and positive health effects (Terry 2014). Blackcurrants are primarily grown in northern latitudes and especially in Europe where 99% of the world production is concentrated (Mitchell et al. 2011). NCGR maintains about 50 unique clonal *Ribes nigrum* accessions as living plants (USDA-NPGS 2016).

There are several viruses affecting the crop (Converse 1987; James and Phelan 2016; Petrzik et al. 2016a, 2016b), with blackcurrant reversion virus, the causal agent of reversion disease, considered to be the most economically important (Susi 2004). As part of our study we identified five new virus species in material exhibiting virus-like symptoms (Fig. 1; Ho and Tzanetakis 2014; Ho et al. 2015b) with this communication focusing on a potentially new member of the genus *Idaeovirus*, tentatively named as blackcurrant idaeovirus (BCIV).

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Ribes nigrum " Belaruskaya Slodkaya (Sweet)" infected with idaeovirus

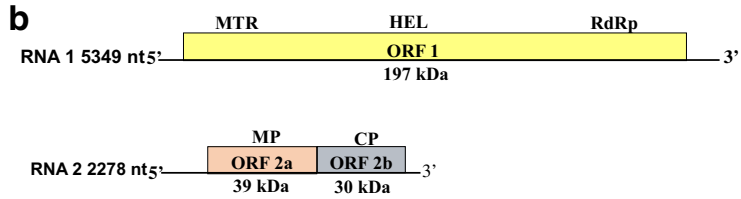

Fig. 1 **a** Blackcurrant NCGR CRIB 1057.001 'Belaruskaya Slodkaya' showing chlorosis infected with blackcurrant idaeovirus (BCIV); **b** Schematic representation of the genomic organization of BCIV. Boxes represent open reading frames (ORFs). Size of each protein products are indicated in kDa. MTR- methyltransferase; HEL- helicase; RdRp- RNA-dependent RNA polymerase; MP- movement protein; CP- coat protein

The unassigned genus *Idaeovirus* belongs to the "alpha-like" virus supergroup and share many features in common with members of the family *Bromoviridae* (Scott 2001). Idaeoviruses are single-stranded, positive-sense RNA viruses with bipartite genomes with RNA 1 encoding a replication-associated protein, whereas RNA 2 codes for the movement and coat proteins (MP and CP respectively). The CP is expressed from a subgenomic RNA (Natsuaki et al. 1991; Quito-Avila et al. 2014). The isometric particles of RBDV virions are reported to contain both the genomic RNAs and CP subgenomic RNA (MacFarlane and McGavin 2009). Currently the genus contains three verified or putative members; raspberry bushy dwarf virus (RBDV, Natsuaki et al. 1991; Ziegler et al. 1992), the type species of the genus; privet leaf blotch-associated virus (PrLBaV; Navarro et al. 2016), and citrus idaeovirus (CIV; Derrick et al. 2006).

For any clonally propagated crop such as currants, the most important attribute for profitable and sustainable production is clean, virus-tested planting materials, free from all targeted systemic pathogens (Gergerich et al. 2015). For this reason, we developed a reliable duplex RT-PCR detection assay to simultaneously detect both genomic segments and minimize the possibility of false negative results due to virus diversity and mis-priming of the detection primers.

The majority of the genome was obtained by large scale sequencing using double-stranded RNA extracted from NCGR accession CRIB 1057.001 'Belaruskaya Slodkaya' essentially as described by Ho and Tzanetakis (2014) and Ho et al. (2015a). The genome areas that were not covered in large scale sequencing were obtained by Sanger sequencing of RT-PCR amplicons obtained using virus specific primers (Supplemental Table). The 5′ ends of the RNAs were obtained using FirstChoice® RLM-RACE Kit (ThermoFisher Scientific, USA). The 3′ ends were obtained using 3′ RACE-RT-PCR on poly-adenylated RNAs (Poly (A) Tailing Kit, Applied biosystems, USA). Three separate RACE-PCRs were carried out for 5′ and 3′ regions of both RNA 1 and RNA 2 (Supplemental Table) and products were sequenced to obtain a three-fold coverage of the regions. The complete nucleotide sequence of BCIV RNA 1 and RNA 2 were deposited in GenBank under accession numbers KY399998 and KY399999 respectively.

Open Reading Frame (ORF) Finder (Wheeler et al. 2003) was used to identify putative ORFs. The possible secondary structures of the 3′ noncoding regions were derived using MFOLD (Zuker 2003). The complete protein sequences of polyprotein, putative MP, CP and conserved methyl transferase (MTR), viral helicase (HEL) and RNA-dependent RNA polymerase (RdRp) domains of PrLBaV and RBDV were obtained from GenBank. The identities among proteins were analyzed using Clustal W in BioEdit (Hall 1999; Thompson et al.

1994). For phylogenetic analysis, the RdRp domain and the MP and CP amino acids sequences of BCIV and other idaeoviruses were aligned with those of the type members of the genera in the family *Bromoviridae.* Phylogenetic trees were constructed using the maximum likelihood method with 1000 bootstrap pseudoreplicates.

For detection purpose, total nucleic acids were extracted and cDNAs were synthesized according to Poudel et al. (2013). Forward primers were designed within the coding regions of each RNA whereas reverse primers were designed from a region of the 3′ end showing 100% nt identity between the RNAs of the virus. The primer combinations that amplified genome segments individually were further evaluated in duplex PCRs for amplification of both genome segments simultaneously. The most reliable results were obtained with forward primers BCIdaeoRNA1F1, 5′-AAGATAGTCTGGAAGAAGTTCGA-3′ and BCIdaeoRNA2F1, 5′-AGGAAGTACTGGGAAGTGATA-3′ for RNA 1 and RNA 2 respectively in combination with reverse primer BCIdaeoR2, 5′-ACAATAGTCCAGATAGCGGAA-3′.

The thermal cycler program consisted of 2 min initial denaturation at 94^0 C, followed by 40 cycles of 20 s denaturation at 94^0 C, 20 s annealing at 52^0 C and 30 s extension at 72^0 C. The PCR mixture at final volume of 25 ul contained equal amount of detection primers (400 nM each), 1× *Taq* buffer, 200 μm dNTPs and 0.5 U of *Taq* DNA polymerase (GenScript, USA).

The BCIV genome consists of two genomic RNAs for a total of 7627 nucleotides (nt) (Fig. 1b). RNA 1 is 5349 nt long with a 5′ and 3′ untranslated regions (UTRs) of 61 and 83 nt respectively and has a single long ORF. RNA 2 is 2278 nt long and codes for two ORFs. The UTRs of RNA 2 were of 320 nt and 84 nt for 5′ and 3′ respectively. The 5′ termini of both RNAs had the identical pentanucleotide (5′-AUAUA-3′) with the first four being in common with the RNAs of RBDV and PrLBaV. The 3′ UTRs of the genomic RNAs were 90% identical and shared a region of 47 identical nt (Fig. 2a) with five cytosine (C) residues at the termini, compared to four C residues at the end of the RBDV and PrLBaV genomes (Natsuaki et al. 1991; Ziegler et al. 1992; Navarro et al. 2016). All three viruses have a conserved hexanucleotide, 5′-AACCCC-3′, at the 3′ termini of both genome segments (with BCIV has an additional C at the end). The 3'UTRs of BCIV and PrLBaV were 64% and 75% identical for RNA 1 and RNA 2 respectively. Interestingly, RNA 2 of both viruses conserved 19 nucleotides at the 3′ termini. In addition, the 3′ UTRs of BCIV and PrLBaV folded into near-identical stem-loop structures leaving three unpaired C residues at the RNA ends (data not shown).

RNA 1 encoded a 197 kDa multifunctional protein showing high amino acid (aa) identity with its orthologs in PrLBaV and RBDV respectively (Table 1). The ORF codes for a 1734 aa replication-associated protein with conserved domains of MTR (aa positions 196–588, Cdd: pfam01660), HEL (aa positions 898–1154, Cdd: pfam01443) and RdRp (aa positions 1282–1715, Cdd: pfam00978). Pairwise comparison of the BCIV conserved domains with the PrLBaV and RBDV orthologs showed the highest identity with the PrLBaV counterparts (Table 1). A second ORF reported in RBDV RNA 1 (Ziegler et al. 1992) was absent in BCIV.

BCIV RNA 2 encoded two proteins with an estimated molecular mass of 39 kDa (352 aa) and a 30 kDa (271 aa). The proteins showed the highest aa identity to the MP and CP of PrLBaV respectively (Table 1). No conserved domains were detected in the BCIV MP whereas the CP contained an RBDV CP superfamily domain (pfam06593). There was no intergenic region between the ORFs. The last nucleotide of the "opal" stop codon of MP ORF was the starting nt for the initiation codon for CP ORF (5′-AUC**UGA**UGUCG-3′; MP stop codon is in bold letters and CP start codon is underlined). This was verified by sequencing both strands of multiple PCR products. In the case of PrLBaV, the two ORFs were separated by an intergenic spacer of a single nucleotide (Navarro et al. 2016).

In phylogenetic analyses using the conserved domains of RdRp and the CP and MP sequences BCIV clustered with other idaeoviruses and always formed a clade with PrLBaV (Fig. 3) confirming its position in the genus and its close evolutionary relationship with PrLBaV. All members of *Bromoviridae* grouped in different clades from idaeoviruses indicating their genetic distance.

Blackcurrant is asexually propagated and it is utmost important to have a virus-tested mother plant for propagation in nurseries to prevent the inadvertent spread of diseases/viruses (Martin et al. 2016). The detection tests developed for BCIV amplify a 329 bp of RNA 1 or a 559 bp of RNA 2 and subgenomic RNA individually or both in a duplex PCR (Fig. 4). We designed for a larger amplicon for RNA 2 as the targeted region is within the CP coding region and therefore primers amplify both genomic

a

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RNA 1-3'UTR 5'-UGUGUUUUGAGAGCUUUUCUCAUGAAAGCUGAA-GCUUUCCGCUAUCUGGACUAUUGUCC
RNA 2-3'UTR 5'-UGAGCAUUGAGAGCUUUUCUCAUGAACUCUUAAUGAGUUCCGCUAUCUGGACUAUUGUCC
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RNA 1-3'UTR AGAUGGUUGCCAAAGGCAACCCCC-3'
RNA 2-3'UTR AGAUGGUUGCCAAAGGCAACCCCC-3'
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b

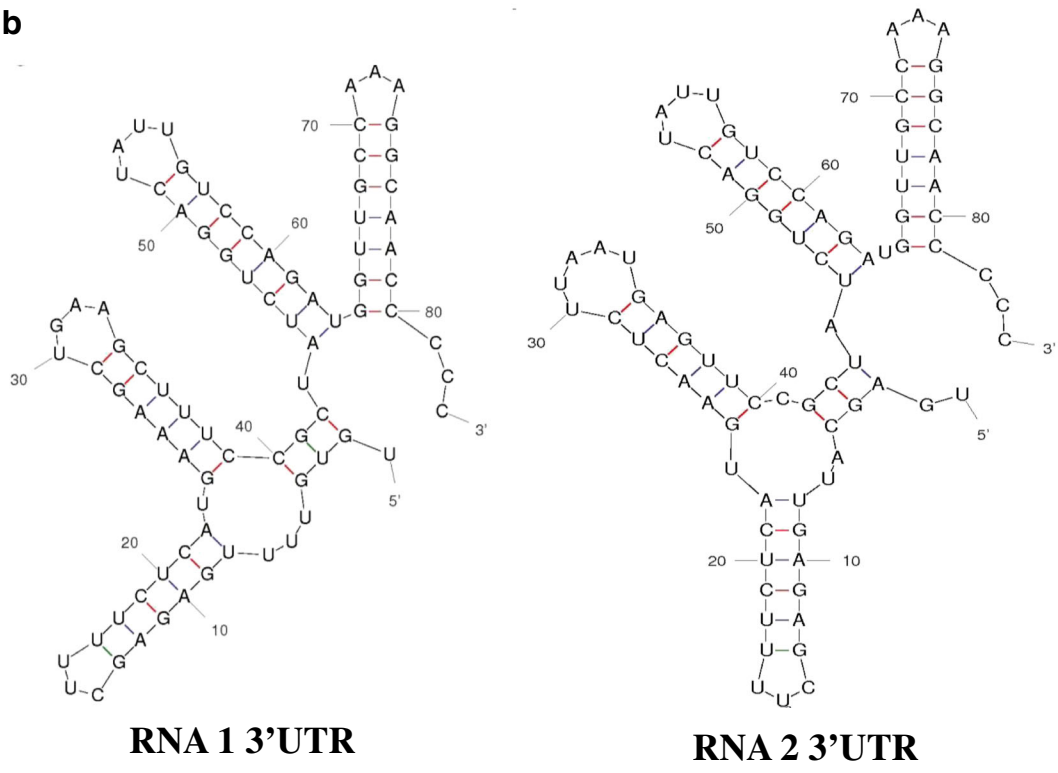

Fig. 2 Comparison of 3′ untranslated regions (UTRs) of genomic RNAs of blackcurrant idaeovirus (BCIV). **a** Conserved nucleotides at the 3′ UTRs. * indicates identical nucleotides. **b** Identical secondary structures at the 3'UTRs of genomic RNA 1 and RNA 2 of BCIV derived by MFOLD (Zuker 2003)

and subgenomic RNAs. Using this approach we aimed to preferentially amplify the RNA1 region (shorter amplicon) as to balance the difference in concentration of the template molecules. The products obtained from single and duplex PCRs were sequenced to verify the viral nature of the products.

BCIV has the typical organization of members of the genus *Idaeovirus* (Fig. 1b) with all three fully characterized viruses (BCIV, PrLBaV and RDBV) having conserved nucleotide regions at the 5′ and 3′ termini and 3′ UTRs folding to similar stem-loop structures (Natsuaki et al. 1991; Ziegler et al. 1992; Navarro et al. 2016). This suggests a vital role for these sequences and structures in protein expression and replication of idaeoviruses. The phylogenetic analysis based on multi gene sequences consistently grouped BCIV with idaeoviruses (RBDV, PrLBaV and CIV) confirming its taxonomic position in the genus (Fig. 3).

There are similarities between members of the genus *Idaeovirus* and family *Bromoviridae* including segmented genomes, protein expression (CP expressed through a subgenomic RNA; Natsuaki et al. 1991; Quito-Avila et al. 2014), particle properties and pollen/seed transmission (Natsuaki et al. 1991). However, phylogenetic

Table 1 Percentage (%) of amino acid identities in different proteins between blackcurrant idaeovirus (BCIV) and other members of the genus *Idaeovirus*

Proteins/Domains	RBDV	PrLBaV	CIV
Polyprotein	46%	66%	NA
MTR	54%	70%	NA
HEL	64%	75%	NA
RdRp	60%	78%	NA
MP	21%	61%	NA
CP	23%	61%	21%

Accession numbers given in the brackets for each virus are for polyprotein, movement protein (MP) and coat protein (CP) sequences respectively

MTR, HEL and RdRp values indicate the amino acid identities at those conserved domains in the polyproteins of respective viruses

RBDV raspberry bushy dwarf virus (NP_620465.1, BAV13382.1 and BAV13380.1), *PrLBaV* privet leaf blotch-associated virus (YP_009305430.1, YP_009305432.1 and YP_009305431.1), *CIV* citrus idaeovirus (CP accession No. AAY98796.1), *MTR* methyltransferase, *HEL* helicase, *RdRp* RNA dependent RNA polymerase, *NA* not available

analysis showed that the two groups are quite distinct in evolutionary terms (Fig. 3) with all idaeoviruses clustering together and forming a monophyletic group unlike the genera in the *Bromoviridae*.

Several NCGR blackcurrant accessions were tested with two found infected with the virus (data not shown). The role of BCIV in disease development is unknown as the accessions infected (CRIB 1057.001 and CRIB 9003.001; USDA-NPGS 2016) were infected by multiple viruses (Ho et al. 2015b). Yet, even if the virus was to be asymptomatic in single infection, the effect of individual species in mixed infections, situations that are rather common in berry crops (Martin and Tzanetakis 2013; Martin et al. 2013) could lead to major losses for producers.

The RT-PCR tests developed from the conserved 3′ UTR simultaneously amplified regions of both genomic RNAs (Fig. 4). The advantage of this approach is that it minimizes the potential of false negatives due to virus diversity as amplification of even one region verifies

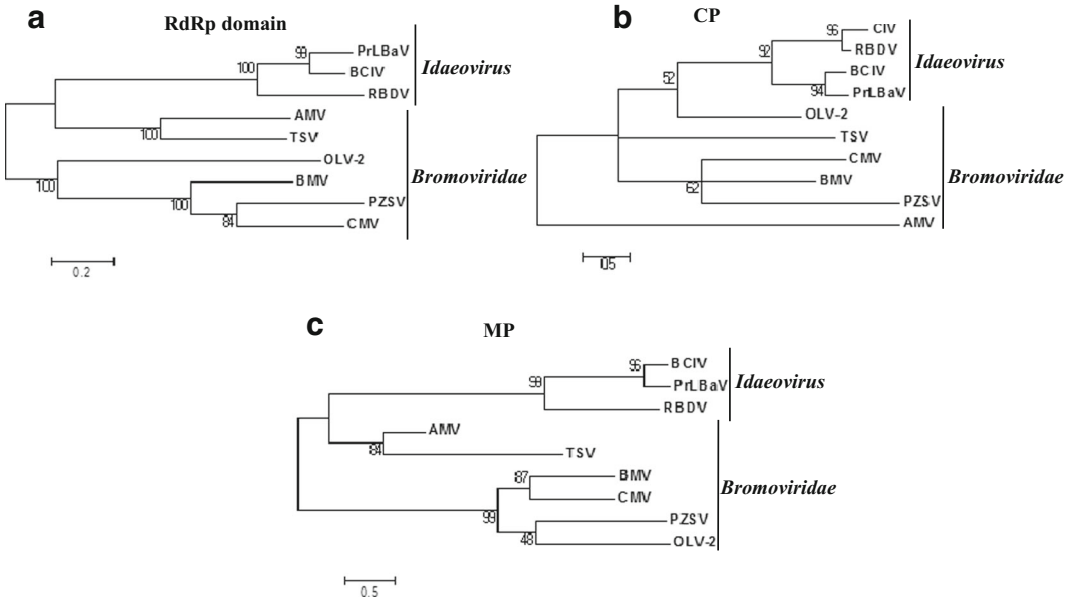

Fig. 3 Phylogenetic relationship of blackcurrant idaeovirus (BCIV) with other members of the genus and representative members of *Bromoviridae* family. Phylogenetic analysis was performed using the conserved domains of RNA-dependent RNA polymerase protein sequences (**a**), coat protein (**b**) and putative movement protein (**c**) sequences. The trees were generated by the maximum likelihood method and bootstrap values (indicated for each branch node) were estimated using 1000 pseudoreplicates. Branch lengths are proportional to genetic distances between sequences and the scale bar represents substitutions per amino acid site. RBDV- raspberry bushy dwarf virus (NP_620465.1, BAV13382.1 and BAV13380.1); PrLBaV- privet leaf blotch-associated virus (YP_009305430.1, YP_009305432.1 and YP_009305431.1); CIV-citrus idaeovirus (CP accession No. AAY98796.1, the only complete protein sequence available in GenBank); BMV- brome mosaic virus (NP_041197.1, AAA46334.1 and BAD83845.1); AMV- alfalfa mosaic virus (ADO85716.1, AAA46297.1 and ADO85717.1); PZSV- pelargonium zonate spot virus (AHG25372.3, AHG25374.4 and AHG25373.3); CMV- cucumber mosaic virus (AEV40477.1, AAX70969.1 and AAL05586.1); TSV- tobacco streak virus (NP_620768.1, ABI81752.1 and AGT58200.1); OLV-2- olive latent virus 2 (Q83944.1, NP_620039.1 and CAM82796.1). Accession numbers given in the brackets for each virus are for the RdRp domain, CP and MP sequences respectively

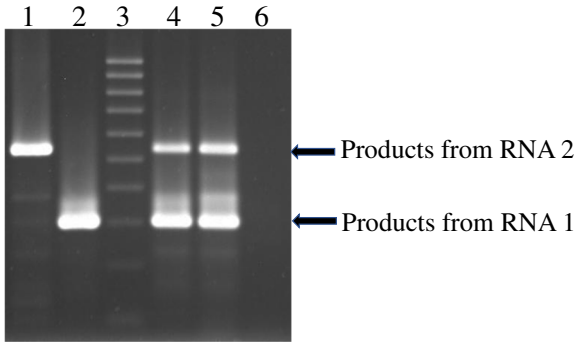

Fig. 4 Reverse transcription-PCR detection of blackcurrant idaeovirus (BCIV) genomic segments using single and duplex PCRs. Lanes 1, 2: PCR amplicons of BCIV RNA 1 (329 bp) and RNA 2/subgenomic RNA (559 bp) respectively; lanes 4, 5: Duplex PCR amplicons from BCIV (cDNAs from two infected plants) using the forward primers for both RNAs and a common reverse primer. Lane 3: 100 bp ladder; lane 6: BCIV-free blackcurrant control

presence of the virus. The possibility of having polymorphisms in both fragments simultaneously is narrow making the test an excellent tool for screening mother plants before they enter the nursery system as well as in production fields. As a putative idaeovirus, BCIV is presumed to be pollen borne and for this reason early detection and roguing of infected material is essential for minimizing the spread in production fields and safeguarding the crop.

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While this paper was accepted with revisions and awaiting the final review by the handling editor, the article 'Complete genome sequence and analysis of blackcurrant leaf chlorosis associated virus, a new member of the genus Idaeovirus' by James and Phelan was published online as an Online First Article in Archives of Virology. After pairwise comparisons we have verified that the two viruses are isolates of the same species.