DNA Barcodes Suggest Cryptic Speciation in Dasineura oxycoccana (Diptera: Cecidomyiidae) on Cranberry, Vaccinium macrocarpon, and Blueberry, V. corymbosum

Authors: Mathur, Snehlata, Cook, Melissa A., Sinclair, Bradley J., and Fitzpatrick, Sheila M.

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DNA BARCODES SUGGEST CRYPTIC SPECIATION IN *DASINEURA OXYCOCCANA* (DIPTERA: CECIDOMYIIDAE) ON CRANBERRY, *VACCINIUM MACROCARPON*, AND BLUEBERRY, *V. CORYMBOSUM*

Snehlata Mathur[1], Melissa A. Cook[2], Bradley J. Sinclair[3] and Sheila M. Fitzpatrick[1]
[1]Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, PO Box 1000, Agassiz, BC, Canada V0M 1A0

[2]Simon Fraser University, Department of Biological Sciences, 8888 University Drive, Burnaby, BC, Canada V5A 1S6

[3]Canadian Food Inspection Agency, Entomology – Ottawa Plant Laboratory, 960 Carling Avenue, Ottawa, ON, Canada KIA 0C6

Abstract

Dasineura oxycoccana (Johnson) (Diptera: Cecidomyiidae) from cranberry *Vaccinium macrocarpon* is similar morphologically to *D. oxycoccana* from blueberry *V. corymbosum*, but a recent study revealed that individuals from cranberry do not mate with those from blueberry. To seek genetic differences between *D. oxycoccana* from cranberry (common name cranberry tipworm) and from blueberry (common name blueberry gall midge), we compared a 559-bp region of the mitochondrial cytochrome oxidase I (COI) gene from 65 individuals. Analysis of the COI sequences based on general time reversible with gamma distribution (GTRþG) distance model revealed 10.7-13.1% divergence between cranberry tipworm and blueberry gall midge, whereas little divergence was observed within cranberry tipworm (0-1.2%) or blueberry gall midge (0-1.3%) sequences. In neighbour-joining analysis, conducted for species identification, blueberry gall midge sequences generated in this study clustered with known *D. oxycoccana* sequences from National Council of Biotechnology (NCBI), but the cranberry tipworm sequences grouped into a separate cluster. To identify and discriminate cranberry tipworm and blueberry gall midge, we developed diagnostic PCR primers based on COI sequence differences. In a duplex PCR assay, these primers successfully discriminated *D. oxycoccana* originating from cranberry or blueberry. The concordance between data from our genetic studies and data from mating experiments by Cook et al. (2011) suggests cryptic speciation in *D. oxycoccana* populations on cranberry and blueberry.

Key Words: cranberry tipworm, blueberry gall midge, mtDNA, cytochrome oxidase subunit I gene, host-associated differentiation

Resumen

Dasineura oxycoccana (Johnson) (Diptera: Cecidomyiidae), una plaga de los arándanos rojo, *Vaccinium macrocarpon*, es similar morfológicamente a *D. oxycoccana* que es una plaga del arándano azul, *V. corymbosum*, pero un estudio reciente reveló que los individuos del arándano rojo no se acoplan con los del arándano azul. Se comparó una región de 559-pb del gene mitocondrial de citocromo oxidasa I (COI) de 65 individuos para buscar diferencias genéticas entre los de *D. oxycoccana* del arándano rojo (con el nombre común del gusano de los brotes de arándano rojo) y los del arándano azul (con el nombre común del cecidomido de agallas de arándano azul). El análisis de las secuencias COI sobre la base de tiempo general reversible con el modelo de distancia de distribución gamma (GTRþG) reveló 10.7-13.1% divergencia entre los *D. oxycoccana* del arándano rojo y los de *D. oxycoccana* del arándano azul, mientras que se observó poca divergencia en las secuencias entre individuos de *D. oxycoccana* del arándano rojo (0-1.2%) o entre los de *D. oxycoccana* del arándano azul (0-1.3%). En el análisis de reunión por vecindad, realizado para la identificación de especies, las secuencias generadas por *D. oxycoccana* del arándano azul en este estudio se agruparon con secuencias conocidas de *D. oxycoccana* del Consejo Nacional de Biotecnología (NCBI), pero las secuencias del *D. oxycoccana* del arándano rojo se agruparon en un grupo aparte. Se desarrollaron PCR primers diagnóstico basados sobre las diferencias en la secuencia de COI para identificar y discriminar los *D. oxycoccana* del arándano rojo y los *D. oxycoccana* del arándano azul. En un ensayo de PCR dúplex, estos primers discriminaron éxitosamente los *D. oxycoccana* procedentes de arándano rojo o de arándano azul. La concordancia entre los datos de nuestros estudios genéticos y los datos de experimentos de apareamiento por Cook et al. (2011) sugiere que hay especiación críptica en las poblaciones de *D. oxycoccana* sobre arándano rojo y del arándano azul.

The gall midge *Dasineura oxycoccana* (Johnson) (Diptera: Cecidomyiidae) has been recorded in North America from cultivated *Vaccinium* species including cranberry *V. macrocarpon* (Gagné 1989; Landry et al. 2002; Mahr 2005); highbush blueberry *V. corymbosum* (Sampson et al. 2002; Yang 2005); rabbiteye blueberry *V. ashei* (Lyrene and Payne 1995; Sarzynski & Liburd 2003); and lowbush blueberry *V. angustifolium* (Dernisky et al. 2005). On cranberry, *D. oxycoccana* is commonly called cranberry tipworm; on blueberry, it is called blueberry gall midge (Sampson et al. 2006; Fitzpatrick 2009). Recently, blueberry gall midge has been reported on highbush blueberries in Italy (Bosio et al. 1998) and in Britain (Collins & Eyre 2010; Collins et al. 2010). On cranberry and blueberry, adult female *D. oxycoccana* oviposit in developing buds, and larvae feed on meristem tissue within the buds. Larval damage to buds can result in serious economic loss to the crop (Sampson et al. 2002; Mahr 2005).

In British Columbia, Canada, the first records of economic damage caused by *D. oxycoccana* came from young highbush blueberry plants east of Mission (49°12'58"N, 122°14'11"W) in 1991 (Fitzpatrick, unpublished). Adults reared from field-collected larvae were identified by R. J. Gagné (R. J. Gagné, USDA, Systematic Entomology Lab c/o Smithsonian Institution, personal communication). In 1991, *D. oxycoccana* was also observed on highbush blueberry in Richmond, British Columbia, Canada, (49°10'16"N, 123°5'40"W) but was not present on nearby cranberry farms (Fitzpatrick, unpublished). In 1998, *D. oxycoccana* was detected for the first time on cranberry in BC, in Pitt Meadows (49°13'15" N, 122°41'25"W) (British Columbia Ministry of Agriculture and Lands 2009). This insect is now widespread on highbush blueberry and cranberry in BC, and is of particular concern to cranberry growers, who wish to know if blueberry farms are the source of *D. oxycoccana* infestation on cranberry.

A recent study of phenology, life history and reproductive behavior of *D. oxycoccana* on cranberry and blueberry in BC revealed differences in characteristics of this insect on the 2 crops (Cook 2011; Cook et al. 2011). Under controlled conditions, *D. oxycoccana* from cranberry (cranberry tipworm) did not mate with *D. oxycoccana* from blueberry (blueberry gall midge) (Cook et al. 2011). This result suggests that, although *D. oxycoccana* infesting cranberry and blueberry are virtually identical morphologically, the 2 populations probably represent cryptic species (Cook et al. 2011); that is, 2 distinct species classified as a single nominal species because they are morphologically indistinguishable (Bickford et al. 2007).

Accurate species identifications are crucial for the identification of invasive and pest species (Bickford et al. 2007). In the case of *D. oxycoccana* in BC, results from Cook et al. (2011) raise the possibility that cranberry tipworm arrived later than blueberry gall midge, possibly as a hitchhiker on plant material from other cranberry-growing regions. To further explore the relationship between *D. oxycoccana* on cranberry and blueberry in British Columbia, we examined the cytochrome oxidase I (COI) region of mitochondrial DNA, which is a popular marker for studying differences among populations or host races (Kambhampati & Smith 1995; Shirota et al. 1999; Sperling et al. 1999). DNA barcodes from the COI region have been used in many studies to successfully diagnose cryptic diversity (Hebert et al. 2004; Smith et al. 2006; Li et al. 2010; Lumley & Sperling 2010).

Here we show differences between the COI region of the mitochondrial gene of *D. oxycoccana* reared from cranberry farms and COI region of *D. oxycoccana* reared from highbush blueberry farms in British Columbia. Based on these genetic differences, we developed diagnostic primers to detect and differentiate *D. oxycoccana* originating from cranberry or blueberry.

Materials and Methods

Insects

Cranberry (*V. macrocarpon*) shoots infested with cranberry tipworm larvae and highbush blueberry (*V. corymbosum*) shoots infested with blueberry gall midge larvae were collected from 3 cranberry and 2 blueberry farms in Pitt Meadows, British Columbia, Canada in 2009 and 2010. To rear cranberry tipworm larvae to adult, infested cranberry shoots were placed individually into containers made from 2 glass scintillation vials, such that the stem end of the shoot pierced a parafilm seal and rested in water, while the infested tip was covered with an inverted scintillation vial. Infested blueberry shoots were placed on moist soil (60% peat 40% perlite) in 1-liter plastic buckets covered with a chiffon screen (mesh approx. 40/cm) and moist paper towel. All reared insects were maintained at 21 ± 2 °C under fluorescent lighting at 16:8 h L:D. Emerged adults were stored in 70% ethanol for identification by Sinclair or in 95% ethanol for DNA extraction by Mathur. Voucher specimens were deposited at the Canadian National Collection (CNC) of Insects, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada.

Isolation of Genomic DNA

Genomic DNA was isolated from individual cranberry tipworm and blueberry gall midge adults with a DNeasy tissue kit® (Qiagen Inc., Mississauga, Ontario). Ten to 16 individuals from each farm (total 34 individuals from cranberry

and 31 from blueberry) were used for DNA extraction. Each insect was air dried to remove ethanol, homogenized using a disposable microtube pestle (Mandel Scientific Company Inc., Guelph, Ontario) in the extraction buffer provided with the kit, and then processed using the manufacturer's proprietary protocol. DNA was eluted in 50 µL eluting buffer and stored at -80 °C for further use.

Polymerase Chain Reaction (PCR) and Sequencing of DNA

The COI region of the mitochondrial genome of cranberry tipworm and blueberry gall midge was amplified by PCR using the universal insect primer set, LCO 1490 (5'GGTCAACAAATCATAAAGATATTGG) and HCO 2198 (5'TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al. 1994). The 50-µL reaction mix contained 1X PCR buffer solution (GeneSys Ltd., Medicorp Inc., Montreal, Quebec), 0.2 mM of dNTPs, 0.2 µM of each primer, 1.25 U of T*aq* DNA polymerase (GeneSys Ltd., Medicorp Inc., Montreal, Quebec), and 30-50 ng of DNA template. DNA amplification was performed using a thermal cycler (iCycler, Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario). The amplification conditions for all PCR reactions were as follows: an initial denaturing step of 2 min at 94 °C followed by 35 cycles consisting of 30 s at 94 °C, 45 s at 50 °C, 60 s at 72 °C, and a final extension step of 7 min at 72 °C. The amplified fragment was analyzed in a 1.5% agarose gel by electrophoresis in 1XTBE buffer (90 mM Tris-borate, 2 mM EDTA), using 2 µL of the PCR mixture, then followed by staining with ethidium bromide (0.5 µg/mL) and visualizing with a UV transilluminator (260 nm). The amplified products were purified with the QIAquick® PCR purification kit (Qiagen Inc., Mississauga, Ontario). Sequencing of the COI region was done by the Nucleic Acid and Protein Synthesis Unit (NAPS) at the University of British Columbia, Vancouver, British Columbia, using an Applied Biosystem sequencer and the universal insect primers described above. All sequences were confirmed by sequencing in both directions. Sequences were checked for errors and for sequence variation within cranberry and blueberry populations. Sequences from cranberry tipworm and blueberry gall midge were deposited in the National Center for Biotechnology Information (NCBI) GenBank with the accession numbers HQ542180-HQ542185 and HQ542186-HQ542191, respectively.

DNA Sequence Analysis

To determine the percentage genetic divergence (%D) within and between sequences from the 2 populations, the sequences generated in this study were compared with those of blueberry gall midge reported from Britain (Collins et al. 2010) and 5 other *Dasineura* species (Table 1) obtained from NCBI. The COI sequences of cranberry tipworm, blueberry gall midge and the other *Dasineura* spp. were aligned using CLUSTAL W (Accelrys Gene 2.5; Accelrys®, Inc., San Diego, California). To determine a model of DNA substitution that fits the COI data set, Modeltest 3.0 (Posada & Crandall 1998) was applied to the data set. The Akaike criteria of Modeltest selected the general time reversible model with gamma distribution (GTRþG) as the best fit for the COI sequence data (base frequencies: $\pi A = 0.3455$, $\pi C = 0.1372$, $\pi G = 0.1118$, $\pi T = 0.4055$; substitution rates: A/C = 9.11, A/G = 55.4044, A/T = 19.8145, C/G = 26.1111, C/T = 55.4044, G/T = 1.0000; gamma distribution shape parameter = 0.2543). The observed distances, obtained from multiple sequence alignments were used to generate a distance matrix using the GTRþG correction model and neighbour-joining (NJ) (Saitou & Nei 1987) distance method. The NJ analysis was performed to determine genetic distances and to construct a phylogenetic tree using PAUP* version 4.0b10b software (Swofford 2002) and 1000 bootstrap replicates (Felsenstein 1985). FigTree v1.1.2 (Rambaut 2007) was used to view the NJ tree.

Primer Design and PCR Analysis

From the differences between cranberry tipworm and blueberry gall midge sequences, diagnostic PCR primer sets were developed that distinguish the two populations. The primer sites were searched based on variable nucleotide positions between sequences of cranberry tipworm and blueberry gall midge, where no variation was observed within cranberry tipworm or blueberry gall midge sequences. From these primer sites, the positions unique for cranberry tipworm or blueberry gall midge were used to develop specific diagnostic primer sets. Several primers were evaluated for suitable base composition, annealing temperature (T_m), and self compatibility using Primer3 (Rozen & Skaletsky 2000). Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa) and the T_m of the primers

Table 1. Cecidomyiids (diptera) used in this study and their accession numbers from the NCBI Genbank.

Genus species	Accession number
Dasineura carbonaria Felt	EU375703
Dasineura folliculi Felt	EU375702
Dasineura oxycoccana Johnson	FR694171
Dasineura oxycoccana Johnson	FR694172
Dasineura oxycoccana Johnson	FR694174
Dasineura rosae Bremi	AB505984
Dasineura strobila Dorchin	EU375687
Dasineura tomentosa Dorchin	EU375694

was determined from the specification sheet provided by IDT. Various primer combinations were first tested for PCR specificity for cranberry tipworm and blueberry gall midge sequences using a singleplex PCR assay. Amplicon size was estimated based on comparison with 100-bp ladder (Invitrogen Canada Inc., Burlington, Ontario). The most suitable primers were then selected based on the annealing temperatures and the fragment size of the amplicons so that they could be used simultaneously in a duplex PCR assay, with the same temperature program (Table 2). PCR conditions and visualization of PCR products were as described previously except that the annealing temperature used was 52 °C.

In a duplex PCR assay, the diagnostic primers specific for cranberry tipworm and blueberry gall midge were mixed in a single reaction tube. PCR conditions and visualization of PCR product were as described previously. The annealing temperature (52 °C) for these 2 primer sets used in combination allowed both primer sets to give specific products for cranberry tipworm or blueberry gall midge DNA.

Results

Analysis of COI Gene from Cranberry Tipworm and Blueberry Gall Midge

DNA from 34 cranberry tipworms and 31 blueberry gall midges successfully amplified with the universal insect primer set. Based on agarose gel separation, all tested specimens produced PCR amplicons of equal size (~700 bp). Sequences generated from these amplicons were 625 bp in size and showed a strong A+T bias (70-71%; Fig. 1). Out of 34 sequences generated from cranberry tipworm and 31 from blueberry gall midge individuals, a total of 12 sequences showing nucleotide variations but originating from different cranberry and blueberry farms in Pitt Meadows, British Columbia, were submitted to NCBI.

Out of 6 sequences from different cranberry farms (HQ542180, HQ542181, HQ542182, HQ542183, HQ542184 and HQ542185), sequences HQ542181 and HQ542183; HQ542180 and HQ542184; and HQ542182 and HQ542185 were, respectively, identical. Out of 6 blueberry gall midge sequences submitted to NCBI (HQ542186, HQ542187, HQ542188, HQ542189, HQ542190 and HQ542191) from different farms, the 3 sequences HQ542187, HQ542188 and HQ542190 were identical. The identical sequences were removed and 3 sequences from cranberry tipworm (HQ542180, HQ542181 and HQ542182) and 4 from blueberry gall midge (HQ542186, HQ542187, HQ542189 and HQ542191) showing nucleotide variations were selected for subsequent analysis. To accommodate the shorter sequences from blueberry gall midge (British population) obtained from NCBI in the alignment for NJ analysis, all other sequences used in this study were trimmed down at the terminal ends. The length of the aligned sequences was 559 bp; the alignment was gap-free.

Alignment of sequences obtained in this study and those obtained from NCBI GenBank showed high percentage genetic divergence (%D = 10.7-13.1%) between cranberry tipworm (Table 3: #1, 2 and 3) and blueberry gall midge (Table 3: #4, 5, 6 and 7) but low divergence within cranberry tipworm (%D = 0-1.2%) and blueberry gall midge (%D = 0-1.3). The population of blueberry gall midge from Pitt Meadows, British Columbia, was genetically similar (%D = 0-1.3%) to the British population of *D. oxycoccana* (Table 3: #8, 9 and 10) on highbush blueberry. Cranberry tipworm and blueberry gall midge sequences showed a high %D (22.3-34.8%) from other species of *Dasineura* (Table 3: #11 to 16) available on NCBI website.

In the neighbor-joining tree, the sequences from cranberry tipworm, blueberry gall midge and other *Dasineura* species from NCBI GenBank separated into 3 distinct clusters: blueberry gall midge from Pitt Meadows, British Columbia, and from Britain; cranberry tipworm from Pitt Meadows, British Columbia; and the 5 other species of *Dasineura* from GenBank (Fig. 2). Distinction of blueberry gall midge from cranberry tipworm was supported by high bootstrap values (100%). Cranberry tipworm and blueberry gall midge separated from other *Dasineura* species with 58% and 84% bootstrap values. Two of the blueberry gall midge sequences reported in this study are identical to the British sequences. The Canadian blueberry gall midge sequence HQ542186 is identical to sequence FR694174 of British blueberry gall midge, and the Canadian blueberry gall midge

Table 2. Specific primers developed in this study to differentiate *D. oxycoccana* from cranberry (CB) or blueberry (BB). F = forward; R = reverse.

Primer	Sequence (5'-3')	Position (bp*)	Annealing temperature (°C)
CBF	TTGTTACTGCTCATGCATTTATT	1638-1660	51.1
CBR	TTGAAAGGTCTACCGAACAC	1907-1888	52.3
BBF	TCGAATAGAATTAAGAGGCGTAA	1578-1600	51.4
BBR	CGGCTAAAACTGGTAATGATAAAAG	2078-2054	52.4

*Position on *Drosophila yakuba*.

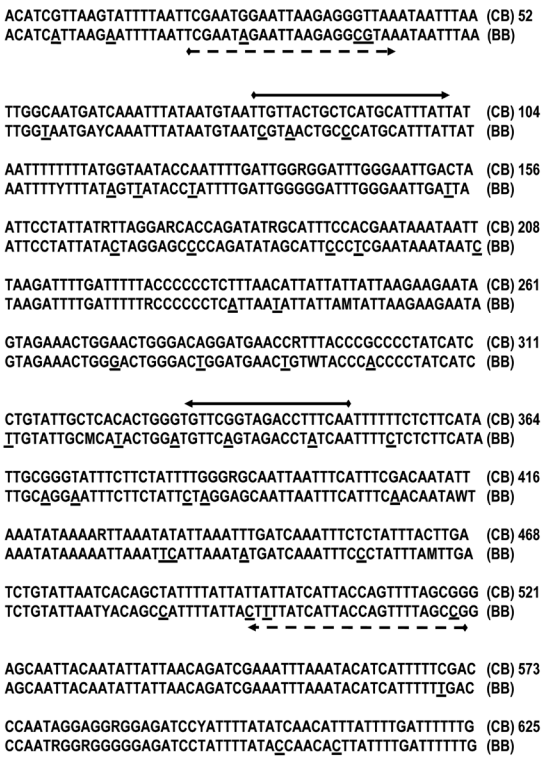

Fig. 1. Alignment (5' to 3') of the partial cytochrome oxidase I region of the mitochondrial gene of *Dasineura oxycoccana*, commonly called cranberry tipworm (CB) on cranberry and blueberry gall midge (BB) on blueberry. Differences (46) in base pairs between CB and BB sequences are underlined. The IUPAC codes (R, Y, W, M) reflect differences (19) within CB or BB sequences. These 19 differences were avoided during primer selection. The solid arrows indicate forward and reverse CB-specific primers; dashed arrows indicate forward and reverse BB-specific primers.

sequence HQ5421191 is identical to the British blueberry gall midge sequence FR694171 (Fig 2).

Molecular Identification of *D. oxycoccana* from Cranberry or Blueberry

The location of the cranberry tipworm and blueberry gall midge specific primers selected in this study are shown (Table 2) in relation to the *Drosophila yakuba* mitochondrial genome (Clary & Wolstenholme 1985). For the insects whose complete mitochondrial region has not been sequenced, mitochondrial genome organization of *D. yakuba* is generally taken as a standard to show the primer positions. When DNA from 34 cranberry tipworms and 31 blueberry gall midges was tested in a singleplex PCR assay, the specific primer set CBF and CBR (Table 2) developed for cranberry tipworm produced a 270-bp fragment from cranberry tipworm DNA, and no fragment from blueberry gall midge DNA. The specific primer set BBF and BBR (Table 2) developed for blueberry gall midge produced a 500-bp fragment from DNA of blueberry gall midge and no fragment from cranberry tipworm DNA. These specific primer sets were selected for a duplex PCR assay because of their similar annealing temperatures and different PCR product size. For the duplex PCR assay, the primer sets CBF-CBR and BBF-BBR were used simultaneously in a PCR mix and tested on DNA from cranberry tipworm and blueberry gall midge individuals from British Columbia. DNA from the cranberry tipworms produced a 270-bp fragment (Fig. 3: lanes 1-5); DNA from the blueberry gall midges produced a 500-bp fragment (Fig. 3: lanes 6-10) in the duplex assay. Using this duplex PCR assay, detection and differentiation of cranberry tipworm from blueberry gall midge can be achieved in a single reaction.

Discussion

There is substantial genetic variability between the mtDNA COI gene region of *D. oxycoccana* collected from British Columbia cranberry farms (cranberry tipworm) and the mtDNA COI gene region of *D. oxycoccana* collected from British Columbia blueberry farms (blueberry gall midge). The separation of cranberry tipworm from blueberry gall midge populations is supported by NJ analysis at high bootstrap value with high average percentage (10.7-13.1%) of genetic divergence between them based on GTRpG distance matrix method. Other species of *Dasineura* separated from both cranberry tipworm and blueberry gall midge sequences, which shows that cranberry tipworm is very different genetically from blueberry gall midge as well as from other *Dasineura* species recorded in the NCBI GenBank. The populations of blueberry gall midge from British Columbia, and from Britain grouped together in the NJ analysis. Some Canadian and British sequences were identical, which strongly suggests that these 2 populations are the same species and confirms the identity of the blueberry gall midge sequences generated in this study.

The host plant of *D. oxycoccana* populations in British Columbia can be identified as cranberry or blueberry through use of the duplex PCR assay developed in the present study, even though adults from the 2 host-plant populations cannot be distinguished on the basis of morphological characters (Sinclair, this study). Genetic separation of the 2 populations supports the conclusion of Cook et al. (2011) that *D. oxycoccana* populations on cranberry and blueberry in British Columbia are reproductively isolated and probably represent cryptic species (per: Bickford et al. 2007; Schonrogge et al. 2002).

Molecular analysis using the COI region of mtDNA has revealed cryptic species in many in-

TABLE 3. PERCENT SEQUENCE DIVERGENCE (%D) BETWEEN SPECIES OF *DASINEURA* (DIPTERA: CECIDOMYIIDAE) BASED ON GENERAL TIME REVERSIBLE WITH GAMMA DISTRIBUTION MODEL (GTRÞG) FOR COI MTDNA SEQUENCES.

		% Divergence between numbered accessions														
Source	Numbered accessions*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cranberry Canada	1. DO HQ542180	0.0														
	2. DO HQ542181	1.2	0.0													
	3. DO HQ542182	0.6	0.6	0.0												
Blueberry Canada	4. DO HQ542186	11.2	10.7	10.9	0.0											
	5. DO HQ542187	13.1	12.5	12.8	1.3	0.0										
	6. DO HQ542189	12.5	11.9	12.2	1.1	0.9	0.0									
	7. DO HQ542191	12.6	12.0	12.3	0.9	0.7	0.2	0.0								
Blueberry Britain	8. DO FR694171	13.0	12.4	12.7	1.1	0.9	0.4	0.2	0.0							
	9. DO FR694172	12.6	12.0	12.3	0.9	0.7	0.2	0.0	0.2	0.0						
	10. DO FR694174	11.2	10.7	10.9	0.0	1.3	1.1	0.9	1.1	0.9	0.0					
Other *Dasineura*	11. DF EU375702	26.5	26.0	25.9	29.0	28.6	28.6	28.6	29.2	28.6	29.0	0.0				
	12. DR AB505984	33.8	34.8	34.3	31.5	31.3	31.3	31.7	31.7	31.7	31.5	32.9	0.0			
	13. DC EU375703	24.1	23.1	23.6	23.1	24.4	24.0	24.1	24.6	24.1	23.1	17.8	30.3	0.0		
	14. DT EU375694	23.9	23.9	24.3	22.4	24.0	23.8	23.4	24.0	23.4	22.4	28.3	33.8	17.3	0.0	
	15. DS EU375687	24.9	24.0	24.5	22.3	23.5	23.9	23.5	24.1	23.5	22.3	28.4	36.2	19.4	9.3	0.0

*DO = *Dasineura oxycoccana*; DF = *D. folliculi*; DR = *D. rosae*; DC = *D. carbonaria*; DT = *D. tomentosa*; DS = *D. strobila*.

sects including mayflies *Baetis vernus* (Stahls & Savolainen 2008) and flower beetle (Blair et al. 2005). Hebert et al. (2004) differentiated 10 cryptic species in a single morphologically identified skipper butterfly species which differed in their larval appearance, food plants and habitat preference. Similarly, Smith et al. (2006) differentiated morphologically indistinguishable parasitoid flies (Tachinidae) to groups of separate host-specific cryptic species.

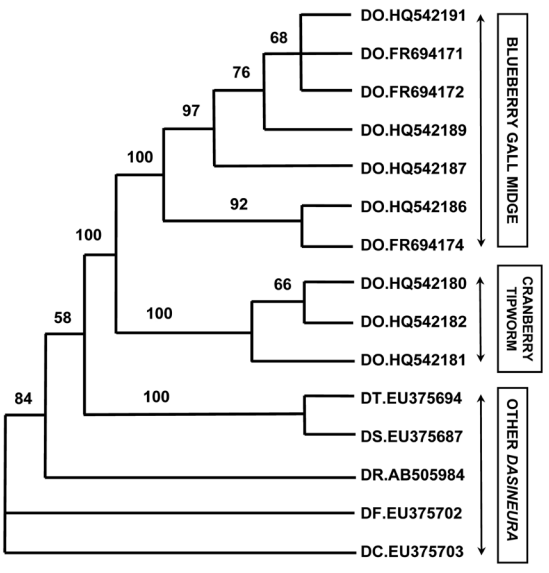

Fig. 2. Neighbor-joining tree based on GTRþG distance of partial COI mtDNA sequences: DO = *Dasineura oxycoccana*; DF = *D. folliculi*; DR = *D. rosae*; DC = *D. carbonaria*; DT = *D. tomentosa*; DS = *D. strobila.* DO accessions containing FR are from Britain; all other DO accessions are from British Columbia, Canada. Percent bootstrap scores are indicated along the branches.

Cook et al. (2011) postulated that *D. oxycoccana* has diverged into 2 specialist species on cranberry and blueberry. Our genetic data support this idea, but do not provide information on the direction or time-frame of divergence. The gall-midge *D. folliculi*, which shows host-associated differentiation (possibly cryptic speciation) on 2 species of goldenrod, is thought to have shifted from *Solidago rugosa* to *S. gigantea*, according to evidence provided by genetic data, oviposition preference, reproductive success and natural-enemy attack (Dorchin et al. 2009). In the case of *D. oxycoccana* in British Columbia, it is extremely unlikely that a host-shift from blueberry to cranberry took place in British Columbia during the 1990's. A more probable scenario is that *D. oxycoccana* on cranberry (cranberry tipworm) is a cryptic invader that arrived on plant material imported from another region of North America. Cranberry is not native to British Columbia, therefore during industry expansion in the 1990's, some new or more vigorous cultivars were imported. Host-divergence or host-shifting among populations of *D. oxycoccana* could have occurred where *V. macrocarpon* and *V. corymbosum* are native, in eastern North America (VanderKloet 1988).

Our study opens the door to a comprehensive examination of genetic and morphological variation among *D. oxycoccana* populations in cranberry- and blueberry-growing regions of North America, particularly the states of Florida, Massachusetts, Michigan, New Jersey, Oregon, Wash-

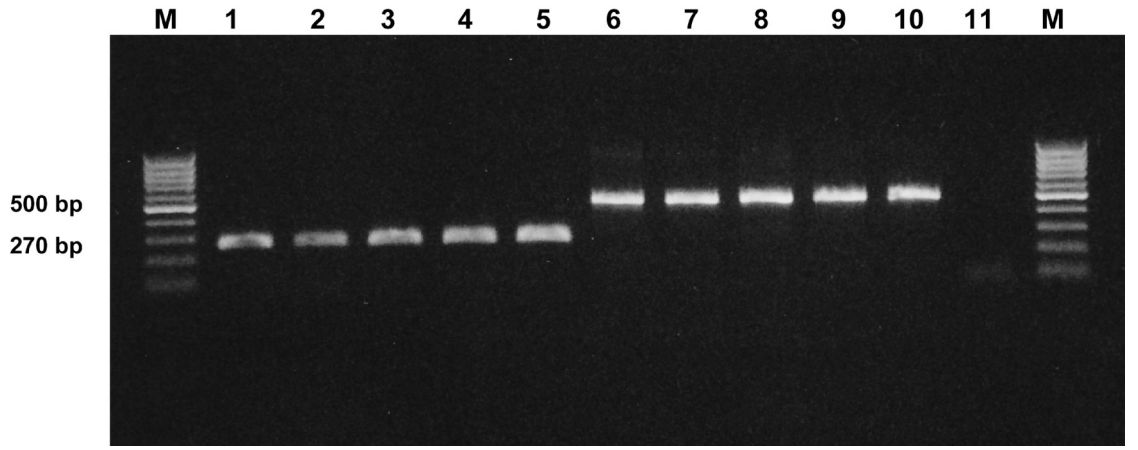

Fig. 3. Specificity of the duplex PCR assay for differentiating cranberry tipworm from blueberry gall midge; DNA from individuals was targeted using 2 primer sets simultaneously that amplified a 270-bp fragment from cranberry tipworm and a 500-bp fragment from blueberry gall midge. Lane M: 100-bp marker; lanes 1-5: individuals from cranberry tipworm; lanes 6-10: individuals from blueberry gall midge; lane 11: blank.

ington, Wisconsin, and the provinces of British Columbia and Quebec. We anticipate greater genetic variability among *D. oxycoccana* from the species and hybrids of commercially harvested blueberry (Hancock et al. 2008) than among *D. oxycoccana* from cranberry cultivars, which are not far removed from native selections (Rodriguez-Saona et al. 2011). Our duplex PCR assay will be a valuable tool, and we recognize that the use of other molecules, like nuclear genes, would provide additional information on the relationships between *D. oxycoccana* on *Vaccinium* host plants.

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