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SHORT COMMUNICATION

Relationships among blueberry species within the section Cyanococcus of the *Vaccinium* genus based on EST-PCR markers

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Abstract: Commercial blueberry species of North America belong to the *Vaccinium* genus, section Cyanococcus. Phylogenetic relationships of 50 accessions of different ploidy levels within Cyanococcus were investigated using 249 expressed sequence tag-polymerase chain reaction markers and standard clustering methods. Of the commercial species, tetraploid *V. corymbosum* grouped most closely with the diploids, *V. fuscatum* and *V. caesariense*, followed by the diploid *V. elliottii*. Tetraploid *V. angustifolium* grouped with the diploids, *V. boreale* and *V. myrtilloides*. Hexaploid *V. virgatum* grouped most closely with the diploid *V. tenellum*, thus shedding light on the origins of these polyploid species.

Key words: DNA markers, dendrograms, evolutionary relationships, expressed sequence tag-polymerase chain reaction, phylogenetic relationships.

Résumé : En Amérique du Nord, les espèces commerciales de bleuet appartiennent à la section *Cyanococcus* du genre *Vaccinium*. Les auteurs ont examiné la phylogenèse de 50 obtentions à ploïdie variable de la section *Cyanococcus* en utilisant 249 étiquettes de séquence transcrite ou marqueurs de la réaction en chaîne à la polymérase et la méthode du regroupement. Parmi les espèces commerciales, l'espèce tétraploïde *V. corymbosum* se rapproche le plus des espèces diploïdes *V. fuscatum* et *V. caesariense*, puis de l'espèce diploïde *V. elliottii*. L'espèce tétraploïde *V. angustifolium* se retrouve avec les espèces diploïdes *V. boreale* et *V. myrtilloides*. Enfin, l'espèce hexaploïde *V. virgatum* est la plus proche de l'espèce diploïde *V. tenellum*, Ces résultats nous éclairent sur l'origine de ces espèces polyploïdes. [Traduit par la Rédaction]

Mots-clés : marqueurs génétiques, dendrogrammes, liens évolutifs, étiquette de séquence transcrite-réaction en chaîne de la polymérase, phylogenèse.

Introduction

Blueberry is a woody perennial shrub that belongs to the *Vaccinium* genus of the Ericaceae (heath) family. Blueberry is comprised of many species, but all the commercial types of North America belong to the Cyanococcus section (known as the true cluster-fruited blueberries) of *Vaccinium*. These commercially important species include the tetraploid highbush blueberry, *Vaccinium corymbosum* L. (syn. *V. formosum* Andrews), which can be subdivided into northern and southern highbush cultivars, the hexaploid rabbiteye blueberry, *V. virgatum* Aiton (syn. *V. ashei* Reade) cultivars, and the wild tetraploid lowbush blueberry, *V. angustifolium* Aiton. The United States is the largest producer of blueberries and grows all four commercial types in 26 states. Canada is the second largest producer of blueberries worldwide and the first largest producer of wild blueberries. Canada grows mainly two types, the wild, lowbush blueberry in the provinces of Nova Scotia, New Brunswick, Prince Edward Island, and Quebec, and northern highbush blueberry cultivars in British Columbia (Galletta and Ballington 1996).

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There are many other species within the Cyanococcus section; the total includes six to eight diploids, six tetraploids, and two hexaploids, depending on the classification used (Camp 1945; Vander Kloet 1983; Galletta and Ballington 1996). To date, the genetic relationships of the commercial species to the other species in this section have not been well studied using molecular markers. Most genetic diversity studies in blueberry using molecular markers have focused on examining the genetic relationships among the commercial types, mostly southern and northern highbush cultivars and some rabbiteye cultivars (Aruna et al. 1993; Rowland et al. 2003; Boches et al. 2006; Brevis et al. 2008; Bian et al. 2014). Two studies have reported on the genetic relationships among only the diploid species of the Cyanococcus section, one using allozyme data (Bruederle and Vorsa 1994) and another using expressed sequence tag-polymerase chain reaction (EST-PCR) marker data (Rowland et al. 2012).

Our laboratory generated the first expressed sequence tags (ESTs) from blueberry, initially by Sanger sequencing (Dhanaraj et al. 2004) and later by 454 sequencing (Rowland et al. 2012). By designing PCR primers near the ends of the ESTs, we developed EST-PCR markers (Rowland et al. 2003), which have proven useful for developing a genetic linkage map of diploid blueberry and identifying quantitative trait loci (QTL) for chilling requirement and cold hardiness (Rowland et al. 2014), analyzing genetic relationships among a collection of blueberry cultivars (Rowland et al. 2003), and characterizing spatial genetic structure of wild lowbush blueberry fields (Bell et al. 2009), among other endeavors. Here, we have extended our use of EST-PCR markers further to examine the phylogenetic relationships of the diploid, tetraploid, and hexaploid species within the Cyanococcus section. This study provides insight into the possible origins of the polyploid species.

Materials and Methods

Plant material

In an attempt to include at least 2–4 wild representatives from each of the diploid, tetraploid, and hexaploid species of the Cyanococcus section in this study, requests were made to several different sources for plant material. Plant material was received from the National Clonal Germplasm Repository (NCGR) in Covallis, OR, USA; Jim Ballington at North Carolina State University, NC, USA; and Nick Vorsa at the Blueberry and Cranberry Research Station, Chatsworth, NJ, USA. Two samples were also collected by Daniel Bell, while working jointly for the University of Maine, Bangor, ME, USA and the USDA-ARS, Beltsville, MD, USA. A list of all the plants used in the study and their sources are shown in Supplementary Table S1[1]. These included 50 *Vaccinium* accessions from the diploid species *V. fuscatum* Aiton [syn. *V. atrococcum* (A. Gray) A. Heller], *V. boreale* I.V. Hall & Aalders, *V. caesariense* Mack., *V. darrowii* Camp, *V. elliottii* Chapm., *V. myrtilloides* Michx., and *V. tenellum* Aiton; the tetraploid species *V. angustifolium*, *V. corymbosum*, *V. hirsutum* Buckley, *V. myrsinites* Lam., *V. pallidum* Aiton, and *V. simulatum* Small; the hexaploid species *V. constablaei* A. Gray and *V. virgatum*; and one accession from *Gaylussacia brachycera* (Michx.) A. Gray to serve as an outlier.

DNA extraction and genotyping

Young, fresh leaf tissue samples were received on wet ice or ice packs. DNA was extracted from 2–3 g of leaf tissue from each of the individual plants using a modified CTAB procedure (Rowland et al. 2003). To develop the EST-PCR markers for the study, forward and reverse PCR primer pairs were designed near the ends of available blueberry ESTs, generated either by Sanger sequencing (available in the EST database of GenBank/NCBI) or by 454 sequencing [available on our website (http://bioinformatics.towson.edu/BBGD454/) and in the sequence read archive of GenBank under accessions SRX100856, SRX100859, and SRX100861-SRX100867]. Primers were designed, EST-PCR markers amplified, and the products separated on agarose gels as described previously (Rowland et al. 2003). The EST-PCR primer sequences and annealing temperatures are given in Supplementary Table S2[1].

Phylogenetic analyses

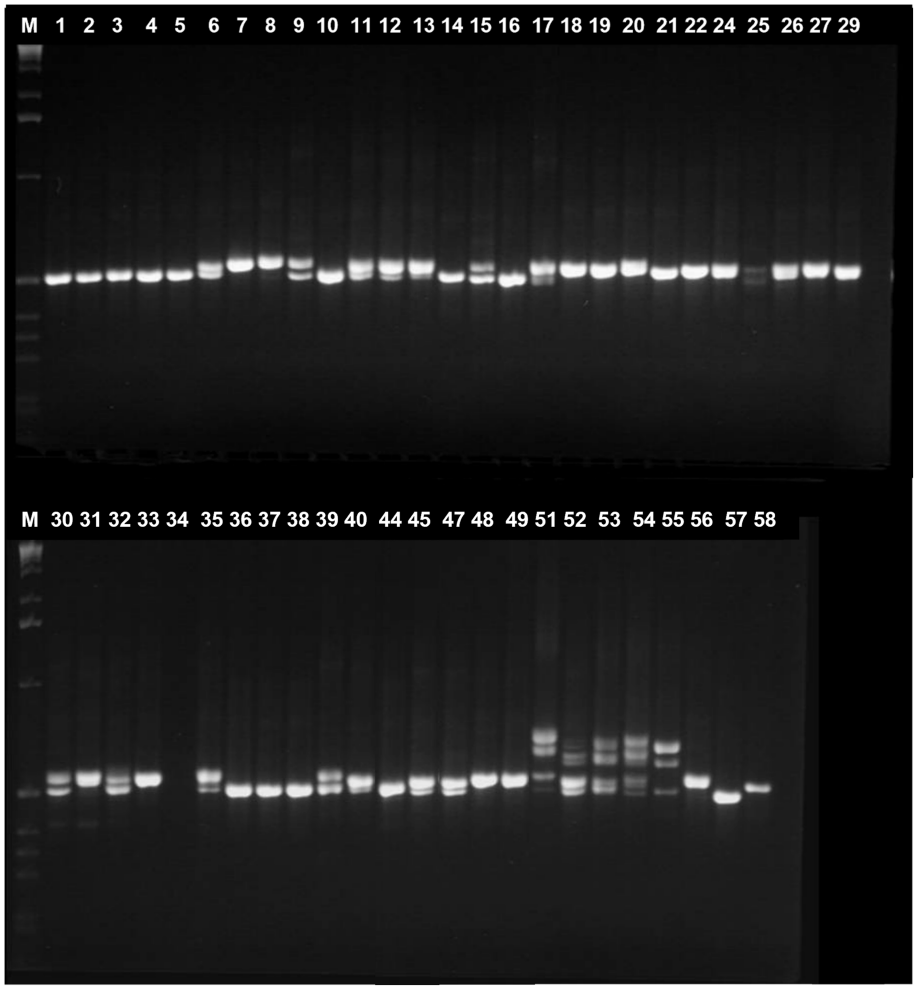
Polymorphic EST-PCR markers were scored as present or absent. The resulting dataset (Supplementary Table S3[1]) was analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-PC version 2.2, Exeter Software, Setauket, NY, USA). Dendrograms were constructed using the Unweighted Pair-Group (UPGMA) and Neighbor Joining (NJ) clustering methods from DICE similarity and dissimilarity matrices, respectively.

Results and Discussion

Here we used EST-PCR markers to examine the evolutionary relationships among the different blueberry species within the Cyanococcus section of *Vaccinium*. Two to five representatives from seven diploid, six tetraploid, and two hexaploid species were included in the final analyses (Supplementary Table S1[1]). Representatives originally thought to be diploid *V. pallidum* were excluded from the study because, after further investigation, they either appeared to have been misclassified or enough high quality DNA was not extracted for PCR amplification. From 62 primer pairs (Supplementary Table S2[1]), 249 polymorphic EST-PCR markers were scored across 50 blueberry accessions and one

[1]Supplementary data are available with the article at https://doi.org/10.1139/cjps-2021-0221.

Fig. 1. A representative gel photo showing EST-PCR amplification products from one pair of EST-PCR primers from EST 661. Molecular weight markers are shown in lanes with the letter M. Lanes 1–58 refer to the genotypes used in this study and are listed in Supplementary Table S1[1], column A.

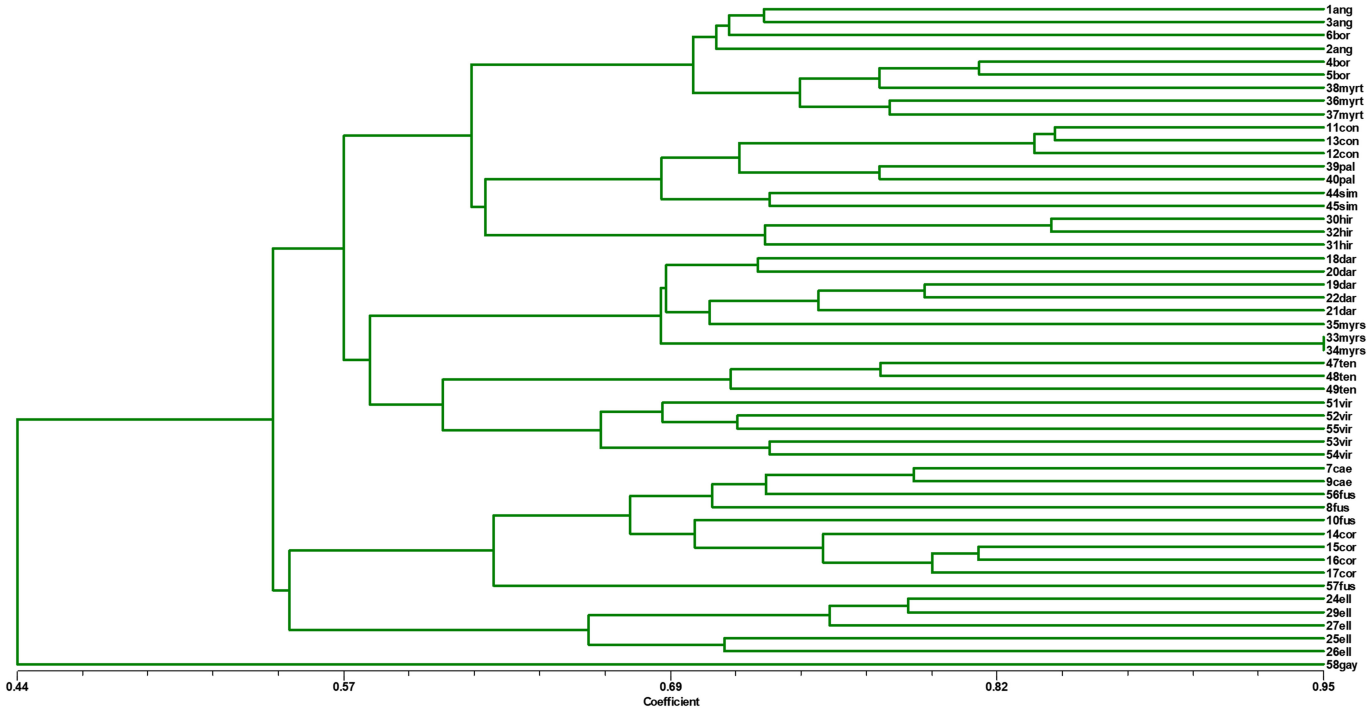


G. brachycera accession, which served as an outlier in the analyses (Supplementary Table S3[1]). A representative gel photo of the EST-PCR amplification products from the EST 661 primer pair is shown in Fig. 1. The marker data file (Supplementary Table S3[1]) was used to generate similarity and dissimilarity matrices (Supplementary Tables S4 and S5[1]), which were then used to construct UPGMA (Fig. 2) and NJ dendrograms (Supplementary Fig. S1[1]). Very similar results were obtained with both clustering methods, thus, we focus on discussing results from only the UPGMA dendrogram below.

First, at the top of the UPGMA tree (Fig. 2), it can be seen that the commercial wild tetraploid lowbush species *V. angustifolium* grouped most closely with the diploid species, *V. boreale* and *V. myrtilloides*. The diploids *V. boreale* and *V. myrtilloides* grouped closely together in the other two studies as well, those focused on only diploids using allozymes (Bruederle and Vorsa 1994) and EST-PCR marker data (Rowland et al. 2012). Galletta and Ballington (1996) have summarized the presumed origins of several of the Cyanococcus species and indicate that *V. angustifolium* is a proposed allotetraploid of either *V. boreale* × *V. myrtilloides* or *V. boreale* × *V. pallidum*. Our results suggest that it may well be an allotetraploid of *V. boreale* × *V. myrtilloides*.

As can be seen at the bottom of the tree (Fig. 2), the most important commercial species, tetraploid highbush *V. corymbosum*, grouped most closely with the diploid species, *V. fuscatum* and *V. caesariense*, followed by the diploid species *V. elliottii*. Vander Kloet (1983) suggested that all these diploid species be lumped together as diploid *V. corymbosum*. However, Bruederle and Vorsa (1994) and Galletta and Ballington (1996) proposed that *V. elliottii* be treated as a separate species based on allozymes, anthocyanins, and other data. Our EST-PCR data [herein and from our previous diploid study (Rowland et al. 2012)] supports maintaining *V. elliottii* as a separate species as well. It is also noteworthy that accession 57 (from Marion Co., FL), which is listed as *V. fuscatum*, and is included in the *V. caesariense*,

Fig. 2. The dendrogram resulting from the Unweighted Pair Group (UPGMA) clustering method using the DICE similarity matrix. Names of the genotypes are abbreviated according to the numbers (column A) given in Supplementary Table S1[1], followed by the first three or four letters of the species name. [Colour online.]



V. fuscatum and *V. corymbosum* cluster, does not appear to be closely related to the other accessions in this cluster. This probably indicates that accession 57 has a more complex origin.

Galletta and Ballington (1996) describe the origin of the tetraploid *V. corymbosum* as likely being an autotetraploid of diploid *V. corymbosum*, but indicate that introgression with other species is often evident. Recent sequence analysis of the tetraploid *V. corymbosum* genome 'Draper' suggests that highbush blueberry is unlikely an autopolyploid resulting from somatic doubling or failure during meiosis of a single parent (Colle et al. 2019). It is worth noting here that 'Draper' has a fairly complex background that includes *V. darrowii*. Colle et al. (2019) suggest instead that *V. corymbosum* is either an allotetraploid derived from two closely related species or an autotetraploid from hybridization of two highly divergent populations of a single species. If *V. caesariense* and *V. fuscatum* are treated as a single species, then our EST-PCR data suggests that perhaps the latter option is the most likely.

From the tree (Fig. 2), the commercial hexaploid rabbiteye species *V. virgatum* grouped most closely with the diploid *V. tenellum* species. Galletta and Ballington (1996) described the origin of *V. virgatum* as "complex". Later, Ballington (2006) proposed that *V. virgatum* is basically derived from *V. tenellum*, because it is clearly closest to *V. tenellum* morphologically, but with the extreme variation observed in the species likely resulting from introgression from other species in section Cyanococcus. Thus, our data provides support for *V. virgatum* being derived primarily from *V. tenellum*. Interestingly, the other hexaploid species *V. constablaei* clustered most closely with the tetraploids *V. pallidum* and *V. simulatum*. Indeed, Galletta and Ballington (1996) describe the proposed origin of *V. constablaei* as tetraploid *V. pallidum* × *V. simulatum* through the production of unreduced gametes from one parent. This is entirely consistent with our results.

Also, from our tree (Fig. 2), the tetraploid species *V. myrsinites* grouped most closely with the diploid species *V. darrowii*. Galletta and Ballington (1996) describe the proposed origin of *V. myrsinites* as an allotetraploid of *V. darrowii* × *V. tenellum*. Our results do not indicate a particularly close relationship between *V. myrsinites* and *V. tenellum*, however, suggesting *V. myrsinites* could have arisen instead as an autotetraploid of *V. darrowii*. Finally, the tetraploid *V. hirsutum* accessions grouped together but quite distantly from all the other species. In the UPGMA dendrogram, they clustered outside the *V. constablaei*, *V. pallidum*, and *V. simulatum* group. In the NJ dendrogram, they clustered distinctly outside all the other Cyanococcus groups.

The use of these EST-PCR markers to examine the phylogenetic relationships of the diploid, tetraploid, and hexaploid species of the Cyanococcus section provides valuable insight into the origins of the polyploid species. These relationships and proposed origins can

now be tested further using other markers, like simple sequence repeats or single nucleotide polymorphisms, and even through whole genome sequencing.

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