NRC Research Press

Genetic structure of the American ginseng (*Panax quinquefolius* L.) in Eastern Canada using reduced-representation high-throughput sequencing

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Abstract: The American ginseng (*Panax quinquefolius* L.) has been used for a wide range of medicinal purposes for more than 300 years, and is at risk in most of its range because of harvesting in natural populations, herbivory, and habitat loss. Its genetic structure is largely unknown in the previously glaciated areas of Eastern Canada, although such information could provide useful information for restoration strategies. We generated and analysed data from a reduced-representation high-throughput sequencing approach with a BAMOVA population model to partition the genetic variation within and among six natural populations of American ginseng in Eastern Canada. We found that an important and significant fraction of the genetic variation was structured among populations ($\phi_{ST} = 42\%$; $F_{ST} = 34\%$) at the geographical scale of the study (<250 km). No clear evidence of isolation-by-distance was observed. This important genetic structure observed among American ginseng populations from a region that was covered by ice during the last glaciations is similar to what had been found in previous studies on southern populations or throughout the species range.

Key words: next-generation sequencing (NGS), genetic structure, medicinal herb, conservation, genetic diversity.

Résumé : Le ginseng à cinq folioles (*Panax quinquefolius* L.) est utilisé comme plante médicinale depuis plus de 300 ans et est vulnérable dans l'ensemble de son territoire dû à la récolte des racines, au broutage et à la perte d'habitat. La structure génétique du ginseng est peu connue dans les régions affectées par les dernières glaciations dans l'est du Canada, bien qu'une telle information puisse aider à développer des stratégies de restauration. Dans cette étude, nous avons généré et analysé des données de séquençage à haut débit d'une fraction réduite de son génome à l'aide d'une approche BAMOVA par population afin de partitionner la variation génétique en fractions intra- et inter-populations. Nous avons observé qu'une fraction importante et significative de la variation était structurée entre les populations ($\phi_{ST} = 42$ %; $F_{ST} = 34$ %) à l'échelle de l'étude (<250 km). Aucune évidence de patron d'isolation par distance n'a été observée. Cette structure importante observée entre les populations du ginseng à cinq folioles situées dans la partie de l'aire de répartition recouverte de glace lors des dernières glaciations est similaire à ce qui a été observée dans des études antérieures effectuées dans le sud ou dans l'ensemble de l'aire de répartition de l'espèce.

Mots-clés : séquençage nouvelle génération, structure génétique, plante médicinale, conservation, diversité génétique.

Introduction

American ginseng (*Panax quinquefolius* L.; Araliaceae) is a long-lived understorey herb of the eastern deciduous forest of North America. It has been harvested for its fleshy root, which has been used for a wide range of medicinal purposes for more than 300 years (Robbins 1998; McGraw et al. 2013), and concerns over the harvesting in wild populations were raised as early as 1770 (Kalm 1987). This non-clonal species is indeed highly sensitive to harvest;

collecting roots of about 5% of productive plants (i.e., with at least three leaves) every year is enough to induce population decline (Nantel et al. 1996). Similarly, harvesting also impacts genetic diversity and structure (Cruse-Sanders and Hamrick 2004). Despite the fact that the American ginseng is listed in the *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES), legally protected in Canada (Species at Risk Act (SARA); Environment Canada 2015) and in the USA (McGraw et al. 2013), it is still threatened by legal and illicit harvesting

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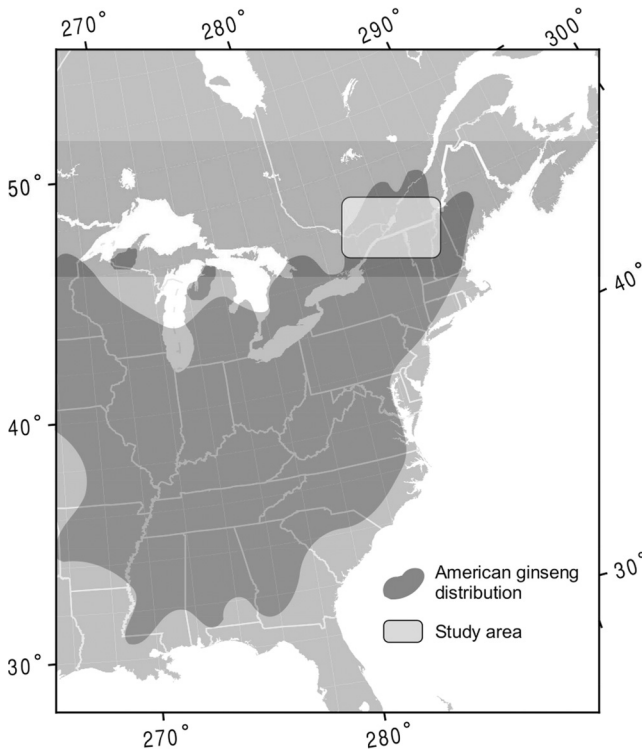**A. Archambault.** Quebec Centre for Biodiversity Science, 1205 Dr Penfield, Montreal, QC H3A 1B1, Canada.
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Fig. 1. American ginseng (*Panax quinquefolius*) distribution (modified from Argus and White 1987; © 1987 Canadian Museum of Nature) and study region from where the six populations were sampled. The map was generated using the Generic Mapping Tools software (Wessel et al. 2013).

Table 1. Information on the populations sampled.

Accession No.	Locality
CDPNQ 3677	MRC Le Haut-Richelieu, Quebec
CDPNQ 3690	MRC Deux-Montagnes, Quebec
CDPNQ 3684	MRC La Vallée-du-Richelieu, Quebec
CDPNQ 3715	MRC Rouville, Quebec
CDPNQ 18468	MRC Brome-Missisquoi, Quebec
ON*	Leeds and Grenville United Counties, Ontario

Note: CDPNQ, Centre de données sur le patrimoine naturel du Québec; MRC, Municipalité régionale de comté.

*The Ontario population does not have a record from the Ontario Ministry of Natural Resources and Forestry; locality information is available upon request.

as well as herbivory and habitat loss (McGraw and Furedi 2005; Souther and McGraw 2014).

Restoration efforts to preserve wild populations of American ginseng have been going on in Canada for the last 20 years (Environment Canada 2015). Because of the potential impact restoration or reintroduction programs can have on natural populations (IUCN 1987; Broadhurst et al. 2008), such efforts in the province of Quebec were always performed using seeds from the target or the nearest viable population (A. Nault, unpublished data). This was done to minimise the risk of losing locally adapted genetic diversity and minimize outbreeding depression (Broadhurst et al. 2008), especially given that populations targeted for restoration typically have small sizes as they are the ones suspected to lack sustainability. Yet, this assumes genetic structure among populations, at least for certain regions of the genome, but virtually nothing is known of local population structure of this tetraploid species in Eastern Canada. The few studies that investigated genetic variation in natural populations of American ginseng have generally found a strong population structure, but these were either performed over large geographical areas (Boehm et al. 1999; Grubbs and Case 2004; Cruse-Sanders and Hamrick 2004) or on populations

from the centre of the species' distribution (West Virginia; Obae and West 2011). These results can hardly be extrapolated to populations from previously glaciated areas, which are known to have distinct diversity patterns because of their demographic histories (Hewitt 2000; Davis and Shaw 2001). Two studies assessed the genetic diversity of American ginseng in the previously glaciated portion of its range using random amplified polymorphic DNA (RAPD). One studied nine populations from New York State (Lim et al. 2007) and the other three from the Quebec province (Schluter and Punja 2002). Although both found considerable within-population variation, neither found compelling evidence of population structure nor quantified the amount of variation partitioned among populations.

We present the results of a pilot study where the objective was to quantify the genetic population structure at a local scale among American ginseng populations in Eastern Canada. The project, initiated in 2010, aimed at testing a high-throughput reduced-representation sequencing approach at the population level (DNA pooling) on a non-model polyploid organism. The results demonstrate that an important and significant amount of genetic variation is partitioned among populations of the American ginseng in Eastern Canada.

Materials and methods

Plant material

Five sustainable American ginseng populations were sampled from Southern Quebec and one from Eastern Ontario (Fig. 1); the most distant populations were 250 km apart (Supplementary data, Table S1[1]). Populations were selected among those that were less likely to have been affected by human populations, either by harvesting or reintroductions (Désilets et al. 2011). Their precise locations cannot be published for protection purposes, but county information and governmental record numbers are given in Table 1, and geographic distances in Table S1. For each population, leaf material was sampled from 10 large, distant individuals to cover all occupied area.

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2016-0144.

Leaves were immediately dried in silica gel for molecular work.

Molecular work, sequencing, and bioinformatics

Detailed methods used for the molecular work, sequencing, and bioinformatics analyses are available in the Supplementary data. Briefly, we used a complexity reduction of polymorphic sequences (CRoPS) strategy (Davey et al. 2011) for investigating genetic variation in populations. Total DNA was digested with restriction enzymes and adaptors were ligated to the fragments. Selective primers then amplified a fraction of the fragments and the pool of amplified fragments was sequenced on one quarter of a Roche 454 run (Génome Québec Innovation Centre, Montreal, Quebec, Canada). We followed Gompert et al. (2010) in using a population level approach in which all individuals from a population were marked with the same common barcode, quantified, and pooled together in equal amount of DNA prior to sequencing. Although individual genotype information is lost with this approach, population allele frequencies and population structure can be estimated (Gompert et al. 2010). Moreover, de Vriendt et al. (2016) have shown that allelic frequencies obtained with population level DNA pooling were highly similar to that obtained with an individual-based genotyping approach.

Sequences were filtered for adaptors and poor quality nucleotides prior to de-novo contig assembly in Geneious (Drummond et al. 2014) with the following parameters: min. overlap, 30 bp; min. overlap identity, 97%; word length, 10; max. ambiguity, 16; reanalyse threshold, 2; max. size of gap, 3. To test whether the assembly algorithm affects the results, contigs were also assembled in Seq-Man NGen (DNAStar Inc., Madison, Wisconsin, USA; see the Supplementary data for the parameters) and gave the same results (data not shown). To eliminate biases in subsequent analyses, we used a BLAST approach to identify and discard contigs of chloroplast, mitochondrial, ribosomal, or bacterial origin, as well as putative transposon sequences. We also discarded over-represented contigs that could represent putative duplicated genes or large gene families. Finally, we removed contig regions with low sequence coverage and used a Coalescent theory approach (using evidence for intra-specific and inter-specific divergence times among sequences in a contig; see the Supplementary data) to remove contigs containing gene paralogs. This last step is important because the American ginseng is an ancient tetraploid (Lee and Wen 2004). Finally, only contigs represented by at least two sequences in each population were retained.

Population genomics

Population structure was estimated with the Bayesian approach of Gompert et al. (2010) implemented in BAMOVA (Gompert and Buerkle 2011). It uses a Bayesian hierarchical model to estimate locus specific and genome wide ϕ_{ST} (Excoffier et al. 1992), which is the amount of molecular variance partitioned among populations. Five independent chains of 500 000 generations were run with the parameters "–l 1 –v 0.2 –D 1 –a 0 –w 2000 –c 0.8", which were found to give the best chain mixing. The chain was sampled every 100 generations and the first 200 000 generations were discarded as burnin. Convergence and mixing of the chains was assessed visually and statistically using the *coda* package (Plummer et al. 2006) in R (R Core Team 2015).

We also estimated F_{ST} based on nucleotide polymorphisms, as in Nordborg et al. (2005, see the Supplementary data for details). A global F_{ST} value was estimated among all populations, as well pairwise F_{ST} values between all pairs of populations. These latter values were used to reconstruct a population network using the NeighborNet algorithm (Bryant and Moulton 2004) in SplitsTree4 (Huson and Bryant 2006). F_{ST} statistics and networks were also estimated on the chloroplast and mitochondrial markers that were set-aside during the filtering steps.

We finally tested for a pattern of isolation-by-distance in the data using a distance-based redundancy analysis (dbRDA; Legendre and Anderson 1999). The response matrix consisted of the vector coordinates of a principal coordinate analysis of the F_{ST} matrix and the predictors were the latitude and longitude of populations. The significance of the models was tested by ANOVA and adjusted R^2 were reported. Analyses were performed with the R package *vegan* (Oksanen et al. 2012).

Results

A total of 248 740 reads were obtained, ranging from 34 836 to 51 899 (mean = 41 457) per population (raw data was deposited in the NCBI SRA archive [SRR4436938–SRR4436943] and processed data was deposited on Figshare [doi:10.6084/m9.figshare.3412990]). The raw sequence lengths varied from 0 to 648 bp (median [N50] = 71). A total of 134 509 reads, out of 248 740, were assembled to produce 13 235 contigs, varying in length between 30 and 813 bp (median [N50]: 214, mean: 145).

In total, 153 contigs were identified to be of chloroplast or mitochondrion origin, 120 contained ribosomal sequences, and 91 were potentially identified as transposons. No sequences were found to be of bacterial or fungal origin, but 435 contigs were identified as representing gene families or containing paralogs. Ultimately, 751 contigs passed all filtering criteria and 46 had coverage of at least two in each population and were used in the subsequent population genomics analyses. Similarly, 79 chloroplast and 11 mitochondrial contigs were included in further analyses.

The five BAMOVA runs converged for all variables. The potential scale reduction factor (PSRF) was below 1.03 for all variables and was 1 for ϕ_{ST}, the variable of interest. Effective sample sizes (corrected for chain autocorrelation) were above 1000 for all parameters. The posterior distribution of ϕ_{ST} shows that genetic variation is strongly

partitioned between populations, with 41.9% of the variation being explained among populations (95% confidence interval: 33.8%–49.3%). The global F_{ST} estimate was 0.34, which supported the BAMOVA results in suggesting an important differentiation among populations. The pairwise population F_{ST} were all between 0.12 and 0.15, and the population network shows an absence of structure (Fig. 2A). There was no evidence of an isolation-by-distance pattern ($R^2_{adj} = 0.028$; ANOVA F stat. $p = 0.30$), although this result should be taken with caution because of the small number of populations included.

The global population structure obtained for chloroplast and mitochondrial markers were about half that of the nuclear markers (chloroplast $F_{ST} = 0.18$; mitochondrial $F_{ST} = 0.17$). A lower value is expected because the mutation rates of the organellar genomes are lower than for the nuclear genome in plants. However, because the exact mutation rates are unknown for ginseng, a direct comparison of population structure is not possible between the genomes. There was no evidence of isolation-by-distance for the chloroplast data ($R^2_{adj} = -0.066$, ANOVA F stat. $p = 0.58$), whereas a marginally significant isolation-by-distance signal was found with the mitochondrial data ($R^2_{adj} = 0.66$, ANOVA F stat. $p = 0.031$). The population networks estimated from pairwise population organellar F_{ST} values illustrate these results. The network of the chloroplast data showed that two populations (CDPNQ 3677 and CDPNQ 3690) were slightly more distant from the others (Fig. 2B), but they were not particularly close geographically to each other (Fig. 2D). In contrast, the mitochondrial population network illustrated why an isolation-by-distance was detected, as the two most distant populations at the genetic level (ON and CDPNQ 18468) were also the most geographically distant populations (Figs. 2C and 2D).
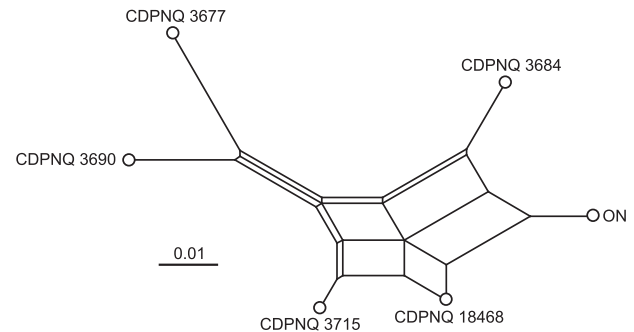
Discussion

We found that a strong and significant amount of the genetic variation is partitioned among studied populations of the American ginseng in Eastern Canada, both in terms of ϕ_{ST} (42%) and F_{ST} (34%; nuclear genome). However, we did not observe a clear genetic structure among the populations studied, perhaps because of their small geographic extent. The genetic variation explained among populations is similar to what has been found across a slightly greater region in West Virginia with RAPD markers (47.3%; Obae and West 2011) and concurs with studies that investigated population structure across the species' range (Grubbs and Case 2004; Cruse-Sanders and Hamrick 2004). Notably, it is the first time that a strong and significant population structure has been estimated among populations found in the previously glaciated portion its range. Indeed, previous studies in this region using polymorphic RAPD markers did not show clear evidence of genetic structure among populations or did not quantify the proportion of genetic variation parti-

Fig. 2. Networks representing the pairwise F_{ST} distances between populations for (A) the nuclear genome, (B) the chloroplast genome, and (C) the mitochondrial genome, as well as the pairwise geographic distances between populations (D). Boxes in the network indicate conflicting signal in the data. Bars indicate F_{ST} distances (A, B, C) or geographic distances in kilometres (D).
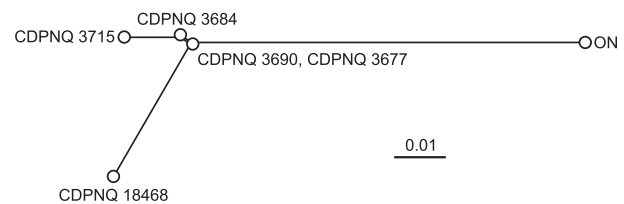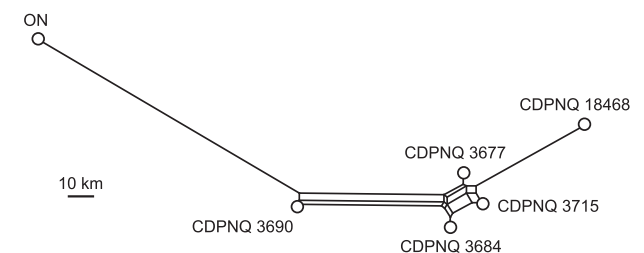
tioned among populations (Schluter and Punja 2002; Lim et al. 2007). These results are important, as recent population expansions such as the one that occurred in American ginseng following glacier retreats at the end of the Pleistocene have important population genetic consequences that prevent the extrapolation of results from previous studies to previously glaciated regions. These peculiar genetic conditions are caused, firstly, by the spread of rare mutations on the migration front that increase genetic differentiation (Edmonds et al. 2004) and, secondly, by lowering effective population size and therefore reducing genetic diversity (Hewitt 2000).

The significant population structure observed here provides relevant information for the management of the American ginseng in Eastern Canada. First, this population structure at this geographical scale might be seen as supporting actual restoration strategies that use seeds from neighbouring populations in restoration projects. The idea behind this is to minimize the impact on local genotypes given the small population sizes, maximise local adaptation, and minimize outbreeding depression (Broadhurst et al. 2008). Whether this strategy is the best one, however, is debatable (Jones 2013) and should be investigated further for the American ginseng. Indeed, a large proportion of the markers supporting this structure might be neutral and thus may not indicate locally adapted genotypes. The absence of isolation-by-distance patterns in the data might be interpreted as an indirect evidence of local adaptation, but this is highly speculative because of the small number of populations involved and of the highly fragmented nature of the landscape between these populations. However, even in the presence of locally adapted genotypes, favouring limited gene flow among populations may help spread useful mutations or reduce inbreeding depression and consequently increase mean population fitness (Broadhurst et al. 2008). Future studies are needed to decide on the most appropriate strategies for preserving this species.

Besides harvesting, threats to local diversity can also come from ginseng cultivation in natural ecosystems; a practice recently gaining popularity (Nadeau and Olivier 2003). Indeed, forest farming practices could threaten the native variation (Environment Canada 2015) if little care is given to seed provenance (no regulation currently exists for this issue in Canada and in the USA), and whether cultivated areas are established near wild populations. The close proximity of natural and cultivated populations could result in introgression of foreign genes into the indigenous genetic background and contribute to the loss of local diversity, if this process is important (McGraw et al. 2013).

While our results were statistically supported, we acknowledge that the sample sizes used in this pilot investigation were suboptimal, even if they are similar to previous studies that used a DNA-pooling approach (e.g., Gompert et al. 2010 used 15 individuals per population). Nevertheless, the Bayesian BAMOVA approach accounts for the error involved in sampling individuals from populations (Gompert et al. 2010). As such, the confidence intervals obtained partly reflect this uncertainty and reinforces the strong signal of genetic structure in the data regardless of the limited sampling.

Despite our efforts in selecting the size of fragments to be sequenced, many short contigs were obtained, which resulted in a decreased sequencing depth (number of reads per marker). Nevertheless, the number of markers we obtained was sufficient to assess the neutral genetic structure of American ginseng. Since the start of this project in 2010, other reduced representation sequencing approaches have gained in popularity, such as RAD-sequencing or Genotyping by Sequencing (Baird et al. 2008; Elshire et al. 2011), which allow individual genotyping for more loci and at lower cost than the technology used in this study. Sequencing depth should not affect our main conclusions because the BAMOVA approach accounts for the uncertainty in sampling sequences from individuals in DNA pools (Gompert et al. 2010). Nevertheless, the new techniques mentioned above appear more appropriate for subsequent investigations. In the future, the combination of such genomic approaches, combined with appropriate trait and fitness measures of plants, will allow us to design better conservation strategies for the American ginseng.

Acknowledgements

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