RESEARCH ARTICLE

Morphological and genetic polymorphism in two North American reindeer lichens: *Cladonia arbuscula s. l.* and *C. rangiferina*

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ABSTRACT

Cladonia arbuscula and *C. rangiferina* are two reindeer lichen species, which are widely distributed in northern climates and have not been shown to be monophyletic. The wide species distribution may suggest an outcrossing reproductive life style, which may contribute to paraphyly and success of these two species. The current study examined the genetic diversity of mycobiont ascospore colonies within and between apothecia of two species, *C. rangiferina* and *C. arbuscula s. l.* using Randomly Amplified Polymorphic DNA (RAPD)-PCR. It also estimated gene flow, morphological variability, and colony growth rates. *Cladonia rangiferina* showed higher rates of growth than *C. arbuscula* but *C. arbuscula* showed more variability in growth between apothecia than *C. rangiferina*. Both species showed levels of genetic variation within and among apothecia that was consistent with heterothallism and recombination. AMOVA analyses provided evidence for gene flow among apothecia in both species. It further hypothesized that higher genetic variation in *C. arbuscula* than in *C. rangiferina* may give *C. arbuscula* an adaptive advantage over *C. rangiferina*.

Keywords: Ascospore colony, *Cladonia arbuscula*, *Cladonia rangiferina,* RAPDs, reindeer lichen, North America

INTRODUCTION

The genus *Cladonia* is a large genus of terricolous lichen-forming fungi including the highly branched reindeer lichens, which are known from the segregate genus *Cladina* (Ahti, 2000; Ahti and DePriest, 2001). *Cladina* is most abundant in the coniferous belt of the Northern Hemisphere and because it covers large portions of northern areas it forms the main source of winter food for northern caribou and reindeer (Rominger *et al.,* 1996). The widespread success of *Cladina* might be explained by the adaptive potential of the lichens, which is reflected in the level of genetic diversity. Lower levels of genetic diversity may reduce the capacity of the population to adapt to changing climate (Pauls *et al.,* 2013) if allelic variation is insufficient to respond to the environmental changes. Allelic variation is introduced by mutations and is shuffled among populations through sexual reproduction and dispersal (gene flow). Climate change in northern North America (Schiermeier, 2007) may influence the adaptive potential of the vegetatively reproducing species more than the sexually reproducing species, where a greater diversity of alleles are available for adapting to changing conditions. Sexually reproducing species may be heterothallic (cross fertilization) where successful mating requires genetically different individuals, or homothallic (self-fertilization) where mating

requires genetically similar individuals. Heterothallic and homothallic species have been investigated in other lichen species (Murtagh *et al.,* 1999, 2000; Dyer *et al.,* 2001; Seymour *et al.,* 2005*a*; 2005*b*) using discordance between dendrograms or by direct sequencing of mating type alleles (Scherrer *et al.,* 2005). Two groups of genetic variants were distinguished by comparing RAPD variation among 59 single spore cultures of *Xanthoria parietina* (L.) Th. Fr. (Honegger *et al.,* 2004) and polymorphism among RAPD loci was reported for single spore isolates of some species of Parmeliaceae, Ramalinaceae and Physciaceae (Honegger and Zippler, 2007). RAPD and Amplified Fragment Length Polymorphism (AFLP) variation was also compared showing genetic polymorphism within single apothecia of three *Cladonia* species (Seymour *et al.,* 2005). The high levels of polymorphism among apothecial colonies supported the contention that heterothallism is more common than homothallism in the species examined.

Sexual reproduction is rarely reported in mat-forming species of *Cladonia* (Formerly *Cladina*; Jahns *et al.,* 2004). Sexually reproducing reindeer lichens can be detected by production of apothecia. *Cladonia arbuscula* (Wallr.) Flotow and *C. rangiferina*, (L.) F. H. Wigg. which are widely distributed in northern ecosystems,are two species

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that have been reported to produce apothecia frequently in Manitoba (Athukorala *et al.,* 2014; Piercey-Normore, 2004; Robertson and Piercey-Normore, 2007). Although Jahns *et al.,* (2004) investigated the sexual reproduction and vegetative growth-pattern of *C. rangiferina,* neither mating type nor genetic diversity has been examined within and between apothecia of this important and widespread species. Studies on genetic diversity in *C. arbuscula* (Myllys *et al.,* 2003; Piercey-Normore *et al.,* 2010, Robertson and Piercey-Normore, 2007) showed some evidence for gene flow between *C. arbuscula* and its segregate called *C. mitis.*

A previous study carried out by Athukorala *et al.,* 2016 showed that *Cladonia arbuscula* and *C. rangiferina* are not monophyletic and they produced reticulate haplotype networks. Therefore, both species are expected to show a level of genetic diversity consistent with a hypothesis of heterothallism and high levels of gene flow within and between individuals.

The level of genetic diversity will depend on the gene region examined such as nucleotide sequence comparison of single or multiple genes (Myllys *et al.,* 2003; Piercey-Normore *et al.,* 2010; Printzen and Ekman, 2003), presence or absence of group 1 introns (Robertson and Piercey-Normore, 2007), Restriction Fragment Length Polymorphism (RFLP) which depends on a prior knowledge of specific genes (Beard and De Priest, 1996), or the development of Simple Sequence Repeat (SSR) microsatellite primers (Walser *et al.,* 2003), and Randomly Amplified Polymorphic DNA (RAPD) (Dyer *et al.,* 2001). Methods such as RAPDs that use random priming of multiple loci within the genome offer a sensitive method to elucidate variation, but RAPD requires careful lab practices in order to maintain reproducibility of results (Weising *et al.,* 1995) and cultivation of the fungal partner to separate the genome from other symbionts or contaminating organisms. The culturing of lichen fungi has a long history (Bonnier, 1887, 1889; Crittenden *et al.,* 1995; Stahl, 1877; Jahns, 1993; Oliver *et al.,* 1989; McDonald *et al.,* 2013; Sangvichien *et al.,* 2011; Stocker-Wörgötter, 2001, 2002; Yamamoto *et al.,* 1993). However, it has not been intensively performed due to challenges such as low growth rate and a high chance of contamination from faster growing fungi and bacteria (McDonald *et al.,* 2013). Few studies have examined the characteristics of single spore cultures. Werner (1930, 1964) compared ascospore germination patterns in lichenized ascomycetes. Ahmadjian (1964) studied the morphology, size, and pigmentation of 700 single spore cultures of *Cladonia cristatella* Tuck., while Pfister (1996) reported morphological characteristics of single

spore cultures of 15 *Cladonia* species. More recently, studies have been quantitative and genetic in their approach.

The goal of this study was to further examine variability among single spore isolates of two common and apotheciate North American reindeer lichen species, *Cladonia arbuscula s. l.* and *C. rangiferina*. The objectives were: 1) to compare colony growth formed by ascospores, 2) to compare colony morphology among apothecia, and 3) to compare genetic diversity of colonies within and among apothecia and different thalli of the same species.

MATERIALS AND METHODS

Mycobiont spore cultures - Single spores were isolated from samples of *C. rangiferina* and samples of *C. arbuscula* (*C. mitis*) (Supplementary Table 1) according to the method described by Athukorala *et al.,* (2014). From each sample of lichen thallus, 10 apothecia were removed and separately attached to petroleum jelly on the underside of Petri dish lids. Each lid contained five apothecia and spores were allowed to eject onto solid 1.5% water agar (Sigma-Aldrich Co., Missouri, USA) and stored at 20 °C in the dark. Beginning after 24 hours, the plates were observed under a dissecting microscope for spore ejection. After spore ejection at least three to eight spores or spore clusters per apothecium were transferred to 1.5% Malt Yeast extract agar (MYA: 20 g malt extract, 2 g yeast extract, 15 g agar, 1 L distilled water) plates, and incubated at 20 °C in the dark.

Colony characteristics (colour, texture, shape, diameter, colony margin, surrounding medium colour) were recorded for each colony for three months at two week intervals. The colony characteristics were described according to standard fungal colony morphology descriptions (Forbes *et al.*, 1998) and are presented in Supplementary Table 2. The diameter of colonies was measured to the nearest mm by taking the average of two perpendicular measurements per colony from the bottom of the petri plate.

Total cellular DNA was extracted from each colony using a modified protocol of Grube *et al.* (1995). Amplification and sequencing of the ITS rDNA region was performed using primers and protocols used by Athukorala *et al.,* (2014) to confirm the identity of colonies. Amplification of DNA from the mycelial colonies was conducted using RAPD primers UBC 31 (ccggccttc c), UBC 34 (ccggcccca a), UBC 60 (ttggcc gag c) and UBC 184 (caaacggca c) on 24 colonies of *C. rangiferina* (SA22); and using UBC 31, UBC 60, UBC 90 (gggggt tag g) and UBC 122 (gtagac gag c) on 22 colonies of *C.*

arbuscula (Athukorala 7) and *C. arbuscula/mitis* (Nomore 9468, Athukorala 12). RAPD-PCR was performed using 0.4 to 2.0 ng DNA per reaction. Twenty μl PCR reactions contained 1X PCR buffer (50 mMKCl, 20mM Tris), 1μM primer (1.1 μM for UBC 184), 2.0 mM of MgCl$_2$, 200 mM of each dATP, dCTP, dGTP and dTTP (Invitrogen Life Technologies, California, USA), and 0.15 U of Native Taq polymerase (Invitrogen Life Technologies, California, USA). Amplification was carried out in a T100^{TM} thermal cycler (Bio-Rad Laboratories, Hercules, California, USA). Amplified products were run on 1 % agarose gels and detected by staining with ethidium bromide (0.5 μg/μl). The presence and absence of fragments for each single spore culture were scored manually as '1' or '0', respectively. The strength of the fragments present on the gel was determined with reference to the 1650 bp fragment in the 1 Kb DNA plus ladder (Invitrogen Life Technologies, California, USA) and the bands that were greater than 4 ng (compared to 1650 bp fragment) were used to score presence or absence of the PCR product.

Data analysis

The variability among colony diameters within and between apothecia that were produced by different branches of the same thallus. Similarly, colony diameter was averaged for each sample of the same species and compared between samples to determine whether there was significant variation in colony diameter between different thalli of the same species. Finally, colony diameter was compared between species, *C. rangiferina* and *C. arbuscula*. Data were analysed by one way ANOVA using colony diameter as the continuous dependent variable and the colony, apothecium, sample or species as the categorical independent variable. Data were tested for normality using Test for Normality Test/Goodness-of-fit Test. The comparison among means of each treatment was done using Tukey-Kramer HSD test. The analyses were performed using JMP® software version 11 (SAS Institute Inc.).

The RAPD bands obtained with all eight primers were scored as present or absent, representing binary data sets which were subjected to eight separate neighbour joining (NJ) analyses using mean character difference in PAUP* 4.0b10 (Swofford, 2003). Bootstrap support was estimated using 1000 re-samplings (Felsenstein, 1985) to determine support for the tree topology. Genetic variation was analysed by AMOVA using GenAlEx ver. 6.5 (Peakall and Smouse, 2012). One population was defined as spore colonies from a single apothecium (2 – 8 colonies per apothecium). Partitioning of the observed genetic variation and calculation of the corresponding PhiPT (Φ_{PT}) values were carried out by means of analysis of

molecular variance (AMOVA) with 999 permutations (Excoffier *et al.,* 1992), which gave two levels of partitioning: (1) differences within colonies of the same apothecium (within population), and (2) differences among colonies from different apothecia (among populations). Pairwise comparisons between apothecia were also performed. Percent polymorphism of colonies within each apothecium was calculated manually using the formula: number of polymorphic loci/total number of amplified loci x 100. Polymorphism was calculated using each RAPD primer matrix separately and using the combined matrix. The number of haplotypes recorded for each apothecium was determined with regards to each primer matrix and combined matrix of both species using the Haplotype Analysis version 1.04 program (Eliades and Eliades, 2009).

RESULTS

Sequence identity and mycobiont colony characteristics

The ITS rDNA sequences obtained from colonies of Athukorala 7 were identical between culture and corresponding lichen sample, and showed high similarity to GenBank Accession no. GU169281 (*C. arbuscula*), with 99% similarity and 0.0 e-score. Those from colonies of Athukorala 12, Normore 9403, Nomore 9468 and Normore 9642 showed high similarity to *C. arbuscula* subsp. *beringiana* (GenBank Accession no. GU169249) and *C. mitis* (GenBank Accession no. GU169228), both with 99% similarity and 0.0 e-score. The ITS rDNA sequences obtained from samples Athukorala 24, Athukorala 22, Athukorala 17 and Athukorala 16 showed high similarity to GenBank Accession no. DQ394367 (*C. rangiferina*), with 98% similarity and 0.0 e-score. All sequences generated in the current study were deposited in GenBank and the relevant accession numbers are listed in Table 1.

Ascospores of *C. arbuscula* and *C. rangiferina* were observed on the growth medium 21-28 days after preparation of the cultures, and germination was observed within 24 hours after spore discharge (Fig. 1). Each colony resulted from one to eight ascospores from a single ascus. Colony growth was observed within one week after the spores were transferred to MY agar, and the colonies were between 2 to 5 mm in diameter after 1 month (Fig. 2). Colony diameter continued to increase after 4 weeks and differences between colonies and species became more evident after 8 to 12 weeks, where *C. rangiferina* grew faster than *C. arbuscula* (Fig. 2).

All colonies of *C. rangiferina* changed from pale-

pink to pale-brown during the 12 week period. Colonies were raised and sometimes hollow after 12 weeks, with undulate or umbonate margins, and the colony surface was wrinkled with concentric markings. Slight morphological differences in the colour, surface texture, and margin shape were observed among colonies within and between apothecia (Fig. 3). All colonies of *C. arbuscula* started as white colonies and developed dark coloration in the colony centre which covered the whole colony by 12 weeks and were just as variable in morphology as *C. rangiferina*.

Table 1. Collection location, number of samples used in the study, and the GenBank accession numbers obtained for ITS rDNA sequences for some of the colonies.

| Species	Collection information	ITS rDNA accession number
C. arbuscula	Canada, Manitoba, Spruce Woods, Normore 9642 (n = 76)	KP031551
	Canada, Manitoba, near Leaf Rapids, MN 9403 (n = 68)	KP031550
	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 7 (n = 20)	KP001207
C. arbuscula/mitis	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 12 (n = 12)	KP001209
	Canada, Manitoba, between Ponton and Leaf Rapids, MN 9468 (n = 26)	KP001206
C. rangiferina	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 16 (n = 06)	KP031549
	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 17 (n = 05)	KP001202
	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 22 (n = 34)	KP001200
	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 24 (n = 06)	KP001201
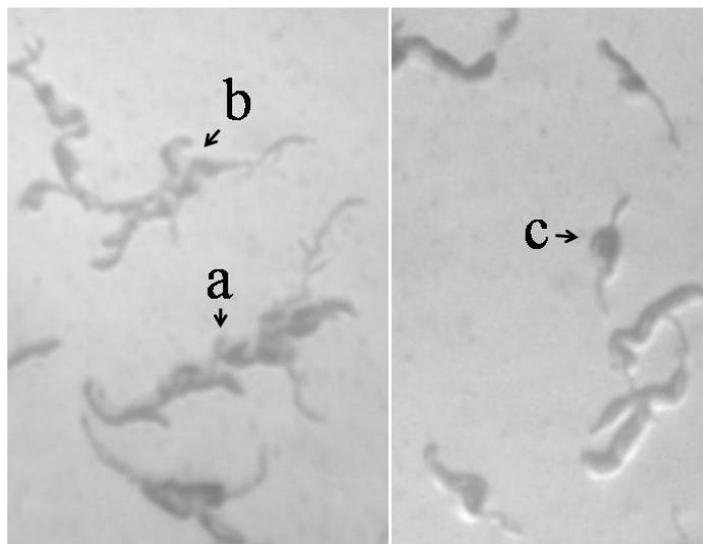
Figure 1. Germination of ascospores ejected from *C. arbuscula* (Normore 9403) showing a set of eight spores (a) or less than eight (b) or as a single ascospore (c).
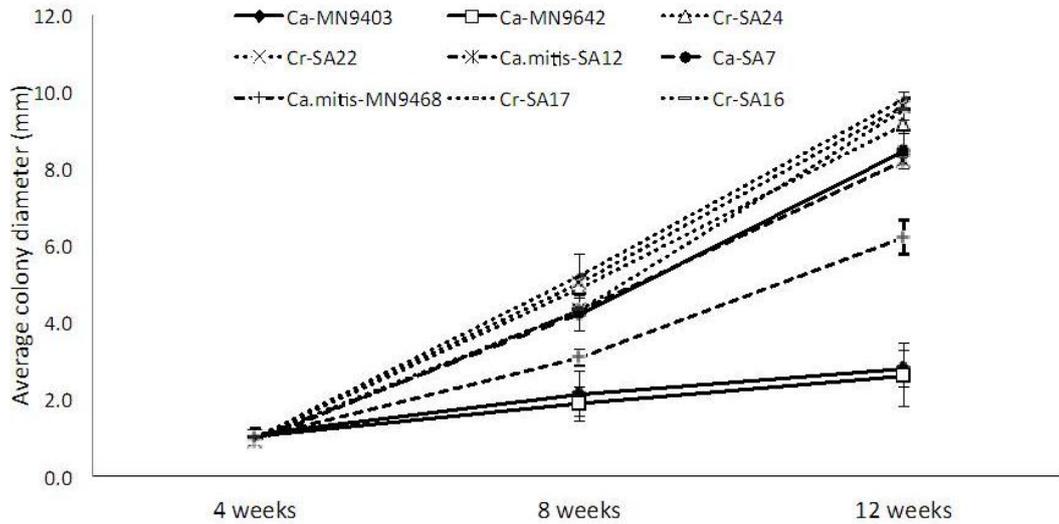
Figure 2. Change in colony diameter from one to three months on malt yeast extract agar for five *C. arbuscula/mitis* and four *C. rangiferina* samples. Specimen names with SA represent collection by Athukorala and MN represents collection by Normore.
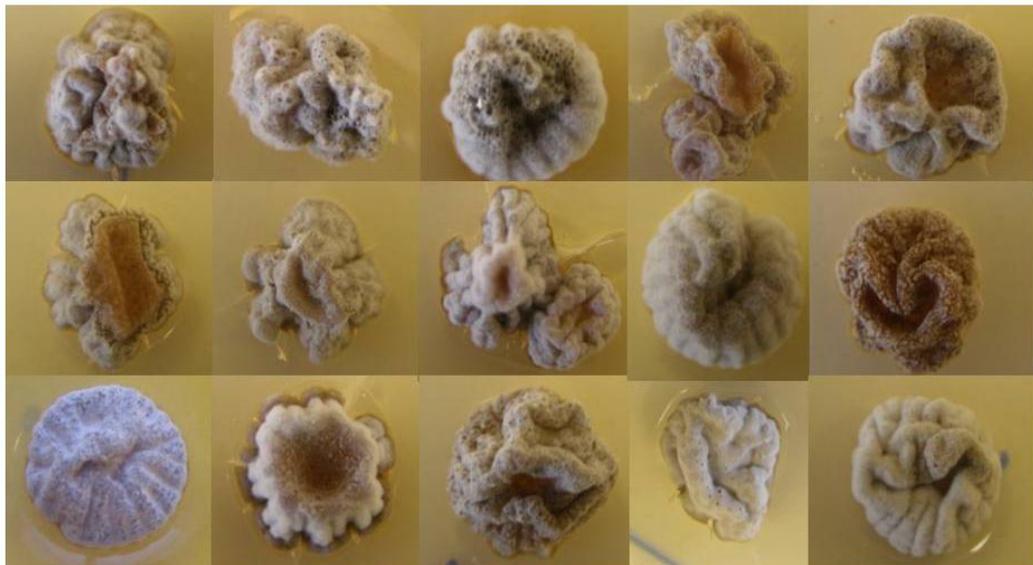

Figure 3. Variation in the colony morphology shown by different spore cultures of *C. rangiferina* at three months incubation at 20˚C on malt yeast extract agar medium.

There was an overall significant difference (P<0.001) in colony diameters between the three species at 12 weeks (Fig. 2). Average colony diameter of *C. rangiferina* was not significantly different (P=0.7773) between specimens (Fig. 4A) of the same species. However, average colony diameter of *C. arbuscula* was significantly different between specimens (Fig. 4b).

Genetic variation - Four RAPD primers exhibited variability among spore cultures of the 24 colonies from *C. rangiferina* and 22 colonies from *C.*

arbuscula (Fig. 5). The eight (four primers for each species) primers amplified fragments with a range of 375–3000 bp in length resulting in a total of 47 fragments for *C. arbuscula* and 52 fragments for *C. rangiferina*. There were 1 to 5 haplotypes of *C. rangiferina* and 1 to 8 haplotypes of *C. arbuscula* depending on the primer. The percent polymorphism ranged from 33 to 100% for *C. arbuscula* and 0 to 91% for *C. rangiferina* (Table 2).

The AMOVA analysis showed 58% variation within a single apothecium and 42% variation among apothecia, respectively for *C. arbuscula*. There was 45% variation within apothecia and 55%

variation among apothecia for *C. rangiferina* (Table 3). The pairwise species comparison of Φ_{PT} showed that four of six comparisons for *C. arbuscula* were significantly different (Table 4) and seven of 28 comparisons for *C. rangiferina* were significantly different from one another (Table 5).

The combined NJ phenograms produced by four RAPD primers revealed a separation of some of the apothecia (SA22(2)-I, II, and V; Fig. 6) into different clades. The combined analysis of *C. arbuscula s. l.* showed that all colonies from Normore 9468, except one colony, formed a weakly supported clade and the SA7-III colonies clustered outside all others with support of 80%.

Table 2. Genetic diversity of colonies representing apothecia from two species, *C. arbuscula* and *C. rangiferina* for primers, showing both separate and combined results. SA represents collection by Athukorala and MN represents collection by Normore.

Species and collection no.	Primer UBC 31	Primer UBC 60	Primer UBC 90	Primer UBC 122	Combined primers
C. arbuscula/ mitis					
SA7-I, n=3	33% (3/9) 3	0% (0/4) 1	No bands	No bands	54% (15/28) 3
SA13-I, n=6	100% (6/6) 5	No bands	60% (3/5) 2	100% (7/7) 6	100% (18/18) 6
MN9468-I, n=8	83% (5/6) 7	100% (10/10) 5	91% (10/11) 7	75% (6/8) 3	91% (29/32) 8
MN9468-II, n=3	80% (4/5) 3	No bands	80% (8/10) 2	No bands	80% (12/15) 2

C. rangiferina	Primer UBC 31	Primer UBC 34	Primer UBC 60	Primer UBC 184	Combined primers
SA22(1)-I, n=3	91% (10/11) 3	33% (2/6) 2	83% (5/6) 3	75% (3/4) 2	74% (20/27) 3
SA22(1)-II, n=4	14% (1/7) 2	70% (7/10) 4	83% (5/6) 4	33% (1/3) 2	52% (13/25) 4
SA22(1)-III, n=2	37.5% (3/8) 2	60% (6/10) 2	40% (2/5) 2	0% (0/4) 1	41% (11/27) 2
SA22(2)-I, n=3	37.5% (3/8) 3	29% (2/7) 2	75% (3/4) 3	67% (2/3) 3	45% (10/22) 3
SA22(2)-II, n=2	14% (1/7) 2	44% (4/9) 2	No bands	0% (0/4) 1	37.5% (9/24) 2
SA22(2)-IV, n=2	22% (2/9) 2	12.5% (1/8) 2	75% (3/4) 2	0% (0/4) 1	17% (4/24) 2
SA22(2)-V, n=5	14% (1/7) 2	17% (1/6) 2	100% (8/8) 5	75% (3/4) 5	52% (13/25) 5
SA22(2)-VI, n=2	14% (1/7) 2	57% (4/7) 2	50% (3/6) 2	0% (0/2) 1	36% (8/22) 2

* Numbers in the first column are the collection number, apothecium number (I-VI), and the number of colonies (n) tested. The *C. rangiferina* samples have an additional thallus number in parentheses (1 – 2) after the collection number. Numbers in each column represent % polymorphism, (number of polymorphic loci/total number of loci), and number of haplotypes.

Table 3. The partitioning of the total diversity in 22 *C. rangiferina* and 19 *C. arbuscula* spore colonies.

Species	Variance component	df	SS	Variance	% total variance	P	PhiPT
C. rangiferina	Among population	7	126.0	4.97	55	0.554	0.000
	Within population	15	59.9	3.99	45	-	-
C. arbuscula	Among population	3	70.5	3.85	42	0.417	0.000
	Within population	16	86.3	5.39	58	-	-

Among populations = among apothecia; within population = within apothecia; df = degrees of freedom, SS = sum of squares

Table 4. Pairwise comparisons of φ_{PT} and probability (P) values for four apothecia from *C. arbuscula* showing sample collection number, apothecium (I – III), and number of colonies (n) tested. SA represents collection by Athukorala and MN represents collection by Normore.

Specimen-apothecial no.	SA7-III	MN9468-I	MN9468-II	SA13-I
SA7-III (n=3)	\	0.012	0.104	0.006
MN9468-I (n=8)	**0.622**	\	0.038	0.004
MN9468-II (n=3)	0.594	**0.172**	\	0.410
SA13-I (n=6)	**0.631**	**0.188**	0.043	\

*PhiPT values are shown below diagonal. Probability, based on 999 permutations is shown above diagonal. Values shown in bold are significant at α = 0.05.

Table 5. Pairwise comparisons of φ_{PT} and probability (P) values for eight apothecia from *C. rangiferina* sample SA22 showing two podetia (1 and 2 in parentheses), the apothecial number (I to VI), and number of colonies (n) tested. φ_{PT} values are shown below diagonal. SA represents collection by Athukorala.

Specimen (podetium)-apothecium	SA22 (1)-I	SA22 (1)-II	SA22 (1)-III	SA22 (2)-I	SA22 (2)-II	SA22 (2)-IV	SA22 (2)-V	SA22 (2)-VI
SA22(1)-I (n=3)	\	0.060	0.097	0.095	0.103	0.111	0.021	0.104
SA22(1)-II (n=4)	0.152	\	0.058	0.029	0.062	0.061	0.009	0.058
SA22(1)-III (n=2)	0.381	**0.449**	\	0.096	0.340	0.342	0.063	0.338
SA22(2)-I (n=3)	0.404	**0.542**	0.511	\	0.103	0.105	0.015	0.096
SA22(2)-II (n=2)	0.445	0.540	0.535	0.609	\	0.329	0.042	0.331
SA22(2)-IV (n=2)	0.546	0.622	0.571	0.750	0.639	\	0.052	0.335
SA22(2)-V (n=5)	**0.478**	**0.656**	0.633	**0.519**	**0.661**	0.782	\	0.043
SA22(2)-VI (n=2)	0.245	0.491	0.406	0.580	0.485	0.721	**0.618**	\

*Probability, based on 999 permutations is shown above the diagonal. Values shown in bold are significant at P = 0.05.

(a)   (b)
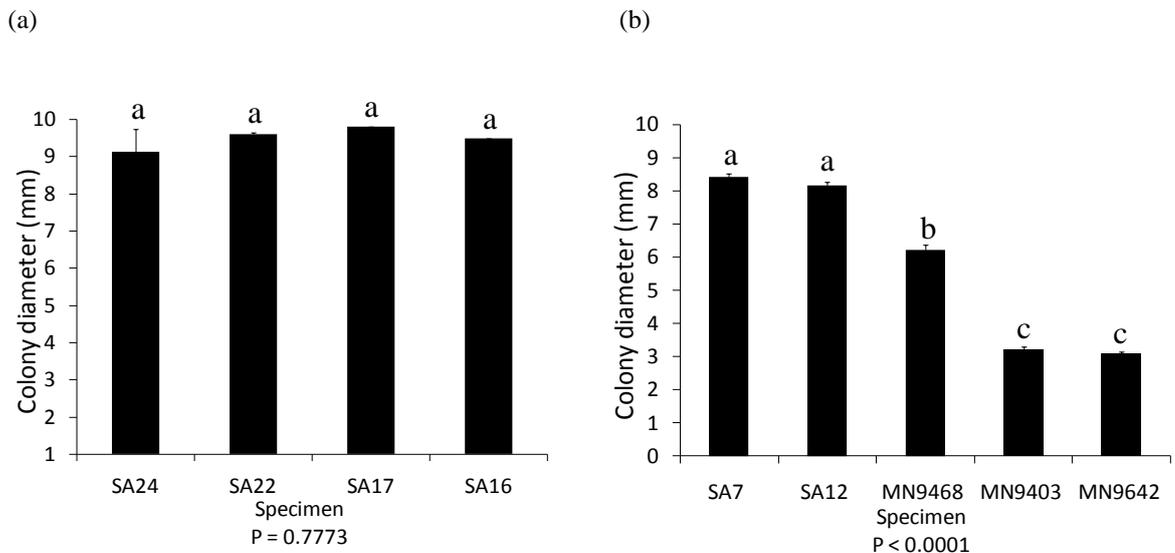
Figure 4. Comparison of average colony diameter between specimens of a) *C. rangiferina;* and b) *C. arbuscula.* Bars with different letters are significantly different. Specimen names with SA represent collection by Athukorala and MN represents collection by Piercey-Normore.
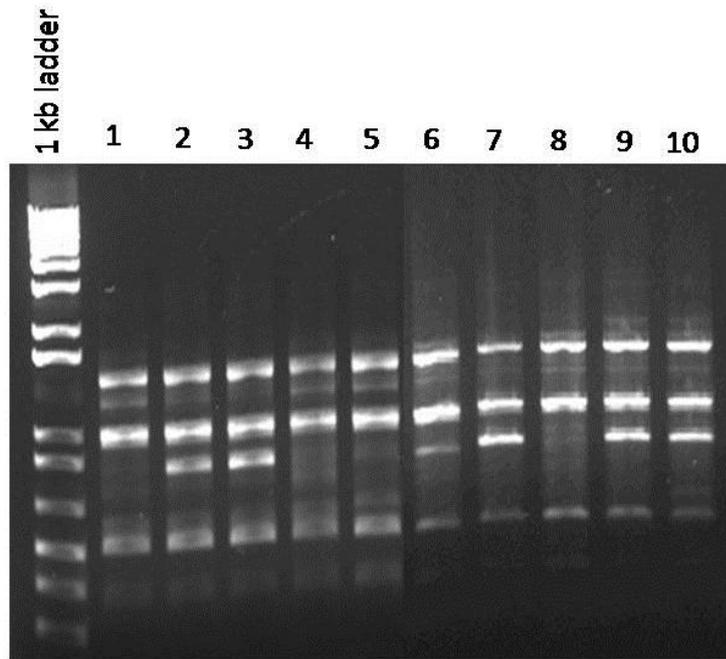
Figure 5. RAPD-PCR agarose gel image obtained from spore cultures of *C. rangiferina* with the primer UBC 31. Comparison of RAPD-PCR band patterns between three apothecia of *C. rangiferina* sample Athukorala 22 [(SA22(2)]. Each column represents a spore colony. Lanes 1 – 5 are from apothecium V, lanes 6 – 8 from apothecium I, and lanes 9 – 10 from apothecium IV. The far left lane is the 1Kb DNA ladder showing fragment lengths from 500 to 1600 bp.
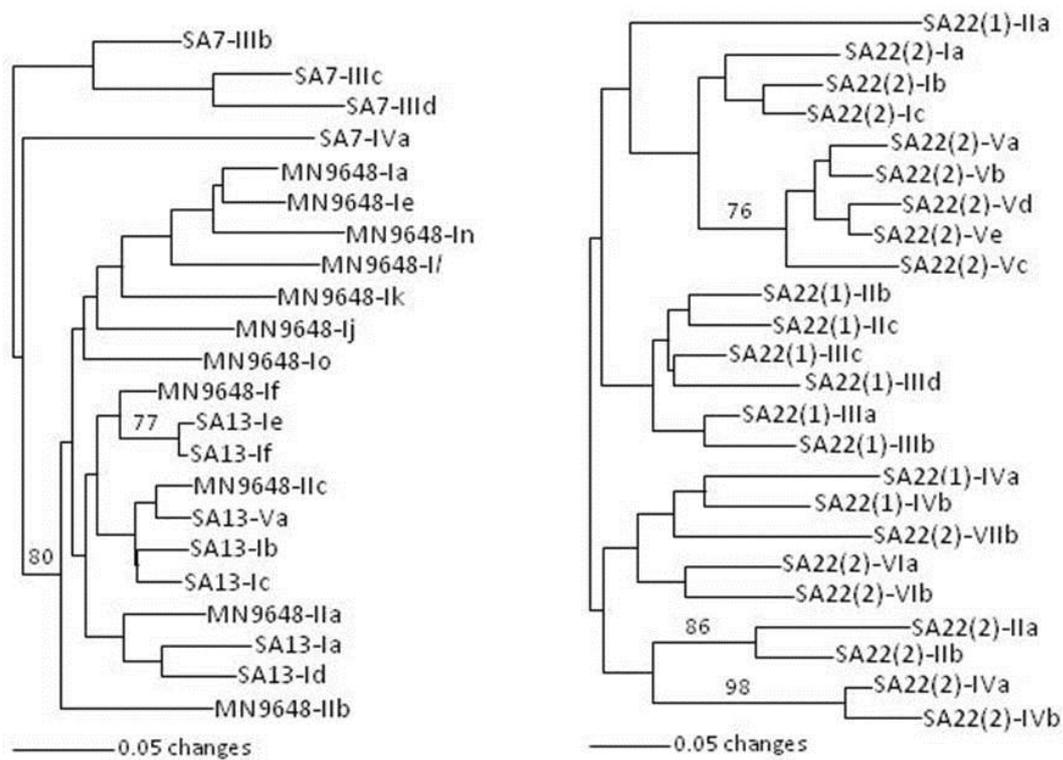

Figure 6. NJ phenograms derived from combined RAPD patterns obtained from mycobiont colonies (lower case letters) from apothecia (Roman numerals) of A) *C. arbuscula* and B) *C. rangiferina*. Sample Athukorala 22 shows two podetia (1 and 2) in parentheses. Bootstrap values greater than 70% are shown on the branches. SA represents Athukorala and MN represents Normore sample numbers.

DISCUSSION

Cladonia arbuscula is more genetically diverse than *C. rangiferina* – The greater morphological and genetic diversity in *C. arbuscula* than *C. rangiferina* may imply that *C. arbuscula* has an advantage over *C. rangiferina*. Greater genetic diversity may facilitate greater adaptive potential for the large range of habitats which is reflected in its wide geographic distribution in both hemispheres (Ruoss, 1987; Ruoss and Ahti, 1989). Studies on *C. furcata* and *C. rangiferina* suggested that a successfully fertilized ascogonium may give rise to several apothecial discs containing asci and ascospores (Jahns, 1973; Jahns *et al.,* 1978, 2004). This may explain why the genetic variation within and among apothecia of *C. rangiferina* was not very high (Table 3). The same phenomenon may also occur in *C. arbuscula*. Even though *C. arbuscula* shows a higher range of genetic and morphological polymorphism than *C. rangiferina* in terms of growth rates (Fig. 2) and colony diameter (Fig. 4), the current study shows comparable numbers of haplotypes (18 for *C. rangiferina* and 15 for *C. arbuscula*), which were also reported in other studies. Eleven genotypes were detected for *C. arbuscula* within a 2 km range in northern Manitoba using RFLP and SSU rDNA (Robertson and Piercey-Normore 2007). Twenty-seven haplotypes were reported in four loci from 30 samples of *Lobaria pulmonaria* collected from different continents using SSR (Walser *et al.,* 2003). Lindblom and Ekman (2006) found 10 intergenic spacer (IGS) and 16 ITS haplotypes in *Xanthoria parietina* in a 3 km range. Each study used markers that were different from those used in this study and from one another; therefore, direct comparisons are difficult to make.

C. arbuscula and C. rangiferina may be heterothallic - Environmental conditions including illumination, humidity or nutrient status have been postulated as an explanation for the high variability among thalli of *C. arbuscula* (Culberson and Armaleo, 1992; Hawksworth, 1976; Leuckert *et al.,* 1990; Rikkinen, 1997). However, the colony variation shown in this study with constant culture conditions suggests that genetic inheritance also plays a role in variability. The variation in ascospore colony diameter (Fig. 2) and level of gene flow (Table 3) within and between apothecia of *C. arbuscula* and *C. rangiferina* after sexual reproduction has occurred, may be explained by recombination events and heterothallism (Honegger *et al.,* 2004). Since genetically isolated populations should form strong clades in combined gene trees, the low levels of clade support and poor resolution in combined trees in this study may imply sexual recombination following the phylogenetic species recognition (PSR) (Taylor *et al.,* 2000). Heterothallism was detected in

Xanthoria spp. where RAPD polymorphism corresponded with phenotypic characters leading to the hypothesis that five *Xanthoria* species were heterothallic. Honegger and Zippler (2007) reported all 16 species examined to be heterothallic. The variation in colony morphology (Fig. 3) and growth rates (Fig. 2) in this study also supports heterothallism (Honegger and Zippler, 2007).

Species differ in gene flow and growth - High Φ_{PT} values shown by *C. rangiferina* (7 of 28 pairwise comparisons) suggests more population subdivision than in *C. arbuscula*. Kotelko *et al.,* (2008) reported significant population subdivision in *C. arbuscula* collected from the same study site, while Robertson and Piercey-Normore (2007) reported low levels of population subdivision in *C. arbuscula* using a different marker in a different study. The combination of low levels of recombination events and gene flow in *C. rangiferina*, which might be a result of less frequent sexual reproduction, may be a trade-off for the faster growth rates observed (Fig. 2). Jahns *et al.,* (1998, 2004) suggested that the induction of sexual reproduction depends on the synchronizing between microclimate and fruiting body development initiation capacity of the lichen and they observed high sexual reproduction in *C. rangiferina* under favourable microclimatic conditions. The vegetative growth was not terminated by the development of apothecia further suggesting the importance of vegetative growth to *C. rangiferina* over sexual reproduction. Prominent vegetative growth shown by *C. rangiferina* supports the low levels of genetic variation observed in the current study. Some lichen fungal populations with low levels of variation have been hypothesized as clonal (Beard and De Priest, 1996), while the others with higher levels of variation were hypothesized to show sexual reproduction (De Priest, 1993; Zoller *et al.,* 1999). The comparison of colony growth between the species is consistent with literature findings in field studies where the biomass of *C. rangiferina* was higher than that of *C. arbuscula* in a bog habitat. The growth rate of *C. rangiferina* was reported to fall within the same range (3.9–7.7 mm/yr and 4.6–7.5 mm/yr, respectively) by Vasander (1981). However, the growth rates were different (3.9–4.3 mm/yr for *C. rangiferina* and 3.0-3.5 mm/yr for *C. mitis*) as reported by Helle *et al.,* (1983). These results are somewhat consistent with the faster growth of *C. rangiferina* in this study, recognizing that the growth under laboratory conditions may not be representative of field conditions.

In summary, the high levels of gene flow and the incongruence in the NJ dendrograms within and between individuals of the same species for both *C.*

arbuscula and *C. rangiferina* in the current study suggested heterothallism. The faster colony growth and lower range of variation with time in mycobiont spore cultures of *C. rangiferina* than *C. arbuscula* was consistent with the smaller range of colony diameter and number of haplotypes in *C. rangiferina* than *C. arbuscula*. The wider range of variability in *C. arbuscula* may give it an adaptive advantage over *C. rangiferina*. However, the success and widespread distribution of both species in northern regions may be explained by a heterothallic lifestyle and high levels of diversity, which may facilitate their adaptation to changing environmental conditions.

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