Embryological and phenological comparison between *Arceuthobium americanum* (the lodgepole pine dwarf mistletoe) growing on *Pinus contorta* var. *latifolia* in British Columbia and on *P. banksiana* in Manitoba.

Abstract

The genus *Arceuthobium* comprises angiosperms that are aerial parasites on Pinaceae and Cupressaceae. These parasites are serious forest pests in North America, where they damage timber trees. In Canada, *Arceuthobium americanum* (the lodgepole pine dwarf mistletoe) is principally found on *Pinus contorta* var. *latifolia* (lodgepole pine) in British Columbia (BC) and Alberta as well as on *Pinus banksiana* (jack pine) in Alberta, Saskatchewan and Manitoba. Although widespread, *A. americanum* seems morphologically uniform in its range. However, the taxon has been divided into two distinct genetic races based on the variation observed in amplified fragment length polymorphism (AFLP) data. This led us to predict that there would be some variation in anatomical, as opposed to external morphological form. Therefore, the objective of this study was to determine if the embryological anatomy of *A. americanum* growing on *P. contorta* var. *latifolia* in BC differed from that growing on *P. banksiana* in Manitoba. Samples of female flowers were collected from both BC and Manitoba throughout the growing season and prepared for routine light microscopy. Key embryological stages were observed, and the relative timing of these stages were compared between the BC and Manitoba collections. Our results were in direct contrast with our prediction, which had to be falsified: hundreds of prepared sections revealed that major embryological events, including double fertilization and zygotic division, are indistinguishable and occur at essentially the same time. This

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first study on emrbyology of two plants growing on the two pine speces at the extreme geographic ranges shows that the development is similar. Despite climate, host, ecology, and geography, which remain variables of undetermined relative significance, the mechanism for embryological development is likely highly conserved for *A. americanum* across Canada.

Introduction

Arceuthobium americanum (the lodgepole pine dwarf mistletoe) has the most extensive distribution of any North American dwarf mistletoe. In Canada, these aerial parasites are principally found on *Pinus contorta* var. *latifolia* (lodgepole pine) in BC and Alberta as well as on *Pinus banksiana* (jack pine) in Alberta, Saskatchewan and Manitoba (Hawksworth and Wiens 1996). Jerome and Ford (2002) argued that amplified fragment length polymorphism (AFLP) data showed enough variation to justify dividing the taxon into distinct genetic races, even though there are no apparent morphological or phenological differences among *A. americanum* growing in the different regions or on different hosts (Figure 1). We tested Jerome and Ford's hypothesis for genetic races, by looking for variation in anatomical, as opposed to external morphological, form. Such anatomical variation might be detected via an embryological study.

Differences in embryological development are often used as taxonomic characters (Friedman 2001; Verdu 2006; Raghavan 2006). Ross and Sumner (2005a, 2005b) made detailed descriptions of key early embryological events and their timing as they occurred in *A. americanum* infecting *P. banksiana* in Manitoba. These events included double fertilization, subtension of the embryo by three endosperm cells, transverse zygotic division and formation of a two-celled embryo, formation of the four-celled embryo, and formation of the eight-celled embryo.

Therefore, the objective of this study was to observe and map the relative timing of these same embryological stages in *A. americanum* growing on *P. contorta* var. *latifolia* in BC and compare the results to those obtained in Manitoba to determine if there was anatomical variation. While site differences might generate variation in timing, major discrepancies in comparative embryology could point to a either a host influence and (or) differences in the genetic pathway for embryology

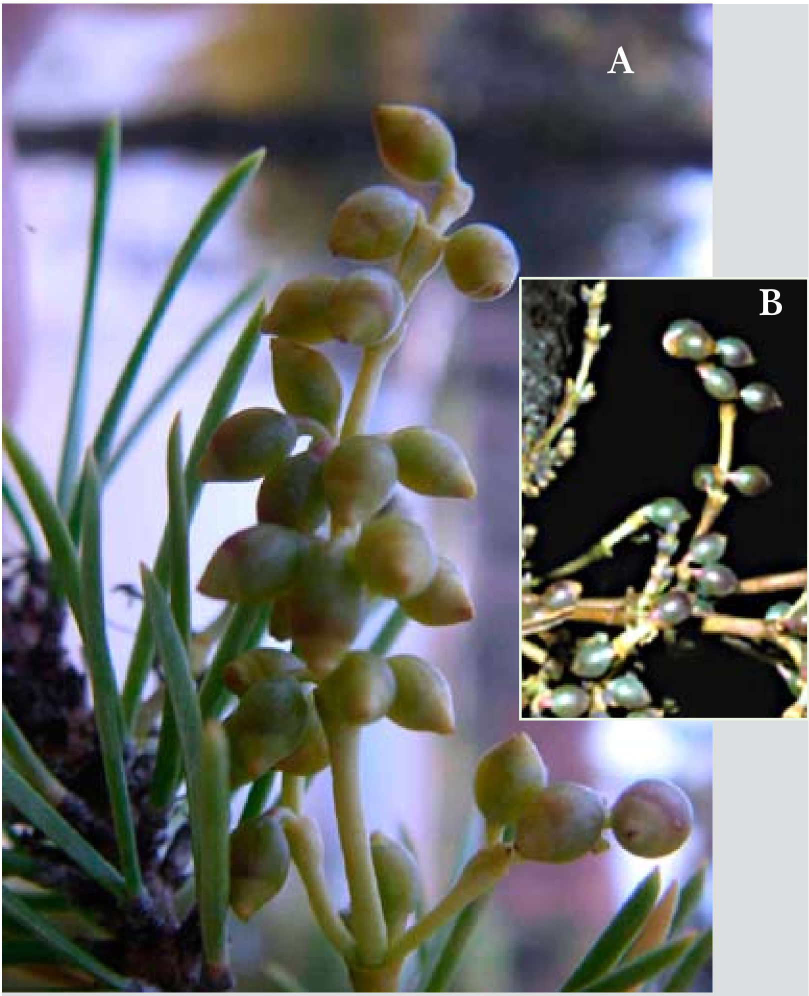

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Figure 1: Pistillate plants of *A. americanum* with fruit growing on (A) *P. latifolia var. contorta*; (B) *P. banksiana*. These plants were sampled at the same time in the year (mid August), are at the same stage of development, and are essentially indistinguishable.

between the two groups. This would provide more support for Jerome and Ford's (2002) division of the taxon into distinct races. This is also the first comparison of the embryology of any *Arceuthobium* species growing on two different hosts.

Methods

Study Sites and Collection

At least thirty pistillate aerial shoots of *Arceuthobium americanum* were sampled from infected *Pinus contorta* var. *latifolia* in an area adjacent to Stake Lake (located 30 km south of Kamloops, BC at 50° 31' N, 120°28' W) over one growing season daily from May 1, 2005 through Sept 1, 2005. Similarly, thirty pistillate aerial shoots had previously been collected from infected P. banksiana in the Belair Provincial Forest (within 5 km of Belair, Manitoba at 50°36' N, 96°32' W) biweekly from March 15, 1995 through March 15, 1997 (Ross and Sumner 2005a, 2005b). At each site, the shoots were placed into a fixative consisting of 2% paraformaldehyde + 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 6.8). The material was taken immediately to the laboratory and placed at 4 °C overnight for fixation, rinsed in the same buffer, and post fixed with 2% osmium tetroxide in the same buffer for 4 h. The material was dehydrated in an ethanol series and embedded in Spurr's resin (Spurr 1969).

Light Microscopy and Documentation

Sections 2 µm thick were obtained with a Sorvall Porter-Blum JB-4 microtome (Sorvall, Newtown, Connecticut) and affixed to gelatin-coated glass slides (Jensen 1962). The slides were routinely stained with 2% crystal violet in a 0.05 M ammonium oxalate buffer (pH 6.7) and were observed and photographed with a Nikon Optiphot compound light microscope (Nikon, Tokyo, Japan). In order to describe and accurately time the occurrence of key embryological stages, at least fifteen representative sections from fifteen different female flowers displaying the same embryological stage needed to be observed before an event date was ascribed. Relevant temperature data for the two sites were obtained from FORECA's open source climatological data.

Results and Discussion

Based on the variation observed in AFLP data, Jerome and Ford (2002) divided *Arceuthobium americanum* into distinct genetic races. This led us to predict that there would be some variation in anatomical, as opposed to external morphological, form. As patterns of embryology have been used as taxonomic characters (Friedman 2001; Verdu 2006; Raghavan 2006), we thought that perhaps differences in embryology might distinguish *A. americanum* growing on the different hosts in the different regions, even if these differences could not necessarily be disentangled from ecological ones. However, our prediction was shown to be incorrect. There was remarkable similarity not only in the physical attributes of key early embryological stages in *A. americanum* infecting *Pinus contorta* var. *latifolia* in BC and *P. banksiana* in Manitoba (Figure 2), but also in the relative timing of the events (Table 1). Therefore, embryological development in *A. americanum* is likely highly conserved across Canada, despite climate, host, ecology, and geography, which remain variables of undetermined relative significance.

Moreover, even though the sampling from the two sites was accomplished in different years, the calendar month dates of the embryological events were essentially identical (Table 1): for example, double fertilization in BC took place on May 30 in 2005 and similarly occurred in Manitoba on May 30 in 1995, 1996, and 1997. This suggests that *Arceuthobium americanum* embryology is under strict regulation, at least in its Canadian range. As the latitudes of the two sites were essentially the same (50° 31' N for Stake Lake, BC, 50° 36' N for Belair, Manitoba), day length is likely the main controlling factor. Temperature might also play a governing role; the temperature highs and lows were notably similar at both sites on each event date (Table 1).

Our results are significant because they indicate that the embryology of *Arceuthobium americanum* in its Canadian range is predictably uniform, and so embryological patterns could be considered to be reliable characteristics of the species. Species in *Arceuthobium* are notoriously difficult to distinguish from each other by morphology (Hawksworth and Wiens 1996). Perhaps the predictable embryology of A. americanum will help to distinguish it from other members of the genus. While search of the plant and animal literature has yielded no descriptions of the comparative embryology of parasites growing on different hosts and limited embryological comparisons of any one species (parasitic

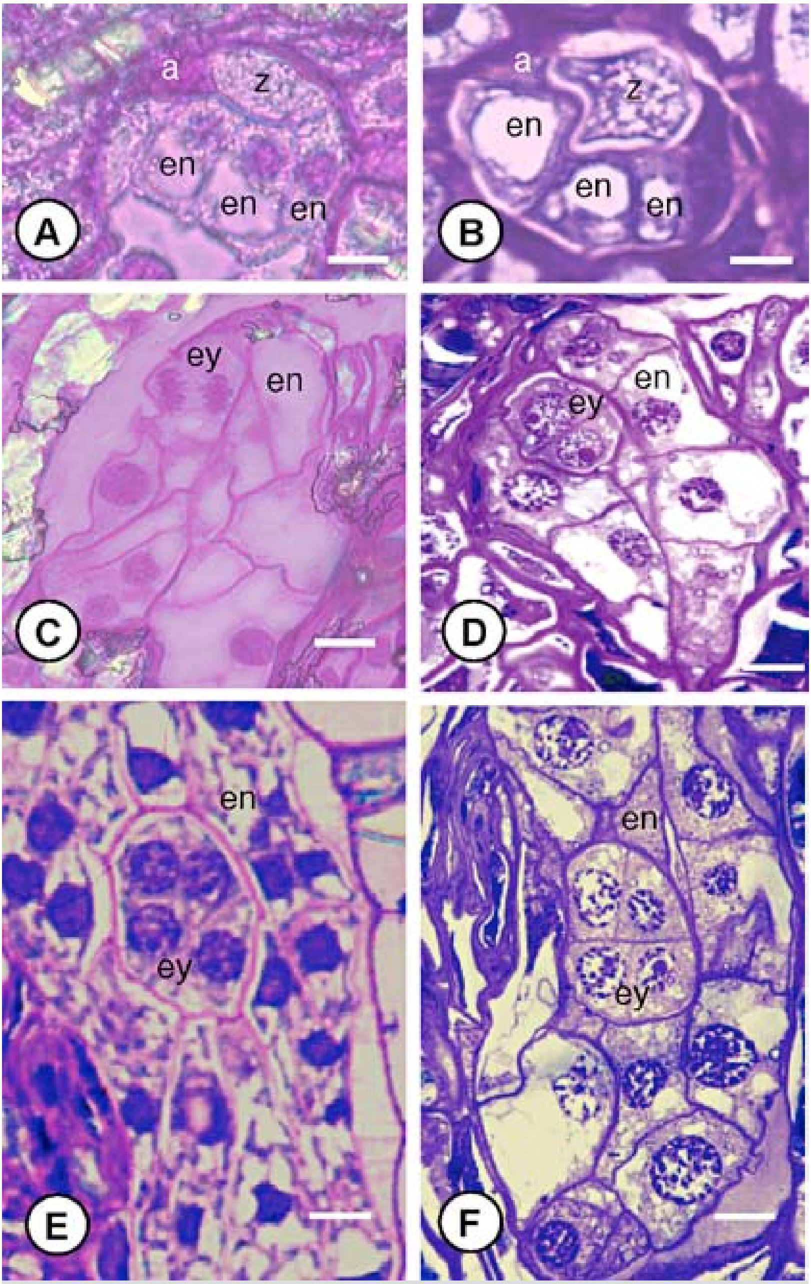

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Figure 2: Some examples showing early embryological stages of *A. americanum* growing on *P. latifolia* var. *contorta* (A, C, E) compared with the same species growing on *P. banksiana* (B, D, F;

these three figures originally appeared in Ross and Sumner 2005b and are reprinted here with permission). (A) Growing on *P. latifolia* var. *contorta*, the *A. americanum* zygote has just been subtended by three endosperm cells, which are beginning to crush the antipodals (July 10 in 2005); (B) as in (A), but on *P. banksiana* (July 10 in 1995, 1996, and 1997); (C) newly formed two-celled *A. americanum* embryo (transverse zygotic division had just taken place) observed on *P. latifolia* var. *contorta* (August 8 in 2005); (D) as in (C), but on *P. banksiana* (August 9 in 1995, 1996, and 1997); (E) Formation of the four-celled *A. americanum* embryo as observed on *P. latifolia* var. *contorta* (August 15 in 2005); (F) as in (E), but on *P. banksiana* (August 15 in 1995, 1996, and 1997). The similarity in the structures, relative timing, and actual calendar date are striking. a = antipodals (crushed), en = endosperm (or cell of), ey = embryo; z = zygote. In (A) and (B), scale bar = 12 µm; in (C) and (D), scale bar = 20 µm; in (E) and (F) scale bar = 15 µm.

or otherwise) across wide geographical ranges, our study is novel, and should be repeated for other parasites and hosts to see if differences can be detected.

If distinct genetic races of *Aceuthobium americanum* do exist (Jerome and Ford 2002), early embryological development cannot be used to tease them apart, and our results do not support the division of the taxon. This first study on the embryos of *A. americanum* growing on two pine species at the extreme geographic ranges of the species reveals that the embryo structures are similar. A useful exercise for the future would be to map and compare embryological stages occurring prior to double fertilization and after formation of the eight-celled embryo, as well as to examine stages of staminate development. It would also be useful to eliminate geography and site differences as variables: the embryology of *A. americanum* could be observed as it occurs in Alberta, where the two host pines are found growing side by side. The rigour shown in embryological development promises potential for widespread disease control, because it is likely that *A. americanum* across Canada reach similar stages of development at similar times; if a certain stage turns out to be vulnerable to a management tool, that stage could be targeted ubiquitously.

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Table 1

Embryological Event in *A. americanum*	On *P. contorta* var. *latifolia* in BC (2005)		On *P. banksiana* in Manitoba (1995, 1996, and 1997)	
	Event Date	†Temperature Range on Event Date (low, high) in °C	Event Date	†Mean Temperature Range on Event Date (low, high) in °C
double fertilization	May 30	10, 23	May 30	10, 20
zygote subtended by three endosperm cells	July 10	14, 28	July 10	16, 26
transverse zygotic division/ two-celled embryo	August 8	14, 28	August 9	15, 26
four-celled embryo	August 15	13, 27	August 15	15, 25

†Temperature data from FORECA (±2°C)

Comparative timeline of key embryological events and temperature data for *A. americanum* infecting *P. contorta* var. *latifolia* in BC and *P. banksiana* in Manitoba. Key events occurred essential on the same calendar date, regardless of the site or year. In order to describe and accurately time the occurrence of key embryological stages, at least fifteen representative sections from fifteen different female flowers displaying the same embryological stage needed to be observed before an event date was ascribed. This was not difficult: in all cases aside from formation of the eight-celled embryo, all fifteen replicates per case were found in order and exclusive of variation. Furthermore, for the eight-celled embryo stage, only two samples differed: one in 1996 in Manitoba, and one in BC, and only by one day later in both cases. The temperature highs and lows were notably similar at both sites on each event date.

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