

ZYMOYPES IN VACCINIUM SECTION CYANOCOCCUS AND RELATED GROUPS.

N.M. HILL and S.P. VANDER KLOET
Department of Biology
Acadia University
Wolfville, N.S., B0P 1X0

The potential use of the isoenzyme technique for resolving species deliniations within *Vaccinium* section *Cyanococcus* was investigated. Only young leaf tissue produced reproducible results upon staining for malate dehydrogenase and phosphoglucose isomerase. These zymograms showed great uniformity among species of *V.* section *Cyanococcus* and species of other sections of the genus. While peroxidase and esterase enzyme banding patterns were not as clear nor reproducible, their zymotypes varied little among species tested. It is hypothesised that the overall similarity among species of *V.* section *Cyanococcus* in isoenzyme patterns reflects the youth of this group.

La possibilité d'utiliser la technique des isoenzymes pour décrire les espèces du genre *Vaccinium* section *Cyanococcus* fut étudiée. Des résultats reproductibles m'ont été obtenus pour les colorations de la malate déshydrogénase et la phosphoglucose isomerase qu'en utilisant des tissus provenant de jeunes feuilles. Les patrons de ces enzymes ont montré l'existence d'une grande uniformité parmi les espèces du genre *Vaccinium* section *Cyanococcus* et parmi les espèces des autres sections de ce genre. Même si les patrons enzymatiques obtenus pour la peroxidase et l' α -esterase ne furent pas aussi précis et reproductibles, ces enzymes ont montré peu de variation parmi les espèces étudiées. Une hypothèse est émise selon laquelle, la ressemblance générale, des patrons enzymatiques des espèces de *Vaccinium* section *Cyanococcus* reflèterait l'apparition récente de ce groupe.

Introduction

* The provenance of all individuals of the species tested is listed in Appendix 1.

Vaccinium section *Cyanococcus* A. Gray consists of nine species, eight of which are restricted to eastern North America, and one, *V. myrtilloides*, is boreal and transcontinental (Vander Kloet, 1982). Species referred to this section have verrucose twigs of the current season; dimorphic perennating buds (viz. floral and vegetative) covered with several (≥ 5) scales; corymbose inflorescences; pedicels articulated with the calyx; awnless stamens included in urceolate or cylindrical corollas; and pseudo-10 loculed berries.

Camp (1942, 1944, and 1945) recognized 24 phylogenetic units, labelled for convenience as species, in *V.* section *Cyanococcus*. Subsequent observations made by Vander Kloet (1972, 1977, 1980, 1982) showed, however, a high incidence of transitions within species of the character states used by Camp for his classification. Vander Kloet (1972, 1977, 1980, 1982) turned to Adansonian principles and numerical taxonomy (Sneath and Sokal 1973) to re-organize the section. The phenetic species defined by these techniques had unique habitat requirements and together with ethological factors gave confidence in their existence as genetic entities (Vander Kloet 1983).

To date biochemical evidence has not been applied to test this systematic rearrangement within *V.* section *Cyanococcus*. Indeed, taxonomically significant biochemical data involving the genus have been scant and thus far restricted to the use of seed material (Frohne and John 1978). Seeds of *Vaccinium* species are small and their high lipid content makes protein extraction troublesome (Vander Kloet, unpublished data). On the other hand, leaves were continuously available from greenhouse plants. This study uses leaf material in order to discover electrophor-

etic differentiation of enzymes not only among species of *Vaccinium* section *Cyanococcus* but also among several sections.

Materials and Methods

Leaves for electrophoretic study were chosen from individuals of species in *V. section Cyanococcus* (viz. *V. boreale*, *V. corymbosum*, *V. darrowii*, *V. myrtilloides* and *V. tenellum*), as well as from members of species in other sections (viz. *V. section Myrtillus*: *V. membranaceum*, *V. myrtillus* and *V. parvifolium*; *V. section Oxycoccus*: *V. macrocarpon*; *V. section Vaccinium*: *V. uliginosum*), which were introduced to make intrageneric comparisons. All plants used were grown from seed at Acadia University greenhouse under uniform conditions.

Ten newly unfurled leaves and ten hardened mature leaves were taken from each plant. These were ground separately with sterilised sea sand and chilled extraction buffer (in the ratio 1 gm tissue (fr. wt.) to 4 ml buffer) in a chilled mortar and pestle. The extraction buffer found to minimize phenolic interference was 0.5 M phosphate buffer at pH 7.0, including the following reagents at indicated concentrations: cysteine HCl, 15 mM; DIECA (diethyldithiocarbamic acid) 11 mM; dithiothreitol, 20 mM; EDTA (ethylenediaminetetraacetic acid), 20 mM; Polyethylene glycol, 6% (wt./vol.) and insoluble PVPP (polyvinylpyrrolidone) at 4% (wt./vol.). This solution was made 24 h before grinding, to hydrate the polymer, PVPP (Loomis and Battaile, 1966).

Once a liquid slurry was reached, leaf extracts were squeezed through four layers of cheesecloth into chilled beakers. Wicks of Whatman No. 1 filter paper were used to absorb this exudate, and these were inserted in 10.5% starch gels. Gels were run at 20 mAmp for 4-5 h.

Two concentrations: A(0.3 M) and B(0.115 M), of tris-citrate electrode buffer were tested at pH 7.5. The corresponding gel buffer in each case was a fivefold dilution of the electrode buffer (adapted from buffer system 1 of Shaw and Prasad, 1970).

Malate dehydrogenase (MDH) and phosphoglucose isomerase (pg 1) were stained according to Schaal and Andersen (1974). The recipe of Shaw and Prasad (1970) was followed for staining α -esterase, and a modification of their recipe for peroxidase staining was made employing o-dianisidine for enzyme staining.

The distance of enzyme band migration from the point of insertion was recorded. Relative migration (RM) was calculated based on the furthest anodal band (RM + 1.0) from the insertion RM 0). Cathodal electromorphs were similarly computed with a negative RM.

Preliminary trials with the following enzyme systems using the recipes of Schaal and Anderson (1974) were unsuccessful: acid phosphatase, alcohol dehydrogenase, alkaline phosphatase, catalase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, glutamate oxaloacetate dehydrogenase, leucine-aminopeptidase, 6-phosphoglucuronate dehydrogenase, and xanthine dehydrogenase.

Results

The extraction technique described herein was found best for preventing browning of extracts and producing reproducible enzyme banding. Interference from phenolics, however, still limited the investigation to four enzyme systems (viz. α -esterase, malate dehydrogenase, peroxidase and phosphoglucose isomerase) and of these, only peroxidase showed activity when mature leaf extracts were tested.

When young flush leaves were used, the effects of phenolic oxidation could still be witnessed unless extractions were kept chilled. In fact one experiment was conducted using young leaf material of *V. corymbosum* (Yar No. 31, 42; HB No. 42 Appendix), in which one set of extracts was allowed to come to 20°C while a duplicate set was kept chilled. The first set at room temperature were visibly browned while the chilled extracts remained green. Browned extracts showed no PGI activity and the typical slow MDH electromorph under buffer system A (see Fig 1) was deleted. α -Esterase zymotypes for browned extracts showed more streaking than did their chilled counterparts, and enhanced migration of peroxidase electromorphs resulted when browned extracts were run.

These effects could be duplicated by the addition of leaves of the evergreen *V. corymbosum* (Fusc. 31, 49) to grinding mixtures of young *V. corymbosum* (Yar 31, 42) leaves.

Conflicting α -esterase and peroxidase zymotypes were obtained for several individuals* of the *V. section Cyanococcus* species in separate electrophoretic trials. α -Esterase and peroxidase zymotypes failed to reveal reproducible intrasectional differences. A three banded peroxidase zymotype (Rm -0.3, +0.25, +1.0) was shared by the majority of individuals (viz. *V. boreale*: 327975, 39976; *V. corymbosum*: Fusc. 11, 15, 31, 49; HB 39; Yar 31, 42, 48, 57, 69, 76; *V. darrowii*; Abs 10; *V. tenellum*: 1428574; 128574; 121675) of *V. section Cyanococcus* species. A four banded α -esterase zymotype (Rm -0.3, -0.2, +0.6, +1.0) was produced by individuals of *V. corymbosum* (Yar 48, 57, 69 and 76) and *V. boreale* (327975, 39976). Other individuals tested demonstrated α -esterase patterns which were assorted combinations of various electromorphs of the four banded zymotype.

The inconsistent nature of the α -esterase and peroxidase results caused us to concentrate instead on the more accountable PG1 and MDH enzyme systems. These were found to be quite susceptible to phenolic interference and even the young leaves of several species (viz. *V. darrowii*, *V. tenellum*, and the evergreen members of *V. corymbosum*) were unsuitable for PGI and MDH electrophoretic study.

All individuals of species of *V. section Cyanococcus* (viz. *V. boreale*: 327975, 39976; *V. corymbosum*: HB 39, 22; Yar 31, 39, 42, 48, 57, 68, 69, 76, 80; *V. darrowii* LII313; *V. myrtilloides* 21881, 517773) and of other sections of the genus viz. *V. section Myrtillus*; *V. myrtillus*; *V. membranaceum*: 42979, 629879; *V. parvifolium*: 229879, 731879; *V. section Oxycoccus*; *V. macrocarpon*: 6121081, 1271081; *V. section Vaccinium*, *V. uliginosum*: 613821) produced a uniform two banded zymotype (Rm. +0.7, +1.0). One individual of *V. darrowii* tested (viz. Abs 6) produced a single slow (Rm +0.7) electromorph. Follow up work was not possible due to the age of the tissues.

All species of the sections tested (viz. *V. section Cyanococcus*; *V. boreale* 327975, 39976; *V. corymbosum*: Yar 31, 39, 42, 48, 57, 68, 69, 76, 80 HB 22, HB 39; *V. darrowii*, Abs LII313; *V. myrtilloides* 21881, 517773; *V. Tenellum* 1428574, 128; *V. Myrtillus*: *V. membranaceum* 629879; *V. myrtillus* 11277; *V. parvifolium*: 731879; *V. Oxycoccus*: *V. macrocarpon* 1271081; *V. Vaccinium*: *V. uliginosum* 613281) except *V. darrowii* (viz, Abs 6, 10) exhibited a monomorphic double banded MDH zymotype, when buffer system "A" was used (e.g. Fig 1). *V. darrowii* individuals, Abs 6 and 10, expressed the single slow MDH band only.

* α -esterase - *V. corymbosum*: Fusc. 9, 11; HB 22; Yar 31, 42; WS 2; *V. darrowii* Abs LII 313; *V. tenellum* 1428574

peroxidase—*V. corymbosum*: Fusc. 9, 15; Yar 48, 57; *V. darrowii*: Abs 6, 10; *V. tenellum* 1428574

Under the more dilute buffer regime "B" it was discovered that the slow MDH electromorph of *V. corymbosum* Yar 31, 42, 48 and 68, see Fig 1) and of *V. myrtillus* (31076) was composed of two electromorphs of very similar mobility (R_m^* .8 and .6, 1.0 and .6 respectively (see Fig 2).

Discussion

Phenolic interference was observed for all enzyme systems investigated. The extraction technique was inadequate for older leaf tissue. Only the peroxidase system was stainable using mature leaf extracts. α -Esterase, MDH, and PGI zymotypes were all susceptible to modification by phenolics. This was suggested by the simple addition of mature leaves to young leaf extracts.

Browning of extract was closely correlated with enzyme banding quality. The slow MDH electromorph was more susceptible to interference than the fast electromorph, and could be deleted while the more resistant fast MDH electromorph was preserved. This situation might easily be misinterpreted without adequate replication with young tissues. A similar case was reported by Townsend (1970) and Dirr et al (1972), who reported no nitrate reductase activity in blueberry leaf homogenates. Enzyme activity could only be detected when *in vivo* techniques were used (Dirr et al 1972).

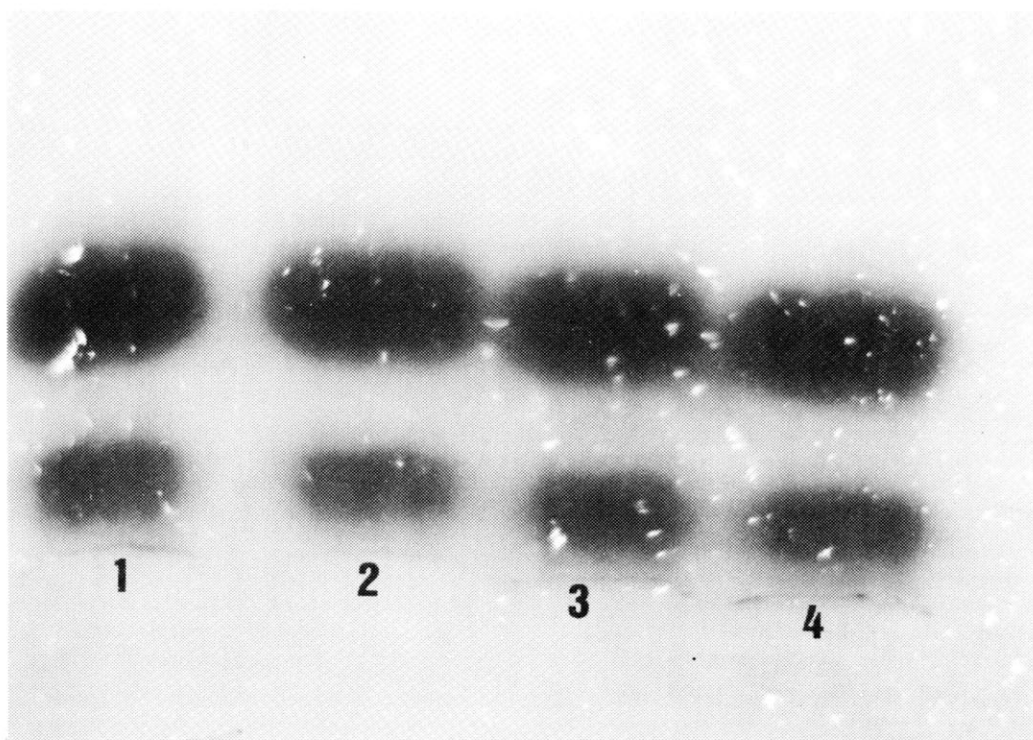


Fig 1 MDH zymotype of *V. corymbosum* using concentrated buffer system "A". (1 = YAR 48; 2 = YAR 57; 3 = YAR 69; 4 = YAR 76).

* R_m based on the fastest of the *V. myrtillus* slow anodal electromorphs.

Soltis et al (1980) simplified the technique of Kelley and Adams (1977) and omitted centrifugation, separatory procedures and the costly germanium dioxide. Similarly we found no benefit from centrifugation or germanium dioxide in this study. However the potential for separatory procedures should be investigated with blueberry leaves, because of the limitations associated with the present extraction process.

These limitations were further manifested in the variable results obtained upon peroxidase and α -esterase staining. In spite of these inconsistencies however, the majority of *V. section Cyanococcus* species tested (viz. *V. boreale*, *V. corymbosum*, *V. darrowii* and *V. tenellum*) exhibited a three banded peroxidase zymotype. Moreover all these species possessed various α -esterase electromorph combinations of the four banded zymotype found for several *V. corymbosum* and *V. boreale* individuals.

The conservative nature of *V. section Cyanococcus* zymotypes was also found for PGI and MDH enzyme systems. These were monomorphic throughout the *V. boreale*, *V. corymbosum* and *V. myrtillus* individuals tested. This conservatism extended to other sections of the genus (viz. *V. section Myrtillus*, *V. section Oxycoccus*, and *V. section Vaccinium*) which shared the same PGI and MDH zymotypes found for *V. section Cyanococcus* species.

When the more dilute buffer system "B" was used, however, *V. corymbosum* (of *V. section Cyanococcus*) and *V. myrtillus* (of *V. section Myrtillus*) were each found to possess an additional slowly migrating MDH electromorph. This could be used to differentiate them from other species as well as from each other. PGI results were unchanged using the different buffer concentrations.

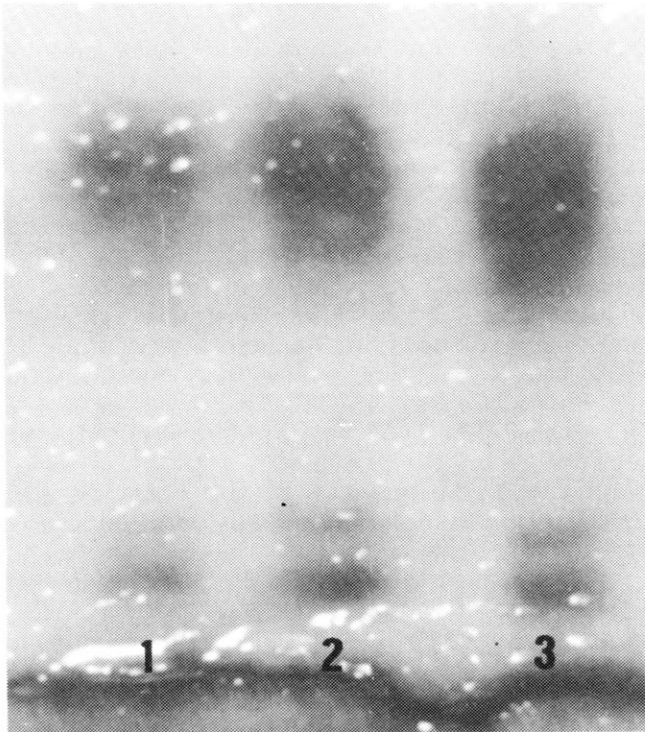


Fig 2 MDH zymotype of *V. myrtillus* using dilute buffer system "B". (1 - 31076; 2 - 31076 x 11277; 3 - 11277).

The overall zymotype similarity observed throughout *V. section Cyanococcus* for all enzyme systems investigated (viz. α -esterase, MDH, peroxidase and PGI) supports the youth of the group. The ancestral *Cyanococcus* is believed to have entered the North American continent via the Proto-Antilles from South America, and to have invaded the sand dunes of peninsular Florida during the Tertiary (Vander Kloet 1982). However despite adaptive radiation, which included the loss of evergreen and xeromorphic characters, homoploids developed no incompatibility systems. In fact there is circumstantial and experimental evidence that *V. corymbosum* resulted from the introgression of *V. tenellum* into the range of *V. darrowii* during the Pleistocene (Vander Kloet 1980).

Various authors (e.g. Soltis, 1982; and Schwaegerle and Schaal, 1979) have discovered low amounts of enzyme polymorphism in groups where extensive colonization has occurred in the post-Wisconsin vacuum. This has been attributed to the processes of founder effect and genetic drift. We venture that a similar situation may have occurred during the divergence of the *Vaccinium section Cyanococcus* group. Moreover speciation in such a group has probably been effected by gene regulatory site changes, as Carson (1976) has argued.

Electrophoretic evidence can only be marshalled to support these hypotheses if appropriate developments are made in the extraction techniques of leaf enzymes. This has been done successfully with many conifers recently (Kelly and Adams 1977, Mitton et al. 1979), work must be done similarly on extraction in ericaceous taxa.

References

- Camp, W.H.** 1942. The structure of populations in the genus *Vaccinium* subgenus *Euvaccinium*. *Brittonia*, 4: 189-204.
- Camp, W.H.** 1944. A preliminary consideration of the biosystematy of *Oxycoccus*. *Bull. Torrey Bot. Club*, 71: 426-437.
- Camp, W.H.** 1945. The North American blueberries with notes on other groups of Vacciniaceae. *Brittonia*, 5: 203-275.
- Carson, H.** 1976. The unit of genetic change in adaptation and speciation. *Ann. Missouri Bot. Gard.*, 63: 210-223.
- Dirr, M.A., Barker, A.V., and Maynard, D.N.** 1972. Nitrate reductase activity in the leaves of the highbush blueberry and other plants. *J. Amer. Soc. Hortic. Sci.*, 97(3): 329-331.
- Frohne, D. and John, J.** 1978. The Primulales: serological contribution to the problem of their systematic position. *Biochem. Syst. Ecol.*, 6: 315-322.
- Kelley, W.A. and Adams, R.P.** 1977. Preparation of extracts from juniper leaves for electrophoresis. *Phytochemistry*, 16: 513-516.
- Loomis, W.O. and Battaile, J.** 1966. Plant phenolic compounds and isolation of plant enzymes. *Phytochemistry*, 5: 423-438.
- Mitton, J.B., Linhart, Y.B., Sturgeon, K.B. and Hamrick, J.L.** 1979. Allozyme polymorphisms detected in mature needle tissue of ponderosa pine. *J. Hered.*, 70: 88-89.
- Townsend, L.R.** 1970. Effect of form of N and pH on nitrate reductase activity in the leaves and roots of the highbush blueberry. *Can. J. Plant Sci.* 49: 333-338.
- Schaal, B.A., Andersen, W.A.** 1974. An outline of techniques for starch gel electrophoresis from American oyster, *Crassostrea virginica* Gmelin. Georgia Marine Science Centre Tech. Rep.—Unpublished manuscript.
- Schwaegerle, K.E. and Schall, B.A.** 1979. Genetic variability and founder effect in the pitcher plant, *Sarracenia purpurea* L. *Evolution*, 33: 1210-1218.
- Shaw, C.R. and Prasad, R.** 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. *Biochem. Gen.*, 4: 297-320.

- Sneath, P.H.A., and Sokal, R.R.** 1973. *Numerical Taxonomy* (the Principles and Practice of Numerical Classification). W.W. Freeman and Co., San Francisco.
- Soltis, D.E., Hanfleur, C.H. and Gastony, G.T.** 1980. Detecting enzyme variation in the fern genus *Bommeria*: an analysis of methodology. *Syst. Bot.*, 5: 30-38.
- Soltis, D.E.** 1982. Allozymic variability in *Sullivantia* (Saxifragaceae). *Syst. Bot.*, 7: 26-34.
- Vander Kloet, S.P.** 1972. *The North American Blueberries Revisited: A Taxonomic study of Vaccinium section Cyanococcus*. Ph.D. thesis, Queens University, Kingston.
- Vander Kloet, S.P.** 1977. The taxonomic status of *Vaccinium pallidum*, the hillside blueberries, including *V. vacillans*. *Can. J. Bot.* 56: 1559-1574.
- Vander Kloet, S.P.** 1980. The taxonomy of the highbush blueberry, *Vaccinium corymbosum*. *Can. J. Bot.* 58: 1187-1201.
- Vander Kloet, S.P.** 1983. The taxonomy of *Vaccinium section Cyanococcus*: A summation. *Can. J. Bot.* 61: 256-266.

Appendix

Provenance of specimens used in the analysis; Vouchers at ACAD.

Vaccinium section Cyanococcus A. Gray

1. *V. corymbosum* L. diploid plants: Fusc 9, 11, 15, 31, 49 and WS2—Archbold Biological Station, 13 km S of Lake Placid, Highlands County, Florida; tetraploid plants: HB 22, 39 and 642—Hebert Bog, Upper Rock Lake, Frontenac County, Ontario; Yar 31, 42, 48, 56, 68, 69, 76 and 80—Lake George and Leap Frog Lake, Yarmouth County, Nova Scotia.
2. *V. darrowii* Camp, ABS6, ABS10, L11313—Archbold Biological Station, 13 km S of Lake Placid, Highlands County, Florida.
3. *V. tenellum* Aiton, 128574—Rowland, Robeson County, North Carolina; 1428574—Myrtle Beach, Horry County, South Carolina; 121675—8 km N of Emporia, on the Greenville-Sussex County Line, Virginia.
4. *V. myrtilloides* Michx. 517773 and 21881—Dominion Atlantic Railway Right-of-Way, Wolfville, Kings County, Nova Scotia.
5. *V. boreale* Hall & Aalders, 327975—Kennington Cove, Cape Breton County, Nova Scotia; 39976—Cape Spear, Avalon Peninsula, Newfoundland.

Vaccinium section Myrtilus Dumortier

6. *V. myrtilus* L. 31076 and 11277—Reycroft Moor, near Bingley, West Yorkshire, England.
7. *V. membranaceum* Douglas, 629879—Mt. Seymour Provincial Park, North Vancouver, British Columbia; 42979—Paradise Meadows, Forbidden Plateau, Vancouver Island, British Columbia.
8. *V. parvifolium* Smith, 229879—Mt. Seymour Provincial Park. North Vancouver, British Columbia; 731879—River Jordan, Vancouver Island, British Columbia.

Vaccinium section Oxycoccus (Hill) K. Koch.

9. *V. macrocarpon* Aiton 6121081—Kennington Cove, Cape Breton County, Nova Scotia; 1271081—Hebert Bog, Upper Rock Lake, Frontenac County, Ontario.

Vaccinium section Vaccinium

10. *V. uliginosum* L. 613281—Cape St. Francis, Avalon Peninsula, Newfoundland.