Reproductive Biology of the Aleutian Shield-fern, *Polystichum aleuticum*

Final Report

To

United States Department of Interior
Fish and Wildlife Service
Anchorage, Alaska

From
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Introduction

The Aleutian shield-fern, *Polystichum aleuticum*, is Alaska’s only endangered plant species and one of the rarest ferns in North America (Smith 1985). Approximately 128 plants grow on the northeast arm of Mt Reed, Adak Island, Aleutians (Talbot, S. U.S. Fish and Wildlife Service, Pers. Comm.). Most plants occur in a narrow microhabitat consisting of rock grottos and moist crevices at an approximate elevation of 360m (Talbot *et al.* 1995). In 1989, research was begun to identify possible causes of this plant’s rarity and to establish methods of propagation for potential use in recovery or germplasm conservation of this endangered species. The purpose of this research was to verify spore viability through germination tests; identify optimum methods of *in vitro* spore germination; and explore environmental physiology of spore germination. Two previous attempts to germinate spores using 12-year-old spores from herbarium specimens and immature fresh spores were unsuccessful (Smith 1985).

In 1992, discussions by the Aleutian shield-fern Recovery Team led to exploratory studies on the genetic variability of the existing fern population through isozyme electrophoresis. The objectives of this program, conducted in association with Dr. Kent Schwaegerle, Institute of Arctic Biology, and the Soltis Laboratory at Washington State University, were to provide direction to the recovery team on possible future avenues of research.

Spore Collection

Populations were observed periodically during July and early August by ecologist, Gerald F. Tande, who identified individual plants in two Adak populations and made recommendations regarding likely candidates for the frond harvest. Patricia Wagner, horticulturist at the Georgeson Botanical Garden, visited Adak and collected a single spore-bearing frond from ten different plants on 29 and 31 August. She observed that several of the plants Dr. Tande had recommended as containing numerous fertile fronds with abundant green indusia, exhibited very few sporangia beneath the indusia in late
August, and most were still green. The majority of the plants, however, had abundant mature brown-colored indusia and sporangia.

Since there was a possibility of inbreeding barriers, more than one plant was sampled, and attempts were made to sample individuals that were not adjacent to one another. Fronds were labeled using Dr. Tande’s numbering scheme for population units (Roman numerals) and individual plants within those units (Arabic numerals). Plants and units sampled were: II-1, V-31, V-16 or 10; VI-16 or 17, VI-21, VIII-4; VIII-8; X-1, X-4, and XI-11. Following collection, all accessible flagging marking individual plants was removed.

**Spore processing and in vitro propagation**

Harvested fronds were placed between folded sheets of smooth paper in manila envelopes and transported to Fairbanks, Alaska. They were air dried at 23°C for 3 days, then refrigerated at 4°C until 18 September. Fronds were, again, air dried at 23°C until 21 September at which time germination tests commenced.

Only spores that had naturally dehisced during the drying process were used for the initial test. Spores were cleaned by hand removal of larger nonspore material then by tilting the paper and tapping it gently to dislodge and remove other nonspore material. Spores were placed into paper packets made of folded filter paper with each packet containing spores from one plant. Packets were soaked for 1 minute in a 5 percent clorox solution to which one drop of the wetting agent, Tween® 20, had been added followed by three rinses in sterile, distilled water. Packets were air dried at room temperature beneath a laminar flow hood. When packets were completely dry, spores were divided among four treatment media with a maximum of 10 jars per medium. Media consisted of:

1) Knop’s liquid nutrient solution with Hoagland’s solution trace elements; 10 g agar and pH adjusted to 6.5

2) modified Hoagland’s solution with 10g agar and pH 6.5.
3) finely-screened, autoclaved medium of 2 parts peat, 1 part fine quartz sand

4) Alaska Soluble Plant Fertilizer (10-52-10 including the following trace elements Fe, 0.1%; Zn, 0.026%; B, 0.008%; Mn, 0.024%; Cu, 0.004% and Mo, 0.0008%) 2 g/l with 15g agar and pH 6.9

Each jar contained 25 ml of sterile agar medium or 50 g of the sterile peat/sand mix. Spores were sown so that each jar received a similar quantity of spores or spore clusters from one plant (average 15.2 spores or clusters per jar). Half of the containers were placed in a growth room at 23°C constant temperature and a 16-hour photoperiod using 40-W fluorescent, cool-white lamps with a photosynthetic photon flux (PPF) of 20 μMm²s⁻¹ measured 30 cm beneath the lamps. The remainder of the jars were placed in a growth chamber at alternating 18/12°C temperatures for 18/6 hour periods. The higher temperature period was illuminated with 40-W fluorescent cool-white lamps with a PPF of 12.3 μMm²s⁻¹ measured 25 cm beneath the lamps. After germination, all prothallia were subcultured every 4-6 weeks onto fresh Hoagland’s No. 2 solution.

In addition to the single-plant cultures, one large petri dish containing 100g of the peat/sand mixture was sown with unsterilized spores from three plants (VIII-8; VI-16 or 17; and XI-1). This container was placed in the growth chamber having the cool, alternating temperatures. This container served as a check for the experimental in vitro procedures described above.

After prothallia were fully developed and some sporophytes had been observed, some of the cultures were transferred to a cool greenhouse with a minimum daytime temperature of 18°C and minimum nighttime temperature of 15°C. Clusters of prothallia, approximately 1 cm² were planted into one of two sterile media: equal parts by volume of Canadian Sphagnum peat and quartz sand, or equal parts of Fison's Sunshine Mix® No 3 and quartz sand. The Sunshine Mix® is a commercial seed-starting mix containing Canadian Sphagnum peat, vermiculite, and a wetting agent. The pH is adjusted to pH 6.5
with dolomitic limestone. Prothallia were embedded into the surface of the medium, watered with distilled water, and the entire flat was covered with a clear polyethylene bag to improve moisture retention. The flats were then placed beneath shade cloth that reduced irradiance by 73%. High-pressure sodium vapor lamps positioned 1.5 m above the bench provided supplemental light for a 16 hr photoperiod daily.

The first prothallia were visible on 20 November, 61 days after spores were sown. On that date, many stages of development were observable from initial filamentous stages to well-developed prothallia. The very first cell divisions probably occurred 7-14 days earlier.

Nearly all of the plants shed spores in clusters rather than individual spores. Even with air drying, many of the spores were released from the sporangium as one unit rather than individual spores. Consequently, germination tended to produce tight clusters of prothallia. Sixty-two percent of the spore clusters that germinated exhibited multiple germination.

Initial germination was observed on spores from several plants: V-16 or 10; VIII-4; VI-16 or 17; V-31; VIII-8. Some spores from all plants except VI-21 eventually germinated, but there was a distinct difference among plants. Spore germination from plants labeled VI-16 or 17 was especially prolific (Table 2).

No germination occurred on the medium containing the commercial plant fertilizer (Alaska Soluble). To date, the most rapid germination of sterile spores occurred on the Hoagland's Solution followed closely by Knop's Solution and the peat/sand mix (Table 1). Germination overall was fairly low which might indicate low spore viability but is probably related to the spore sterilization procedures. One minute soak in chlorox may be too, long for these spores. This observation is substantiated by the fact that the very best germination occurred with unsterilized spores sown on the peat/sand mix. In addition, unacceptable levels of contamination were still present despite
Table 1  Record of successful spore germination using 4 sterile media.

<table>
<thead>
<tr>
<th>Days from Sowing</th>
<th>Cultures with successful germination (%) (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hoagland's Solution</td>
</tr>
<tr>
<td>61</td>
<td>29</td>
</tr>
<tr>
<td>89</td>
<td>43</td>
</tr>
<tr>
<td>120</td>
<td>43</td>
</tr>
<tr>
<td>150</td>
<td>43</td>
</tr>
<tr>
<td>175</td>
<td>50</td>
</tr>
<tr>
<td>205</td>
<td>50</td>
</tr>
</tbody>
</table>

\(^1\)n=20 cultures per solution; percentages corrected for contaminated cultures.

\(^2\)No germination occurred at any time.
the chlorox soak. Therefore, additional studies on spore sterilization techniques are warranted.

Only cultures growing in the cool, alternating temperatures germinated. On 10 December, those cultures growing at the 23°C constant temperature were transferred to the cool growth chamber. On 8 January, germination was noted in three of the transferred jars indicating that they were still viable, but possibly thermodormant.

The first sporophyte was visible on the peat/sand mixture on 28 March, 1990, 188 days after sowing. This first leaf occurred on the population of mixed spores (VIII-8, VI-16 or 17 and XI-1). A second sporophyte appeared on 15 April in this same culture. No further development was evident on any of the cultures until 15 June when three of the agar-based media cultures exhibited the first-leaf stage. All of these cultures had spores from the plant labeled VI-16 or 17. No further sporophytes grew from the original mixed peat/sand culture, however sporophyte development on the agar-based cultures was very rapid.

During June and July, many spore clusters sprouted numerous first leaves indicating either successful apogamy or successful multiple fertilizations. The prothallia were not examined closely to rule out apogamy, but it seems unlikely since sporophytes generated by apogamy tend to appear at the same time. Sporophytes in my cultures appeared over many weeks. It is interesting to note that sporophytes developed from spores taken from a single plant accession as well as populations of mixed prothallia. Unless apogamy is occurring, cross fertilization with other individuals in the population does not appear to be necessary for plant reproduction.

By 1 October, 303 sporophytes had developed in all cultures. Ninety percent of these sporophytes had occurred in cultures that were composed of the plant accession VI-16 or 17 alone or mixed with prothallia from other plants. This accession was by far the most successful in terms of spore germination and subsequent development. Nearly all
spores from this accession germinated, and 84% of those cultures produced sporophytes (Table 2). Four cultures from this accession also exhibited frond development beyond the first leaf stage by 1 October.

Clusters of prothallia with and without sporophytes were transferred to the two greenhouse media on 15 September. As of 1 October, 96% of the cultures were alive; no additional sporophytes had developed; and 4% of the cultures succumbed to mold. All containers were treated with Benomyl fungicide to prevent further disease growth. This initial greenhouse culture was used to learn if the existing environment would be conducive to fern growth and identify any problems that might arise such as diseases.

**Soil Analysis**

As a supplement to the germination studies, analysis of soil samples collected by Gerald Tande was conducted to provide an estimate of certain physical and chemical characteristics of the soils that support fern growth on Adak. This information would facilitate the greenhouse studies by elucidating some characteristics of the greenhouse media on which the sporophytes must be planted. Samples were collected from three population units: VI, V and VIII. Samples were not handled properly following collection, and they remained moist, at room temperature in plastic bags for several months. Analysis consisted of pH; percent sand, silt and clay; percent organic matter, total ppm nitrogen, phosphorus and potassium content; and ppm of ammonium and nitrate nitrogen. All tests were conducted and funded by at the Agricultural and Forestry Experiment Station Tissue and Soil Testing Laboratory, Palmer, Alaska.

Results of the test of Adak soils are listed in Table 3. Since the storage conditions were improper for some analyses, results such as ammonium nitrogen are not accurate. Nevertheless, they provide some clues to the soil environment on which these ferns grow. The data on soil physical composition will be valuable in the future greenhouse media studies.
Table 2. Spore germination and development of 10 different plants collected on Adak Island.

<table>
<thead>
<tr>
<th>Plant Identification</th>
<th>Cultures with successful germination (%)</th>
<th>Number of cultures Oct 1, 1990</th>
<th>Cultures with sporophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-1</td>
<td>66</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>X-1</td>
<td>33</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>V-31</td>
<td>50</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>XI-11</td>
<td>66</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>V-16 or 10</td>
<td>60</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>VI-16 or 17</td>
<td>100</td>
<td>39</td>
<td>33</td>
</tr>
<tr>
<td>VIII-4</td>
<td>80</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>VI-21</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>X-4</td>
<td>25</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>VIII-8</td>
<td>80</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

1Plant identification code according to Gerald Tande
2n=6; percentages corrected for contaminated cultures
Table 3. Soil characteristics of three Adak sites containing the Aleutian shield fern.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Soil pH</th>
<th>NH$_4$ N</th>
<th>NO$_3$ N</th>
<th>P</th>
<th>K</th>
<th>sand</th>
<th>silt</th>
<th>clay</th>
<th>organic matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>6.40</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>84</td>
<td>65.6</td>
<td>26.0</td>
<td>8.4</td>
<td>6.03</td>
</tr>
<tr>
<td>VI</td>
<td>5.64</td>
<td>11</td>
<td>6</td>
<td>2</td>
<td>184</td>
<td>53.6</td>
<td>42.0</td>
<td>4.4</td>
<td>16.75</td>
</tr>
<tr>
<td>VIII</td>
<td>5.92</td>
<td>14</td>
<td>6</td>
<td>4</td>
<td>154</td>
<td>61.6</td>
<td>34.0</td>
<td>4.4</td>
<td>12.06</td>
</tr>
</tbody>
</table>

1Population unit identification by Gerald Tanoe
These studies showed that *Polystichum aleuticum* has viable spores that will germinate in approximately 6-8 weeks under controlled conditions. Most spores are released in clusters, and multiple germination per cluster is common. Germination will occur on Hoagland's or Knop's Solution as well as a peat/sand medium. Spores appear to exhibit a thermodormancy.

Spores from 9 of the 10 plants germinated, but there was a significant difference among plants in germination success. Spores from the plant labeled VI-16 or 17 were especially successful in germination and sporophyte development.

In October 1990, 303 sporophytes had been counted from the masses of one-year-old prothallia. Half of the clusters of prothallia, with or without the first-leaf stage, were removed from the test tube, segmented into approx. 0.5 cm² pieces, and moved to the greenhouse to test existing environmental conditions and transfer techniques. Plants were grown at 18°C minimum day and night temperature with natural light supplemented with high intensity discharge sodium vapor lamps on a 16 hr photoperiod. The containers were cell-packs with 78 cells per flat and clear polyethylene lids. The entire flat plus lid was enclosed in a clear plastic bag to maintain high, constant moisture levels.

Prothallia and sporophytes were planted in 4 different media: *Sphagnum* sp. peat; *Sphagnum* sp. peat/sand (50/50 by vol); a commercial greenhouse seed starting mix, Sunshine® Mix No 3; and Sunshine Mix® No 3/sand (50/50 by vol). The limited quantity of prothallia and the different growth stages did not permit formal experimentation on media types, but observations were made on growth and plant health for future experiments. Plants grew poorly on the peat only medium. True leaves were pale green or whitish-yellow, and some had brown, papery edges. The pH of 4.0 might be too low for this species.
The media with sand was prone to algae growth, and all cultures showed contamination by fungi. All prothallia and sporophytes were treated periodically with Benomyl (Benlate®) fungicide which worked well, but there was some damage to the fern leaves. Many leaves developed light brown edges immediately after treatment, but new growth appeared healthy. The Sunshine Mix® medium seemed to be adequate for plant survival and growth.

The fern was transferred to the greenhouse either as a cluster of prothallia or clusters with at least one "first leaf". Development of true leaves after the first leaf stage was fairly rapid. Consequently, on 1 Feb 1991 there were approximately 36 well-developed, well-rooted plantlets in the greenhouse averaging 2 cm in height. The clusters of prothallia have been much more slow to develop. Approximately half of the clusters showed the first-leaf stage, whereas the remainder showed no signs of sporophytic development. Sporophyte development in the test tube was much more rapid. Therefore, transfer to the greenhouse should be made only after the first leaf is present.

The remaining prothallia and sporophytes generated from the first spore germination attempt were segmented and aseptically subcultured every 4 weeks on Hoagland’s Solution. On 10 Jan the agar concentration in the medium was reduced to 4 g/l. On 1 Feb, 1991 there were 75 containers supporting many development stages from prothallial clusters to well-developed plants with up to 6 true leaves. One hundred and eighty six sporophytes was a conservative estimate of the population size in aseptic culture.

In addition to the agar-based cultures, there were 18 jars of sterile peat/sand mix containing prothallia and sporophytes. Sterile Hoagland’s solution was added to the jars every 6 weeks. Sporophyte development appeared most rapid in jars where the medium was very wet or flooded. These jars contained approximately 46 sporophytes.

On 10 January, 36 cultures on the agar-based medium were transferred to Hoagland’s solution without agar; 21 of the jars were returned to the growth chamber
with the other cultures, whereas 15 jars were placed on a rotating table to maximize oxygen concentration in the liquid. On 1 Feb, 1991 the liquid-filled stationary bottles had 29 sporophytes, and the liquid-filled rotating bottles had 22 sporophytes.

Second spore collection

On 9 September, 1990, Vernon Byrd collected a second set of fronds from the Adak populations following the same harvesting procedures as in 1989. The fronds were received in Fairbanks on 12 September, air dried for 3 days at room temperature, then stored, refrigerated at 4 °C. On 19 Nov 1990, fronds were sterilized for one minute in 5 percent clorox solution plus 2 drops Tween® 20 as a wetting agent. Under aseptic conditions, sporangia and indusia were scraped off the frond and plated onto Hoagland's No 2 solution with 0, 2, 4, 6, or 8 g/l agar. Ten jars, each containing fern spores from a separate Adak population, were sown on each of the 5 agar levels. Jars were placed in a growth chamber in a completely randomized design with an 18 °C constant temperature and an 18 hr photoperiod. On 20 Dec, 31 days after sowing, the first prothallia were visible. The number of jars showing germination were 8, 6, 7, 7, and 3 for the 0, 2, 4, 6 and 8 g/l treatments, respectively. By 3 Jan 1991, all treatments had 8 or 9 containers with successful germinants. Consequently, it appears that germination rate is much more rapid for the agar concentrations below 6 g/l. Germination is not inhibited by higher concentrations of agar, just delayed. The 0 g/l agar treatment appeared to be the most rapid, but since the spores were sown in clusters or in association with other material such as sporangia or indusia, much of the material sank in the liquid. Immersion did not prevent germination, but it might influence future growth unless the liquid is agitated to improve oxygen content.

On 3 October the agar concentration experiment was terminated. Like the appearance of prothallia, the appearance of the first leaf stage was delayed by as much as 8 weeks, but agar concentration did not inhibit fertilization. Many of the new leaves developing in the 0 and 2 g/l agar treatments had dark brown veins and yellow blades.
This condition was non-existent at the higher agar concentrations. Perhaps some component of the Hoagland's solution is available at low agar levels, but is tied up or released slowly to the plant at higher agar concentrations.

Spores were sown as above on half-strength Hoagland's solution and no agar. The pH of the medium was adjusted with 1N NaOH to pH 5.75, 5.98, 6.26, 6.51, 6.7 and 6.92 prior to autoclaving. The experiment, containing 4 single-jar replicates randomized in the growth chamber, was begun on 6 June 1991. There was no difference among treatments in spore germination. By 6 August all jars had prothallia. By 1 October, the first leaf stage was visible on at least one jar of each treatment. It appears that pH is not a significant factor in spore germination *in vitro*.

An experiment was begun on 6 June, 1991 to test the effects of light on spore germination. Spores were sown on Hoagland's solution with 4 g agar. Four single-jar replicates were maintained as before in a well-lighted growth chamber with 18 hr photoperiod and 18°C constant temperature. Four other jars were enclosed in foil to exclude light. Spores in the darkened jars did not germinate by 6 August 1991. All of the light-treated jars showed well-developed prothallia. The darkened jars were returned to the light, and by 1 Oct, all previously-darkened jars showed germination. This experiment was repeated, and both experiments showed that light is required for germination. Darkness inhibits germination, but spores maintain viability. When exposed to light, they too, will germinate.

A cell sieve was purchased in order to attempt to separate individual spores for germination tests. The sieve was autoclaved, and sterilized fronds were rubbed on the screen. The screen successfully separated most of the spores that were used in the light experiment described above. The single prothallia were separated to observe the possibility of self-fertilization and observe any methods of vegetative propagation of prothallia.
On 1 April, 1991 there were 468 shield-fern prothallia or sporophytes in the greenhouse; 189 were sporophytes at the first leaf stage, and 36 plants had well-developed fronds. By 1 Oct, 1991, this number increased to 1009 sporophytes: 730 plants with well-developed fronds and 279 plants at the first-leaf stage of development. No plants had fronds with spores.

At the beginning of June, 1991 plant numbers were approximately 11 percent higher than the October level, but very high air temperatures over 38°C occurred on 15 days in July and early August and contributed to plant losses, especially sporophytes at the first leaf stage. A humidifan was purchased to ameliorate the high temperatures, but it did not arrive until the last week of August. Despite these losses, most of the ferns with well-developed fronds survived. Individual fronds became brown and brittle, but after temperatures moderated in mid August, new fronds from the crown began to appear.

All plants in the greenhouse were grown on Sunshine Mix® No 4. The plants were fertilized weekly with 150 mg/l 15-16-17 soluble Peter’s fertilizer added to the irrigation water. The plants appeared healthy, but this system was not without problems. The high peat content and moisture attracted fungus gnats whose larvae feed on decaying peat and plant roots. Weekly treatment with Gnatrol®, a biological control agent containing Bacillus thuringensis, was very successful in limiting the numbers of insects. After several weeks of treatment, an occasional larva was found in the medium, but the numbers were not sufficient to cause serious damage to all but the smallest ferns. Other fern growers have recommended diazinon as a control for fungus gnats, but it caused too much frond damage on the tiny sporophytes of the Aleutian shield fern. It might be useful on more mature plants.

The high moisture and peaty medium also promoted lush growth of mosses and algae. A suitable chemical control has not yet been found. The moss and algae were removed by hand, and in serious infestations, the ferns were re-potted into new media and sterile containers.
The fronds also supported aphids. An insecticidal soap, Safers®, had no phytotoxic effect on the ferns, and was used for all greenhouse and outdoor control.

This fern might be able to tolerate a drier environment than was previously surmised from conditions existing at its Adak "home". Six plants growing in containers of Sunshine® No 4 mix were enclosed in a zip-loc plastic bag. This bag was not sealed properly, and the containers dried out completely. The fern fronds dried to a papery, brittle condition for at least 2 days. The container was flooded with water, and 24 hrs later the plants had recovered full turgidity and appeared to be thriving. This "accident" demonstrated that we should definitely test the limits of this fern beyond its traditional environment, particularly in regards to moisture and temperature.

On 1 Oct 1991 there were 131 jars containing aseptic cultures of shield fern: 89 jars had prothallia and sporophytes, while the remaining 42 jars contained only prothallia. These jars included 12 from the original spore germination experiments that had not yet developed sporophytes, but the majority were from completed experiments described below. The number of sporophytes was counted in 20 jars, and the average count was 27 ± 12. A conservative estimate of the number of sporophytes in aseptic culture was 324 on 1 Oct 1991.

On 15 February, 1992, the number of Aleutian shield-fern plants in the greenhouse included 1476 sporophytes: 1200 plants with well-developed fronds and 276 plants at the first-leaf stage of development. No plants had fronds with spores. They were still, relatively small (average height 3.5 cm). Since January, fronds had been gradually dying back on approximately 70 percent of the plants. Plants appeared to be entering a dormant phase. Ten crowns of dormant sporophytes were examined by removing all of the medium. Two sporophytes showed no new growth and appeared dead. The remainder had a well-developed pale green crown with tightly curled croziers beneath the medium surface. The original growing point appeared dead at that time.
Sporophytes appeared to be thriving in the humid (70% r.h.) greenhouse (minimum night temperature, 18°C, with supplemental light from fluorescent tubes positioned 1 m above the bench, 45 uM·m⁻²·s⁻¹, 16 hr photoperiod). Scale, a new pest problem, was not severe and caused no visible damage.

On 15 Feb 1992, 155 jars containing prothallia and sporophytes were grown in the controlled environment growth chamber; 20 contained well-developed sporophytes; 90 have sporophytes at the first leaf stage; and the remainder were still in the prothallial stage. Six of the jars from six different individuals were sent to Dr. Kent Schwagerle's laboratory on 26 Feb, 1992 for preliminary experimentation with electrophoresis.

A cell sieve was purchased in order to attempt to separate individual spores for germination tests. The sieve was autoclaved, and sterilized fronds were rubbed on the screen. The screen successfully separated most of the spores that were used in the light experiment described previously. The single prothallia were separated on 31 Oct, 1991 to observe the possibility of self-fertilization and observe any methods of clonal propagation of prothallia. By 28 Feb, 1992 all of the single prothallia had grown into a round cluster indicating clonal reproduction of the haploid prothallia. One of the clusters had two first leaves emerging from the mass of green prothallia. These observations indicated that self fertilization probably is possible with this shield-fern.

In March 1992, plants in the greenhouse that had been grown from spores germinated in 1989 and 1990 appeared dormant and were transferred to a cold room with a constant air temperature of 2°C ± 1°C. Plants were maintained in the cold room until June, then moved outdoors. Because funding was not received until late summer for the outdoor shaded mist bed, spores were kept in the flats covered with clear plastic lids and located beneath large trees in the Georgeson Botanical Garden. Growth after the three-month cold treatment was slow, and many plants did not survive. Three factors were identified as possible reasons for the demise of 820 plants:

1) fungus gnats feeding directly on the fern crown,
2) unidentified fungus infection of crowns, and
3) possible improper moisture levels in the medium.

The remaining sporophytes were returned to the greenhouse in Sept. 1992. Plants continued to be fairly small, approximately 4 cm in height except for one individual from VI 16 or 17. This plant was 5 cm in height and had developed a robust, healthy crown. No sori or indusia were evident on the fronds.

Moisture control appeared to be a critical factor in the success of these ferns. The medium must be evenly moist but very well drained to promote good growth. The high humidity and moist medium contributed to significant moss and liverwort growth that significantly inhibit the growth of the ferns.

Control of pathogens and fungus gnats was also important. We tried two biological controls for fungus gnats during the spring. Both Gnatrol® (a formulation of Bacillus thuringensis) and Exhibit® (a parasitic nematode) reduced levels of fungus gnats, but did not eliminate them. Any level of fungus gnats was too much.

One hundred seventy-five jars containing prothallia and sporophytes were growing in the controlled environment growth chamber; 129 contained well-developed sporophytes, and the remainder were still in the prothallial stage.

In May, 1992 Dr. David Murray received dried, pressed specimens of P. lachenense from two collection locations in Japan: Nantou Co. Chilgishan and Taichung Co., Nanhutashan, collected on 1 Sept. and 9 Sept, 1991, respectively. The herbarium labels noted the plants had been collected in crevices of rocks in open, sunny locations at 3300m (Nantou Co) and 3500m (Taichung Co.) elevation. Spores from the dried fronds were sown onto the Hoagland's No. 2 aseptic medium on May 26, 1992 and germinated.

Spores of the Aleutian shield-fern were examined by scanning electron microscopy to identify any abnormalities in spore structure; and to compare spores of the Aleutian shield-fern with those of the putative relative, Polystichum lachenense. The first spores to be examined were from the 1989 collection. These spores showed a very
distinctive sunken bowl-shape. A second set of spores from the 1991 collection date showed a mixture of globose and bowl-shaped spores. Spores of *P. lachenense*, had only the ellipsoidal or globose shape more consistent with SEM photographs of *Polystichum* spp. published in the literature (Tryon and Lugardon 1990). Observations under a dissecting microscope (approx. 45x) revealed one plant (VIII-4) with what appeared to be bowl-shaped spores.

Spores from the most recent collection were examined under a light microscope at 100x. At this power the bowl shape was clearly visible. Bowled spores also had a dehydrated, wrinkled appearance with cracked, ragged edges. The percentage of bowled spores in a sample from any one individual ranged from 15% to 78% for the 1991 collection (Table 4). At this time we also noted a color change in the 1989 spore collection which might be related to spore longevity.

The sunken shape might be caused by prolonged storage or possibly to the vacuum used to prepare and observe the spores for SEM. Under pressure, spore walls could collapse revealing outlines of the "bowl" of protoplasm that occurs within the spore walls. This option does not seem very likely because of the hard, almost bony nature of the spore covering. Any handling of the spores easily shatters this covering, and any implosion would probably cause cracks rather than bends in the surface.

The bowl-shaped spores could be a normal occurrence in this species. Spores of other species we have observed show a distinctive bowl shape. It is interesting to note though, that in some individuals of the Aleutian shield fern, most of the spores show this bowl configuration (XII-4), while in others, the globose shape was more prevalent (V-7).

In seed-bearing plants, because of problems with sterility or pollination, seeds often develop a seed coat, but have no embryo or endosperm. The seed coat may appear normal or may be misshapen or flattened. These seeds will not germinate under natural conditions. The bowl shape we observed might be deformed because they are sterile or immature.
Table 4. Counts of bowl-shaped versus non-bowl shaped spores for eleven individuals of *Polystichum aleuticum* collected in 1991.

<table>
<thead>
<tr>
<th>Population Unit</th>
<th>Plant Number</th>
<th>Number counted</th>
<th>Percent bowl-shaped spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>9</td>
<td>100</td>
<td>24</td>
</tr>
<tr>
<td>VI</td>
<td>1</td>
<td>100</td>
<td>53</td>
</tr>
<tr>
<td>V</td>
<td>2</td>
<td>46</td>
<td>59</td>
</tr>
<tr>
<td>X</td>
<td>4</td>
<td>100</td>
<td>61</td>
</tr>
<tr>
<td>VI</td>
<td>13</td>
<td>100</td>
<td>78</td>
</tr>
<tr>
<td>VI</td>
<td>7</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td>XII</td>
<td>14</td>
<td>100</td>
<td>73</td>
</tr>
<tr>
<td>VIII</td>
<td>5</td>
<td>100</td>
<td>71</td>
</tr>
<tr>
<td>V</td>
<td>7</td>
<td>100</td>
<td>44</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>V</td>
<td>4</td>
<td>100</td>
<td>63</td>
</tr>
</tbody>
</table>
In Mar 1993, the total number of shield-fern plants growing in the greenhouse was 353. The plants continued to grow in the greenhouse environment, but no mature, spore-bearing fronds had developed. Several other species of *Polystichum* were being maintained in the greenhouse in addition to *P. aleuticum*: *P. lachenense* (two strains from Taiwan) *P. acrostichoides* (a common horticultural fern native to eastern North America) and *P. lonchitis* (spores collected by G. Tande on Adak). These species provided an interesting developmental comparison with *P. aleuticum*. For instance, *P. aleuticum* never produced mature fronds, but *P. lonchitis* did. The other species produced fronds more rapidly and had a more robust growth habit than *P. aleuticum*.

An experiment was initiated in Mar 1993 to determine if soil pH is important in plant growth and development. The project was repeated five times over the next year, but technical problems prevented completion of the experiment. Dolomitic lime was added at varying concentrations to locally-mined Lernaeta peat and later to *Sphagnum* sp. peat to provide a pH range from pH 4.5 to 7.5. Regardless of quantity, pH over time reached the same level, pH 6.7, in all peat mixes. The pH levels also changed almost daily so that stabilizing the peat at one pH level became impossible. Further attempts to complete this project were terminated when all plant materials were shipped to New York.

Control of pathogens and fungus gnats continued to be a major concern with greenhouse cultures. We applied the restricted-use insecticide, Temik®, in Feb 1993 on a sample of plants. The well-established plants with many fronds showed no phytotoxic effects from the Temik®, but younger plants at the first leaf stage or those with one or two true fronds were killed.

We refined the techniques for spore sterilization. In the past, it has been impossible for us to surface-sterilize individual spores. They were simply sown onto a sterile medium, and contamination was significant. We tried making envelopes of filter paper and sterilizing the entire envelope. This method did not permit complete
sterilization, and many of the spores were lost because they would become lodged among the paper fibers. We now use a microfiltration system more commonly used in microbiological work. Fronds are first rubbed against a 200 mesh screen to dislodge and break up the spores. Spores are spread onto the nylon filter and covered with 10 percent clorox solution for one minute. The clorox is removed by vacuum suction, and spores are rinsed in two washes of sterile, distilled water. The nylon filter is removed from the apparatus and placed in a sterile petri dish to dry. The drying period is approximately 5 minutes, whereas paper filters dried in 2-3 hours. Sterile spores are sown onto agar plates for germination. Contamination is minimal with this method.

In 1993 and 1994, we refined techniques for microscopic examination of the spores. The purpose was to develop a chronological sequence of events from initial germination through development of the antheridia and archegonia. It was interesting to note that the spores showed no visible activity for approximately 30 days. Germination, once begun, was very rapid. Prothallia appeared within days, and antheridia and archegonia developed rapidly. The antheridia appeared first as dark knobs scattered among the rhizoids (Figs 1 & 2). Literature on other species has shown that the appearance of antheridia and archegonia is somewhat environmentally controlled. Light and temperature are significant factors as is the amount of crowding of the prothallia.

During the past three seasons, we established a protocol for sporophyte establishment in the greenhouse following transplanting from in vitro culture. Immature sporophytes grew for two years on a commercial peat-lite medium. Problems associated with cultivation included abundant growth of algae and mosses, fungus gnats and aphids. Optimum growing conditions now include a gravel mulch to reduce fungus gnat infestations and treatment with a biocontrol agent, Exhibit® or Gnatrol®. Despite our best efforts, after two years, plants began a steady decline. At the termination of the experiment, fewer than 50 sporophytes were alive from the original populations. This decline was remarkable since we have grown successfully nearly 50 species of ferns
under the same conditions. It is interesting to note that the shield-fern sporophytes never produced spores, whereas *Polystichum lachenense* (Nantou Co., Taiwan) showed development of spores with the second flush of fronds. Aleutian shield-ferns always were small, slow-growing sporophytes, not at all robust, when compared to the many other *Polystichum* species grown concurrently in our greenhouse.

**Environmental Physiology**

This component of the project involved examining environmental factors affecting spore germination. Two projects were conducted: light intensity and temperature effects on spore germination.

Spores were germinated in controlled environment temperature chambers at 4, 8, 10, 12, 14, 16, 18, 20, 24 and 28°C to identify optimum germination conditions. Spores were sown on an agar-based Hoagland's solution and germinated *in vitro*. Counts of prothallia commenced six weeks after sowing and continued daily for 30 days. Spores did not germinate at 4, 8 and 20°C or above. Under these conditions, spores were quiescent. They germinated readily when moved to 16°C. The most rapid and complete germination occurred at 16°C. Germination percentages averaged 30.2 percent at that temperature.

Spores from one clump (II-9) were treated with six levels of light from fluorescent lamps located at the top of the growth chamber. Petri dishes of spores were suspended on fishing line and staggered vertically in the chamber so that each container had a direct, unobscured path of light. Photosynthetic photon flux measured at the six sites varied from 10 to 50 μMm²s⁻¹. Germination did not differ significantly among light levels in the first trial or two subsequent trials. Germination percentages ranged from 10 to 22 percent for all treatments. Although light is necessary for germination, the intensity of light has no effect on germination. Many other fern spores germinate based upon a phytochrome-mediated response. Light quality and not quantity probably is important in shield fern germination.
Isozyme electrophoresis

A preliminary experiment was conducted by Dr. Kent Schwaegerle, UAF Institute of Arctic Biology, to evaluate isozyme patterns in the individual plants of the Aleutian shield-fern. Dr. Schwaegerle used acetate card electrophoresis and found no differences among the individuals tested. In order to verify these results, a second experiment using starch gel electrophoresis was conducted at Washington State University. The results of the starch-gel electrophoresis corroborated the results found earlier by Dr. Schwaegerle. Of the isozyme systems evaluated, no differences were evident among individuals in the Adak island shield-ferns (See Mr. David Boyd’s summary attached). These results have significance for future work in long-term germplasm preservation. Collection of a small sample of spores from a few randomly selected individuals probably will suffice for preservation of the germplasm as opposed to collecting spores from every individual.

This project gained taxonomic significance when we learned that Polystichum aleuticum was classified as *P. lachenense*, its closest Asian relative, by D. Lellinger, Smithsonian Institute. Dr. David Murray, UAF Museum, obtained two herbarium specimens of *P. Lachenense* from Taiwan, both of which were successfully cultured *in vitro*. These specimens, along with several non-related *Polystichum* species which we have grown *in vitro*, were included in the isozyme electrophoresis analysis at Washington State. Of the ten isozymes evaluated, none differed between *P. aleuticum* and *P. lachenense*. It is convenient to conclude that these species are one and the same, but this conclusion is premature. Two samples of *P. lachenense* are insufficient for meaningful comparisons. In addition, evidence from electrophoresis must be evaluated concurrently with morphologic and physiologic evidence to elucidate taxonomic relationships.

A very limited DNA analysis was also conducted by Dr. Pamela Soltis, but results were inconclusive. The quantity of plant material necessary for DNA analysis is substantial, significantly more than that necessary for isozyme analysis. I recommend that
further DNA analysis be suspended until sufficient plant materials can be obtained in culture.

Conclusions

1. *Polystichum aleuticum* has viable spores that will germinate in approximately 6-8 weeks *in vitro*.

2. Most spores are released in clusters, and multiple germination per cluster is common.

3. Germination occurs on Hoagland's or Knop's Solution as well as a peat/sand medium.

4. Optimum agar concentration is 6 g per l and less. Agar concentration does not prevent germination, but concentrations over 6g/l slows it down significantly.

5. Medium pH *in vitro* does not influence spore germination between pH 5.75 and 6.92.

6. Soils at the Adak site are predominantly sandy loam with 6-16 percent organic matter and a pH of 5.6 to 6.4.

7. Spores exhibit a thermodormancy at temperatures below 8°C and above 20°C.

8. Spores require light for germination, but light intensity within the range of 10 - 50 uMm²s⁻¹ has no effect on germination.

9. Sporophytes grown in a greenhouse require sterile, humid conditions. A commercial potting mix, Sunshine® Mix No 3, provides a good growing medium.

10. Mulching with stones reduces attacks by fungus gnats and growth of algae. Other common problems were aphids and liverworts. Biological controls such as Gnatrol® and Exhibit® showed no phytotoxicity, whereas chemical fungicides and insecticides produced damage.

11. Two experiments with isozyme electrophoresis failed to show any differences among individuals in the Adak Island population of Aleutian shield-fern. The 14 individuals tested comprise a single genotypic population with no indication of genetic variation. Banding patterns for isozyme activity were also identical between *P. aleuticum* and the two samples of *P. lachenense* from Taiwan.
Literature Cited


Figures 1 and 2. Reproductive structures of *Polystichum aleuticum* showing antheridia scattered among the rhizoids.