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Independent Gametophytes of *Hymenophyllum wrightii* in North America: Not as Rare as We Thought

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Abstract.—*Hymenophyllum wrightii* is a filmy fern known primarily from Japan and Korea. In North America, it is known as a sporophyte only in Haida Gwaii (Queen Charlotte Islands), British Columbia, Canada. Rare independent, filmy fern gametophytes found from the late 1950s and onward at a handful of locations in British Columbia and southeastern Alaska were presumed to be *H. wrightii*. Our 2006 surveys in southeastern Alaska determined that these gametophytes are common within specific habitats, and our survey in 2008 found gametophyte populations on the Olympic Peninsula in Washington State—the first report of *H. wrightii* in the contiguous United States. Samples from gametophyte populations from southeastern Alaska, British Columbia and Washington show no variation in *rbcL* or *rps4-trnS* sequence and are similar to sequences from Asian *H. wrightii* sporophytes, providing corroborating evidence of the identity of these independent gametophyte populations.

Key Words.—asexual, *Hymenophyllum wrightii*, independent gametophyte, Olympic Peninsula, southeastern Alaska, Vancouver Island

Hymenophyllum wrightii Bosch is a filmy fern (Hymenophyllaceae) found primarily in Japan and Korea, but with small disjunct populations on the northwest coast of North America. In North America, *H. wrightii* sporophytes are known from a few sites in Haida Gwaii (Queen Charlotte Islands), British Columbia, Canada (Iwatsuki, 1961; Persson, 1958; Taylor, 1967). Isolated, apparently asexual gametophyte populations have been reported over a wider area, including one site in southeastern Alaska (Taylor, 1967), six sites in Haida Gwaii and the area near Prince Rupert, British Columbia (Schofield, 1962; Taylor, 1967), and three sites on Vancouver Island (Cordes and Krajina, 1968). Since those initial reports only a few additional gametophytes have been collected (University of British Columbia Herbarium; Figs. 1–3). Because of its apparent rarity, *H. wrightii* was designated as a Sensitive Species by the USDA Forest Service, Alaska Region, during the 1990s (Goldstein *et al.*, 2009).

Identifying ferns to genus and species based on gametophyte morphology is difficult (de Groot *et al.*, 2011; Li *et al.*, 2009; Schneider and Schuettpelz, 2006; Watkins *et al.*, 2007) but identification to genus and subgenus is often possible

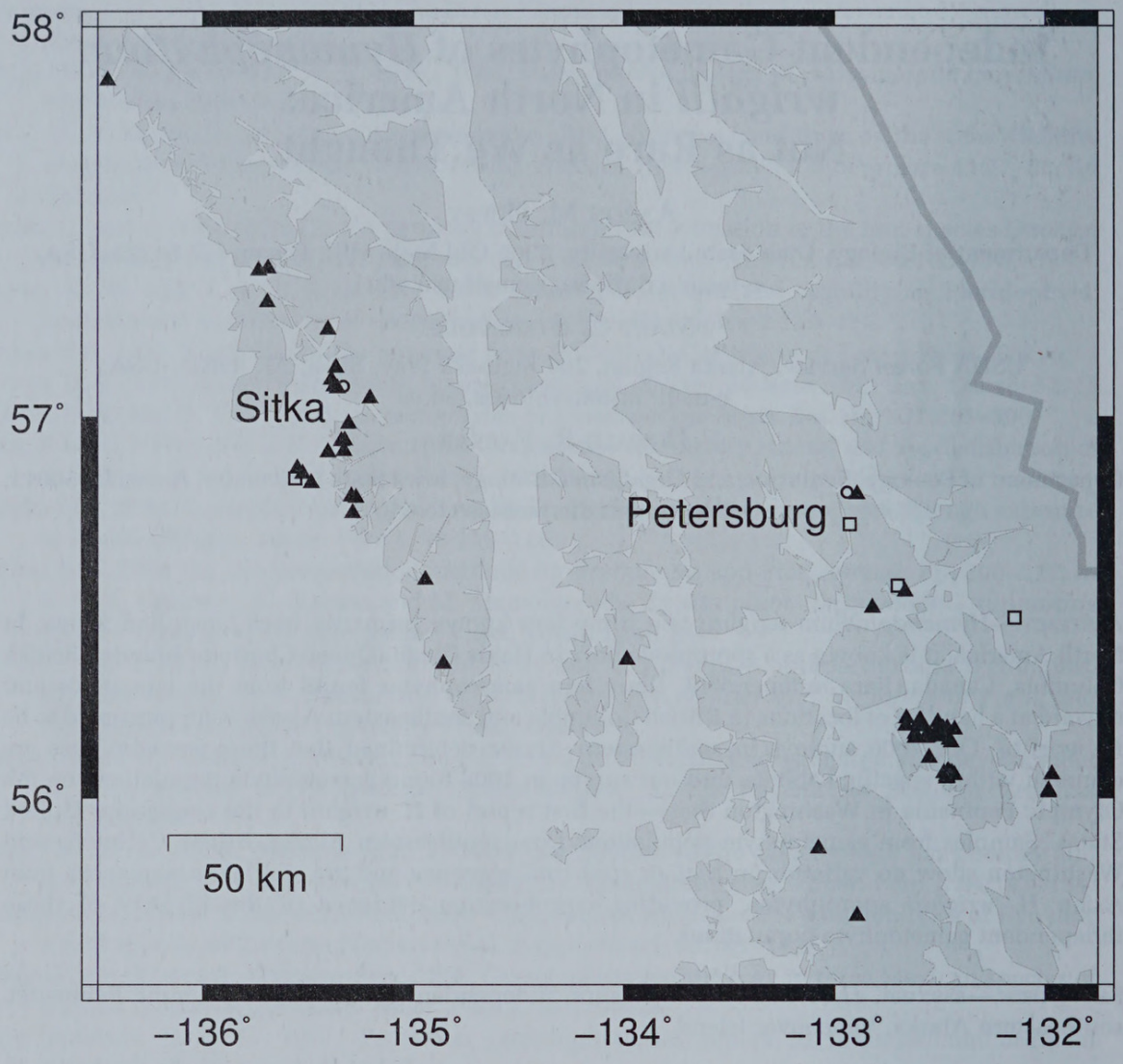

FIG. 1. Map of southeastern Alaska showing locations where *Hymenophyllum wrightii* gametophytes were found during and since 2006 (black triangles) and locations where gametophytes were reported or collected prior to 1980 (open squares).

in taxonomic groups possessing distinctive gametophyte morphologies (Farrar *et al.*, 2008). These populations of gametophytes are assumed to be *H. wrightii* because it is the only filmy fern known from western North America and because the North American gametophytes do not differ morphologically from gametophytes found growing among *H. wrightii* sporophytes from Japan and Haida Gwaii (Taylor, 1967). Furthermore, their gemmae are morphologically diagnostic of subgenus *Mecodium* to which *H. wrightii* belongs (Raine *et al.*, 1991). The gametophytes of these populations are slow-growing, long-lived, branching, ribbon-like thalli, and they produce gemmae at their margins (Fig. 4). They form dense tangled mats, but are small, grow in dark sheltered locations, and are easily overlooked.

Hymenophyllum wrightii grows on a variety of substrates in different parts of its range. Sporophytes in Japan are found on the bases of large trees in humid

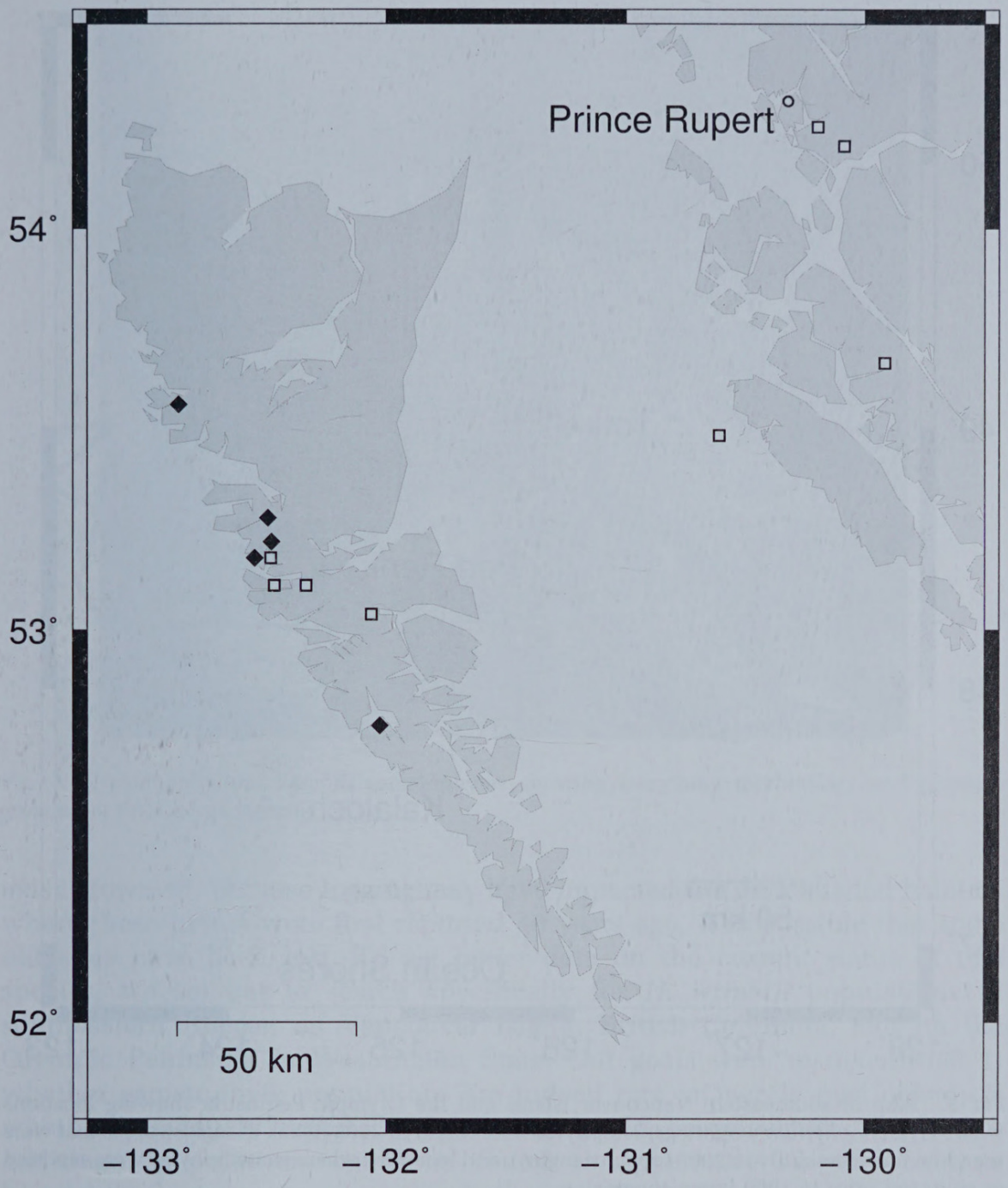

FIG. 2. Map of Haida Gwaii and the area surrounding Prince Rupert, British Columbia showing locations where *Hymenophyllum wrightii* sporophytes have been reported or collected (black diamonds) and *Hymenophyllum wrightii* gametophytes were reported or collected prior to 1980 (open squares).

forests and on moist shaded rocks and cliffs at elevations up to 1000 meters. The sporophyte-producing populations in Haida Gwaii are found on dark, shady, wet cliffs near sea level. The gametophytes collected in the 1960s through 1980s in Haida Gwaii, near Prince Rupert, and in southeastern Alaska were also growing on deeply shaded, wet rock outcroppings beneath

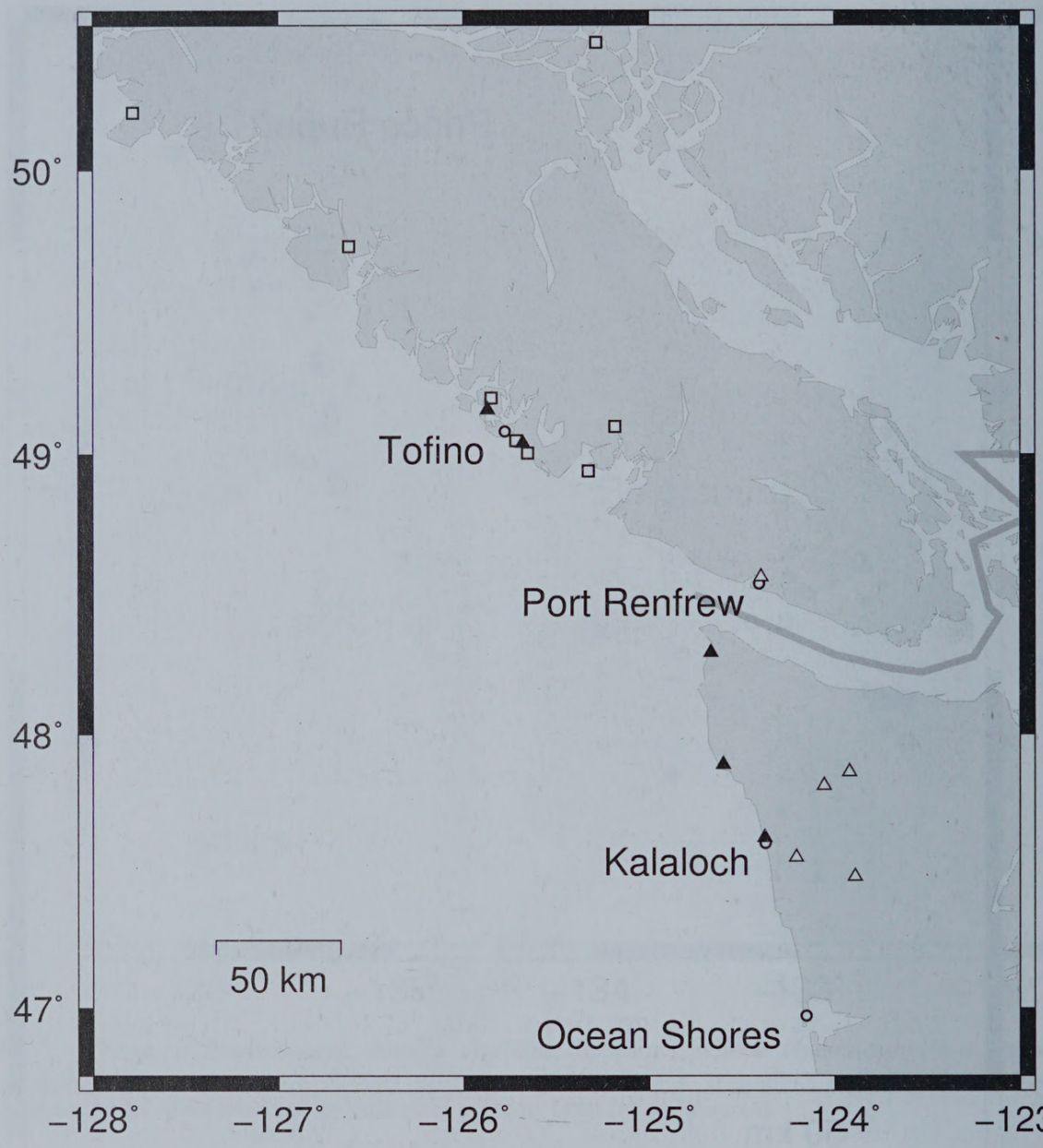

FIG. 3. Map of southeastern Vancouver Island and the Olympic Peninsula showing locations where *Hymenophyllum wrightii* gametophytes were found in 2008 (black triangles), areas that were searched unsuccessfully in 2008 (open triangles), and locations where gametophytes were reported or collected prior to 1980 (open squares).

overhangs or in wet cliff crevices near the coast or in stream canyons, although one collection was found on an overturned stump and another grew on the underside of a fallen Douglas fir, spanning a stream (Taylor, 1967; University of British Columbia Herbarium). On Vancouver Island, gametophytes were found on rotting wood or on old bark of Sitka spruce within 500 m of the coast (Cordes and Krajina, 1968).

Because the potential habitats of *H. wrightii* gametophytes are common throughout the Pacific Northwest, and because the gametophytes are so easily overlooked, it seems likely that many unidentified populations could

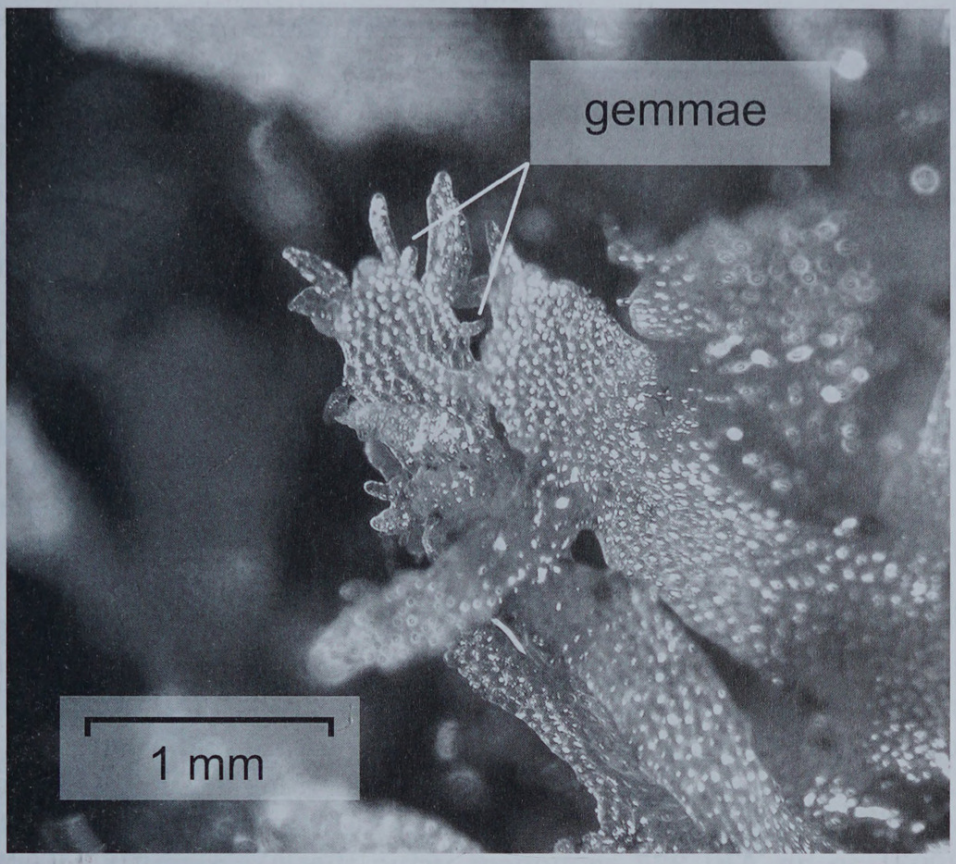

FIG. 4. *Hymenophyllum wrightii* gametophytes showing branching morphology and gemmae growing at the thallus margins.

exist. However, because logging may have impacted the dark shaded habitats where these plants were first reported 40 years ago, it is possible that some old sites have been lost. To get better data on the current status of this species, we set out to search specifically for *H. wrightii* populations in southeastern Alaska, on Vancouver Island, British Columbia, and on the Olympic Peninsula in Washington State. Our goals were to determine: 1) whether gametophyte populations are indeed rare or merely overlooked; 2) whether previously reported populations could be relocated; 3) whether additional populations could be found in apparently suitable habitats beyond the known range, and; 4) whether the North American gametophytes are indeed *H. wrightii*.

MATERIALS AND METHODS

Surveys.—In July of 2006, we intensively surveyed areas in southeastern Alaska near Sitka and Petersburg where gametophytes had previously been reported, as well as in other areas having potential habitats. During July of 2008, we surveyed sites on the west coast of Vancouver Island, British Columbia, including the Tofino area, where gametophytes were previously reported as well as further south on Vancouver Island near Port Renfrew. We

also surveyed the west coast of the Olympic Peninsula in Washington State as far south as Ocean Shores, Grays Harbor County.

Our efforts focused on microhabitats similar to those where gametophytes were previously reported: on wet tree bases, beneath overturned stumps, and on rock outcrops in deeply shaded rainforest. Light levels were so low as to require the use of flashlights and hand-lenses to distinguish *H. wrightii* gametophytes from the surrounding mosses and liverworts. Most areas searched were at low elevations within 1.5 km of the coast. However, on the Olympic Peninsula, we also searched sites up to 30 km inland along the Hoh, Queets, and Quinault Rivers.

DNA extraction, amplification, and sequencing.—A single living gametophyte was separated from each sample, manually cleaned of bryophytes, visible algae, or other contaminants, and washed in distilled water. Each gametophyte was ground with a pestle in a 1.5 μL tube on ice, and genomic DNA was extracted using a CTAB method (Doyle and Doyle, 1987).

We PCR amplified approximately 1300 bp of the *rbcL* gene using primers F1F and F1379R (Little and Barrington, 2003) and approximately 1000 bp of the *rps4* gene and *rps4* to *trnS*-GGA spacer (Small *et al.*, 2005). PCR amplification followed Der *et al.* (2009) for *rps4-trnS*, and Wolf *et al.* (1994) for *rbcL*. Fragments were sequenced in both directions using the PCR primers as well as filmy fern specific internal primers H1R1 and H1F1 for *rbcL* (Ebihara *et al.*, 2003), in an ABI 377 sequencer using the DYEnamic ET Terminator (Amersham Biosciences). Sequences for each individual were joined in Sequencher 4.5 (Gene Codes Corporation) and compared to other *rbcL* and *rps4-trnS* sequences using online BLAST (Altschul *et al.*, 1990).

Other barcoding markers or combinations of markers, such as *rbcL* and *trnL-trnF*, have been proposed as more effective at identifying ferns to species (Chen *et al.*, 2013; de Groot *et al.*, 2011; Li *et al.*, 2009). However, *rbcL* and *rps4-trnS* sequences for many *Hymenophyllum* species were available in GenBank as a result of phylogenetic studies of filmy ferns (Ebihara *et al.*, 2003; Hennequin *et al.*, 2006, 2010), so we selected these markers in order to maximize the number of species comparisons.

Results

Surveys.—We found gametophytes at the locations near Sitka and Petersburg, Alaska, where they had previously been reported as well as at approximately 60 other locations in southeastern Alaska (Fig. 1). Gametophytes were found at two locations near Tofino on Vancouver Island, including Meares Island (Fig. 3), one of the sites where gametophytes had previously been reported (Cordes and Krajina, 1968). On the Olympic Peninsula, we found gametophytes at three sites extending as far south as Kalaloch (Fig. 3). No *H. wrightii* sporophytes were found at any of the survey sites, nor have any sporophytes been produced in any of our live cultures in the years since these collections were made.

TABLE 1. *Hymenophyllum wrightii* gametophyte populations included in DNA sequencing. Because no sequence variation was found among populations only a single *rbcL* and *rps4-trnS* sequence representing each region (southeastern Alaska, Vancouver Island, and the Olympic Peninsula) was deposited in GenBank.

Collection	Location	Voucher	Accession
Duffy 08004	Rain Forest Trail near Tofino, Vancouver Island, British Columbia	UTC255727	
Duffy 08005	Rain Forest Trail near Tofino, Vancouver Island, British Columbia	UTC255728	
Duffy 08008	Rain Forest Trail near Tofino, Vancouver Island, British Columbia	UTC255729	
Duffy 08009	Rain Forest Trail near Tofino, Vancouver Island, British Columbia	UTC255730	
Duffy 08011	Big Trees Trail, Meares Island, British Columbia	UTC255731	JN585965 (*rbcL*), JN585968 (*rps4-trnS*)
Duffy 08013	Shi-Shi Beach Trail near Neah Bay, Washington	UTC255732	JN585966 (*rbcL*), JN585969 (*rps4-trnS*)
Duffy 08014	Shi-Shi Beach Trail near Neah Bay, Washington	UTC255733	
Duffy 08015	Third Beach near La Push, Washington	UTC255734	
Stensvold and Farrar 8319	Mitkof Island (South) near Banana Point, Alaska	UTC256069	
Stensvold and Farrar 8320	Mitkof Island (North) near Petersburg, Alaska	NA	
Stensvold and Farrar 8329	Biorka Island near Symonds Bay, Alaska	UTC256068	
Stensvold and Farrar 8334	Krestof Island near Olga Point, Alaska	UTC256071	
Stensvold and Farrar 8335	Chicagof Island near Bradshaw Cove, Alaska	UTC256070	
Stensvold and Farrar 8338	Kruzof Island near Sukoi Inlet, Alaska	UTC255737	JN585964 (*rbcL*), JN585967 (*rps4-trnS*)

DNA sequencing.—We sequenced full or partial *rbcL* and *rps4-trnS* from six samples from southeastern Alaska, five from Vancouver Island, and three from the Olympic Peninsula (Table 1). These sequences, representing populations from the known northern and southern range extents of *H. wrightii* in North America show no variation at either *rbcL* or *rps4-trnS*.

The *rbcL* sequences of these North American gametophytes differ from an Asian *H. wrightii* sporophyte sample (GenBank: AB083277.1; Ebihara *et al.*, 2003) at only two nucleotide positions. The North American *rps4-trnS* sequences differ from the Asian sample (GenBank: AY775430.1; Hennequin *et al.*, 2006) at only three nucleotide positions. One of those three nucleotides is ambiguous and could not be called with confidence in any of the North American sequences (despite otherwise clean sequence reads). The other two differences are a 'TA' missing from a dinucleotide repeat (six repeats in the North American sequences and seven in the Asian sequence) in a region that appears to be highly variable among *Hymenophyllum* species.

The North American *H. wrightii* gametophytes show more sequence similarity to *H. wrightii* than to sequences in GenBank from other *Hymenophyllum* species. The next closest *rbcL* match is an accession of *H. polyanthos* from Japan (GenBank: AB083276.1; Ebihara *et al.*, 2003) with 12 nucleotide differences and the next closest *rps4-trnS* match is *H. inaequale* from La Réunion (GenBank: AY095122.1; Hennequin *et al.*, 2003) with approximately 30 nucleotide differences.

Discussion

Our survey findings suggest that *H. wrightii* is more common and widespread on the northwest coast of North America than previously thought. We identified many additional gametophyte populations within the previously known range and found populations outside that range. *Hymenophyllum wrightii* gametophytes were located at previously reported sites near Sitka and Petersburg and on Vancouver Island, and found at locations in the surrounding areas. Significantly, populations of *H. wrightii* gametophytes were found on the Olympic Peninsula in Washington State, extending the known range of this species southward by approximately 200 km and providing the first reports of *H. wrightii* in the contiguous United States.

The surveys on Vancouver Island and the Olympic Peninsula were not as intensive or exhaustive as those in southeastern Alaska, so the results in these areas cannot be directly compared and our survey methods were not systematic—the dense clustering in certain areas (Fig. 1) is the result of focused collecting efforts. However, it appears that *H. wrightii* gametophytes are not as common on Vancouver Island or the Olympic Peninsula as they are in Alaska, but the ease with which we found new populations suggests that additional previously unknown populations may be found in appropriate microhabitats.

As a result of these surveys we now have better information on the habitats and microhabitats where *H. wrightii* is most likely to be found. This species grows on wet substrates in dark hollows of rotten fallen trees or at the bases of dead standing trees, and beneath the exposed roots and on the bases of mature conifers. It prefers the darkest areas, where competition with bryophytes is minimal. These habitats occur under the closed canopy of shady rainforests within a few hundred meters of the coast.

We did not search all habitats and sites exhaustively, so we cannot be certain that we did not overlook some *H. wrightii* occurrences, but certain commonalities were observed among the habitats where it was found. Gametophytes were not found at sites over 1.5 km inland, even though these inland sites were chosen specifically because they receive similar amounts of rainfall to southeastern Alaskan sites. Despite the high amount of rainfall in the Hoh, Queets, and Quinault rainforests, the dark inner surfaces of likely looking stumps were dry compared to stumps in coastal forests receiving less rainfall, suggesting that perhaps coastal humidity or temperature moderation play a role in creating suitable habitat for *H. wrightii* gametophytes. Other

environmental factors such as soil type or substrate pH may be important and warrant further investigation. We also did not find gametophytes in sites that had been recently logged or burned. In several areas, we inspected the rotting remains of burned trees but did not find gametophytes. As we moved south on the Olympic Peninsula, patches of wet, dark forest became more difficult to find, but we found gametophytes as far south as Kalaloch, Jefferson County.

Additional populations may exist at microsites further south along the Pacific coast, along parts of Puget Sound, and further west along the Alaskan coast. However, it appears that these gametophytes require high levels of moisture and dark, temperature-moderated environments. Based on the habitats where they have and have not been found, as well as observations of plants maintained in culture, the gametophytes may not tolerate repeated drying and seem to compete poorly with mosses and liverworts in brighter lighting. Other long-lived filmy fern gametophytes are frequently found in both desiccated and frozen states (Farrar, 1998), so a single event may not prevent *H. wrightii* gametophytes from inhabiting a site, but the average conditions in a particular location may not allow sufficient assimilation through photosynthesis to exceed loss due to respiration in the long-term. Suitable habitat likely becomes scarce in Puget Sound or further south along the Pacific coast in Washington due to low moisture levels, and in Alaska west of Kodiak due to a lack of rainforest.

Through DNA sequencing, we confirmed that the *Hymenophyllum wrightii* gametophytes growing on the west coast of North America are genetically similar to Asian *H. wrightii* sporophytes at two chloroplast loci. The sequences from North American and Asian plants are not identical, but they are much more similar to each other than either is to other available *Hymenophyllum* species sequences. This genetic evidence corroborates the previous identification of these North American gametophytes as *H. wrightii* based on their morphology—though it cannot rule out the possibility of hybrid origins or the possibility that they are gametophytes of some *Hymenophyllum* species that is not represented in GenBank. Further study, including sequencing sporophytes from Haida Gwaii is still necessary.

Hymenophyllum wrightii is the only fern reported to form long-lived, gametophyte-only populations in western North America, but other ferns share this unusual lifecycle, including *Crepidomanes intricatum* (Farrar) Ebihara & Weakley (Hymenophyllaceae) and *Vittaria appalachiana* Farrar & Mickel (Vittariaceae) in the eastern United States. However, for those species no sporophytes have been found and it seems likely that these species are no longer (or were never) able to produce them (Farrar, 1990, 1992, 1998; Farrar and Mickel, 1991). *Hymenophyllum tayloriae* Farrar & Raine (Hymenophyllaceae) in the southern Appalachian Mountains and Appalachian plateau produces independent gametophyte colonies, some of which also produce juvenile sporophytes, but mature sporophytes have not been found (Raine *et al.*, 1991). *Callistopteris baueriana* (Endl) Copeland (Hymenophyllaceae) in Hawai'i, is found as dense mats of gametophytes which only produce sporophytes in wetter, higher altitude locations (Dassler and Farrar, 1997).

In the case of *H. wrightii*, it is not clear whether the apparent absence of sporophytes outside of Haida Gwaii is due to some habitat limitation, or whether these gametophytes are genetically incapable of producing sporophytes (as suggested by the lack of sporophytes in culture). This raises questions of how the North American gametophyte populations were established. Are they the result of recent spore dispersal from the sporophyte populations in Haida Gwaii or elsewhere, or are they reproducing only asexually and dispersing via gemmae? If they are currently limited to asexual reproduction, how and when were the populations established? Further work, including DNA sequencing of sporophyte samples from Haida Gwaii and using genetic markers that are variable among populations, will be required to distinguish among these possibilities.

A better understanding of the relationship between the Haida Gwaii sporophytes and the widespread gametophyte populations will be important in management and conservation of this species. Based on our surveys in southeastern Alaska, *H. wrightii* was removed from the Forest Service's Alaska Region Sensitive Species List in 2009 (Goldstein *et al.*, 2009) on account of its great abundance, wide distribution, and stable populations in the Tongass National Forest. In British Columbia, *H. wrightii* is identified as a 'yellow' listed species rated as 'Vulnerable' to 'Apparently Secure' (B. C. Conservation Data Centre). Sporophytes and gametophytes are managed as a single entity, though they differ greatly in their distribution. *Hymenophyllum wrightii* gametophytes on the Olympic Peninsula do not appear to be nearly as common or widespread as in southeastern Alaska, but further study is warranted to determine appropriate protections.

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