

1 **Discovery of two Palearctic *Bootanomyia* Girault (Hymenoptera: Megastigmidae) parasitic**
2 **wasp species introduced to North America**

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4 **Running head title.** Palearctic *Bootanomyia* Parasitic Wasps Introduced to North America

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19

20 **Conflict of Interest**

21 The authors declare no conflict of interest.

22

23

24 **Abstract**

25 Invasive species are among the greatest threats to ecosystems, but our ability to detect species
26 introductions varies across taxa. Parasitoid wasps, though one of the most species-rich groups of
27 all animals, are small and have ephemeral adult stages, such that they often go unnoticed. Here,
28 we document two separate introductions of European parasitoids of oak gall wasps into North
29 America. Both wasps key morphologically to *Bootanomyia dorsalis* (Fabricus), which previous
30 genetic data from Europe suggest comprises two distinct species, *B. dorsalis* sp. 1 and *B.*
31 *dorsalis* sp. 2. We find *B. dorsalis* sp. 1 in oak galls from New York, USA and *B. dorsalis* sp. 2
32 in oak galls from Washington, Oregon, and British Columbia, Canada. All oak gall wasp hosts
33 were North American natives. We detect no genetic variation at the mtCOI locus within *B.*
34 *dorsalis* sp. 2 specimens, suggesting this introduction may have had only a small number of
35 founder individuals. In their native ranges, both species attack several different gall wasp hosts,
36 and we likewise reared both from galls of multiple North American gall wasp hosts, suggesting a
37 potential for widespread impact on North American gall insect communities. These introductions
38 were detected only because our research groups are actively sampling and identifying parasitoid
39 communities across gall habitats. Most parasitoid communities are not regularly sampled across
40 hosts, time and space, or are well characterized, such that many more undetected wasp
41 introductions may be impacting native insects worldwide.

42

43 **Keywords**

44 Cynipidae, gall wasp, *Quercus*, parasitic wasp, non-native, Nearctic

45

46

47 **Introduction**

48 The ability to detect introduced and potentially invasive species varies greatly across
49 different types of organisms (Crystal-Ornelas & Lockwood, 2020). Insects represent the most
50 frequently introduced group of animals to non-native ranges, with documented invasions
51 occurring at similar rates compared to other invertebrates (Pagad et al., 2022; Seebens et al.,
52 2017; Sodhi & Ehrlich, 2010). One category of insects, the parasitoid wasps, may be the most
53 species-rich of all animals (Forbes et al., 2018), but are not commonly cited invaders and often
54 go undescribed even in their native ranges, due to their small size and ephemeral adult stages
55 (Saunders & Ward, 2018). This combination of high species richness with challenging detection
56 suggests there could be extensive undocumented parasitoid wasp introductions. This potential for
57 under-detection is a critical problem given the important role of parasitoids in regulating the
58 population sizes of other insects (Gómez & Zamora, 1994).

59 One low-tech but high-confidence approach to detection of parasitic wasps is to collect
60 potential insect hosts and then rear and identify any wasps that emerge. Our research team
61 collects and rears insects from galls induced on oaks by wasps (Cynipidae: Cynipini) in North
62 America to better understand their diversity and evolution. Oak gall wasps lay eggs into the
63 meristematic tissue of oak trees and induce the tree to form galls—growths of plant tissue that
64 provide food and putative shelter to the developing wasp larvae (Egan et al., 2018). This work
65 has resulted in new species discovery (Sheikh et al., 2022; Ward et al., 2020), species
66 descriptions (Egan et al., 2017; Nastasi et al., 2024), evolutionary patterns (Ward et al., 2020;
67 Ward et al., 2022b; Ward et al., 2024), and patterns in diversity and interactions (Prior et al.,
68 2023). These investigations have also revealed extensive, and previously undocumented,
69 parasitic wasp diversity associated with oak galls (Ward et al., 2022a).

70 Some surprising associates of cynipid oak galls among our collections have been wasps
71 from the genus *Bootanomyia* Girault (Hymenoptera: Megastigmidae). Species in this genus are
72 common parasites of cynipid gall forming wasps on oaks in the Palearctic (Doğanlar, 2011), but
73 there were no published records of Nearctic *Bootanomyia* associated with oak galls prior to our
74 surveys. In two recent papers, we noted the presence of wasps fitting the description of the
75 Palearctic species *Bootanomyia dorsalis* (Fabricus) reared from four different host galls native to
76 the Pacific coast of North America (Ward et al., 2022a; Prior et al., 2023). We considered the
77 possibility that these might represent an introduction (Ward et al., 2022a; Jones, 2023) but did
78 not test that hypothesis. Separately in 2023 and 2024, we reared *Bootanomyia* wasps from native
79 North American oak galls collected at the Stone Barns Center for Food and Agriculture in
80 Westchester Co., NY and near the Brooklyn Bridge Park Conservancy in Brooklyn Co., NY.
81 These new collections, more than three thousand kilometers from the Pacific coast wasps,
82 inspired us to test whether North American *Bootanomyia* from oak galls represent introductions
83 of Palearctic species, or if they instead could be previously undetected species native to the
84 Nearctic.

85

86 **Methods**

87 *Collections and Rearing*

88 We collected Pacific coast (northern CA, OR, WA, and southwest BC) galls exclusively
89 from the Oregon White Oak (*Quercus garryana* Dougl. Ex Hook.), a white oak (section
90 *Quercus*). These collections focused primarily on 23 gall morphotypes (Jones et al., 2022; Prior
91 et al., 2023). Collections from the east coast targeted more than 30 oak species from both
92 sections *Quercus* and *Lobatae* (red oaks) and included more than 200 gall morphotypes (Ward et

93 al., 2022a; Brown et al., unpublished data). All gall wasp and oak tree species were native to
94 North America. We identified galls to morphotype and placed them into containers for rearing in
95 either environmental chambers or under ambient conditions (Westchester Co.). We checked
96 rearing containers once per week for the Pacific collections and daily for the eastern collections
97 for emerging insects. We immediately placed all adult insects into tubes of 95% EtOH. For more
98 collection and rearing details see Jones et al. (2022), Prior et al. (2023), and Ward et al. (2022a).
99 We identified all *Bootanomyia* specimens morphologically using a key in Doğanlar (2011). We
100 photographed representative specimens using a Leica S9i digital stereo microscope (Leica
101 Microsystems, Heerbrugg, Switzerland) with an integrated 10-megapixel camera at 5x
102 magnification. Some point mounted images were stacked using Zerene Stacker (Zerene Systems,
103 LLC, Richland, WA).

104 *Genetic Identification*

105 We sequenced 19 Pacific coast *Bootanomyia* specimens and four NY specimens. West
106 coast samples were destructively extracted using a Qiagen PureGene standard protocol for DNA
107 purification from tissue, excluding the RNase A step. One New York specimen was also
108 destructively extracted, while for the second (Westchester Co.) we removed the legs from one
109 side of the body for destructive extraction and then used a non-destructive method on the
110 remaining tissue. We extracted DNA from both New York samples using a modified CTAB
111 protocol (Chen et al., 2010). Two New York specimens and two Pacific coast specimens were
112 pinned and submitted to the University of Iowa Museum of Natural History (SUI_INS 39342–
113 39345). We PCR-amplified the mitochondrial locus Cytochrome Oxidase Subunit I (mtCOI)
114 using primers for pF2 (5'-ACC WGT AAT RAT AGG DGG DTT TGG DAA-3') and 2437d (5'-
115 GCT ART CAT CTA AAW AYT TTA ATW CCW G-3') (Karttinen et al., 2010; Simon et al.,

116 1994). The conditions for PCR were an initial denaturation at 95°C for 1 min followed by forty
117 repeated cycles of denaturation at 95°C for 30s, annealing at 48°C for 30sec, and elongation at
118 72°C for 30sec. After these cycles, we performed a final elongation at 72°C for 5 min and
119 recovered at 4°C. Amplicons from the Pacific coast samples were sequenced using Sanger
120 Sequencing by Eurofins (Louisville, KY), and reads were processed in Geneious version
121 2024.0.5 (Biomatters, LLC, New York, NY) for final consensus sequences. For PCR on the NY
122 specimens, primers were tagged with distinct 13bp oligonucleotide barcodes designed by
123 Srivathsan et al., (2021) such that each amplicon was distinguishable from others. Primer
124 sequences and their barcodes can be found in Supplemental Table S1. Amplicons were combined
125 into a single library and prepared for sequencing on an Oxford Nanopore MinION Mk1B
126 platform with a V14 (SQK-LSK114) Ligation Sequencing Kit (Wang et al., 2021). We
127 sequenced the library using a R10.3 chemistry Flongle Flow Cell. We demultiplexed reads using
128 a custom Snakemake workflow (Mölder et al., 2021) that implemented the following steps:
129 demultiplexing using minibar v0.24 (*Calacademy-Research/Minibar: Dual Barcode and Primer*
130 *Demultiplexing for MinION Sequenced Reads*) then dereplication, clustering, and chimera
131 removal using VSEARCH v2.30.0 (Rognes et al., 2016). VSEARCH implements the UCHIME2
132 algorithm (Edgar, 2016) for chimera removal.

133 We compared sequences from our specimens to previously published mtCOI sequences
134 from Palearctic *Bootanomyia* associated with oak galls (Stone et al., 2012). These included *B.*
135 *stigmatizans* Fabricus and two distinct species previously identified within the *B. dorsalis*
136 morphology (Nicholls et al., 2010, 2018). We included a *Bortesia* sp. Pagliano & Scaramozzino
137 as an outgroup, as well as five Palearctic *Megastigmus* Dalman species. We aligned all
138 sequences using the MUSCLE algorithm (Edgar, 2004) in Uniprot's Ugene v50 (Okonechnikov

139 et al., 2012). We used IQ-Tree v2.0.7 (Minh et al., 2020) and ModelFinder (Kalyaanamoorthy et
 140 al., 2017) using 5,000 ultra-fast bootstraps and an approximate likelihood ratio test (aLRT;
 141 Guindon et al., 2010) to perform maximum likelihood analysis and infer a phylogeny for
 142 visualizing the relationship between sequences. The unpruned tree (supplementary Figure S1)
 143 contained 19 sequences from the Pacific coast. Several were identical sequences from the same
 144 collection sites, and we pruned some of these sequences from the tree for ease of viewing. Gene
 145 tree pruning/stylizing was completed using the “ape” package in R v4.3.3 (Paradis & Schliep,
 146 2019; R Core Team, 2024). Sequence similarity comparisons, in the form of percent identity,
 147 were made using the “seqidentity” function from the “bio3d” package in R v4.3.3 (Grant et al.,
 148 2006). Data for all samples used in molecular work can be found in Supplemental Table S2.

149

150 **Table 1.** North American oak gall species from which *B. dorsalis* parasites were reared, the
 151 location from which they were collected, the generation of the gall, the tree species from which
 152 they were collected, and the taxonomic section to which the host tree belongs.

<i>Bothanomyia</i> species	Gall Wasp Species	Location	Gall Generation	Host Tree	Tree Section	# Reared	# Sequenced
<i>B. dorsalis</i> sp. 1	<i>Neuroterus quercusbatatus</i> (Fitch)	NY	unisexual	<i>Quercus bicolor</i>	Quercus	4	3
<i>B. dorsalis</i> sp. 1	<i>Acraspis erinacei</i> (Beutenmüller)	NY	unisexual	<i>Quercus alba</i>	Quercus	1	1
<i>B. dorsalis</i> sp. 2	<i>Andricus opertus</i> (Weld)	BC	bisexual	<i>Quercus garryana</i>	Quercus	25	4
<i>B. dorsalis</i> sp. 2	<i>Neuroterus saltatorius</i> Edwards	BC, OR	unisexual	<i>Quercus garryana</i>	Quercus	52	3
<i>B. dorsalis</i> sp. 2	<i>Neuroterus washingtonensis</i> Beutenmüller	WA, BC	bisexual	<i>Quercus garryana</i>	Quercus	2307	12
<i>B. dorsalis</i> sp. 2	<i>Besbicus mirabilis</i> (Kinsey)	WA	unisexual	<i>Quercus garryana</i>	Quercus	1	0

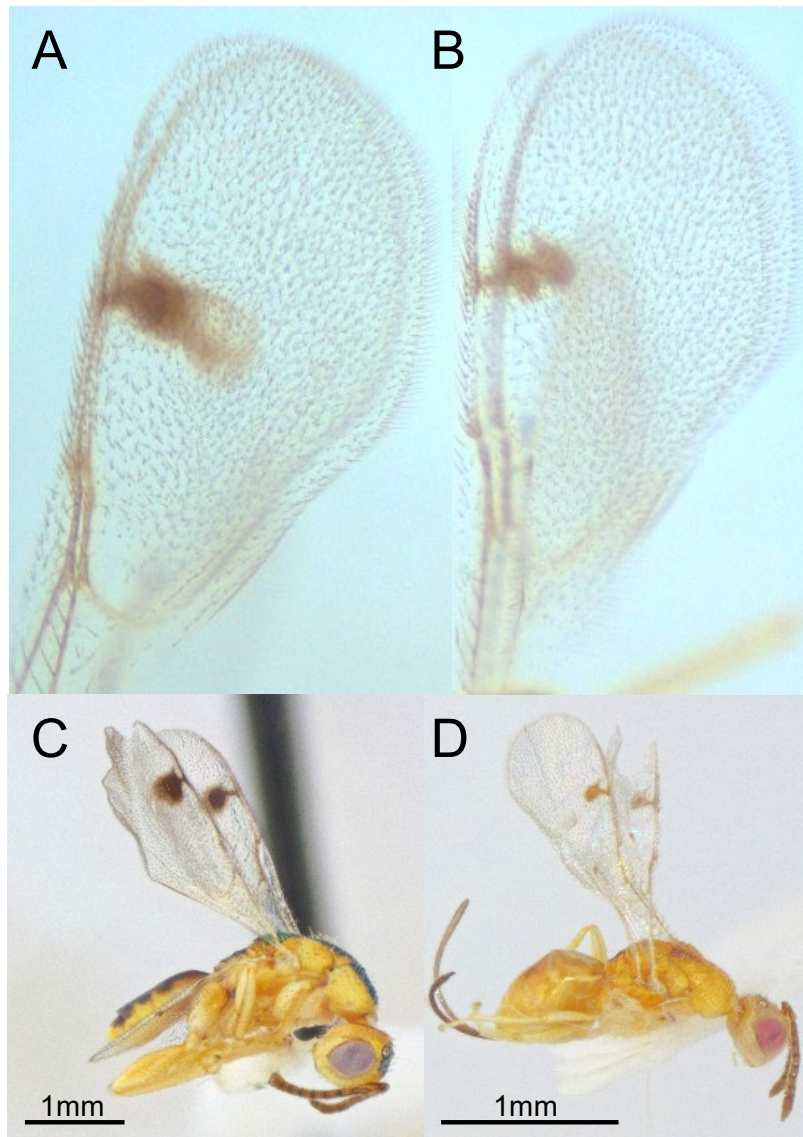
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160 **Figure 1:** Two species of *Bootanomyia dorsalis* wasps introduced to North America from
 161 Europe. **A & B:** Variation in the extent of wing infumation from a single collection of *B.*
 162 *dorsalis* sp. 2 from *Neuroterus washingtonensis* in Metchosin, BC. **C:** A male *B. dorsalis* sp. 1
 163 collected from New York. **D:** A female *B. dorsalis* sp. 2 from the Pacific coast of North
 164 America. Body coloration of both C and D wasps are representative of their respective species
 165 regardless of sex.

166

167

168

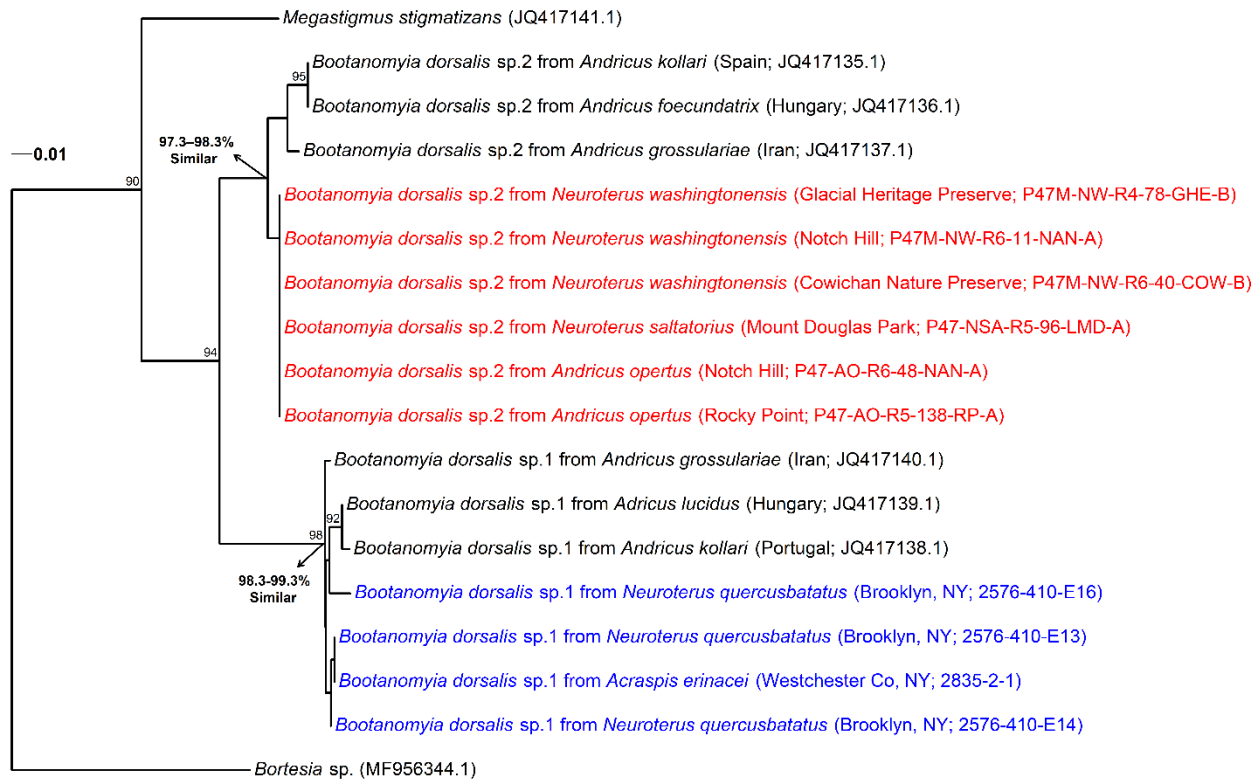
169 **Results**

170 *Collections and Morphological Identification*

171 We reared *Bootanomyia* wasps from six different oak gall wasp morphotypes collected on three
172 oak tree species (Table 1). All 2,431 Pacific coast wasps were associated with galls on *Quercus*
173 *garryana* (the only oak sampled). Five NY wasps emerged from galls on *Quercus bicolor* Willd.
174 and *Quercus alba* L., both in the oak section *Quercus*. Pacific coast samples matched the
175 description of *Bootanomyia dorsalis*, though we noticed variation in the extent of wing
176 infumation, ranging from the typical expected from this species (extending from the stigma to
177 the middle of the wing) to no or hardly any infumation (Figure 1A & B). The Brooklyn, New
178 York collection (2576-410-E14) also keyed to *B. dorsalis*, except the wing infumation, as in
179 some Pacific coast samples, was not as strong as expected for that species. Doğanlar (2011)
180 suggests that *B. dorsalis* has three body color “forms”: “typical”, “pale”, and “dark”. The
181 specimens from the Pacific coast better match the pale form whereas those from NY are closer to
182 the dark form (Figure 1C & D).

183 *Genetic Identification*

184 The mtCOI gene tree sorts the North American *B. dorsalis* into two clades, each also
185 containing sequences from Palearctic *B. dorsalis* (Figure 2). Sequences from NY specimens were
186 98.4–99.4% similar to sequences from *B. dorsalis* sp. 1 from Portugal, Iran, and Italy (Nicholls
187 et al. 2018). Sequences from Pacific coast specimens were 97.9–98.1% similar to sequences
188 from *B. dorsalis* sp. 2 from Spain, Hungary, and Iran. Sequences in the *B. dorsalis* sp. 1 and sp. 2
189 clades are 5.3–6.6% different from each other. Sequences from *B. dorsalis* sp. 1 collected from
190 New York are 98.7%–100% similar to each other. All wasps from the Pacific coast were 100%
191 identical at the mtCOI locus. Genetic sampling from the Pacific coast was more extensive,



194 Figure 2: Pruned and stylized mtCOI maximum likelihood phylogenetic tree of *Bootanomyia*
 195 *dorsalis* and close relatives. Ultra-fast bootstrap scores >90 are shown next to their respective
 196 nodes. Tip labels highlighted in red were collected from the Pacific coast. Sequences from the
 197 same collection site and host gall have been removed for simplicity (see Figure S1 for the
 198 complete tree). Sequences highlighted in blue were collected from samples in New York. All
 199 other sequences were acquired from previously published data. GenBank accession numbers for
 200 these sequences can be found in Table S2. Similarity scores are shown for clades where Nearctic
 201 and Palearctic sequences are both present. The score represents the range of genetic distance
 202 between Nearctic and Palearctic sequences.
 203

208 totaling 19 individuals from three different oak gall hosts from six sites in both the USA and
209 Canada.

210

211 **Discussion**

212 These results suggest that two different species of oak gall-associated *Bootanomyia*
213 wasps have been introduced from the Palearctic to the Nearctic. One introduction, to the Pacific
214 coast of North America shares a single mtCOI haplotype closest to those previously sequenced
215 for *B. dorsalis* sp. 2. The lack of mtCOI genetic variation for the Pacific coast *B. dorsalis*, which
216 are variable in the Palearctic (Nicholls et al., 2018), is a strong indicator of a population
217 bottleneck, consistent with a founder effect upon introduction. The second introduction,
218 discovered via two independent collections in New York, have similar mtCOI haplotypes to
219 Palearctic *B. dorsalis* sp. 1. The New York introduction appears to be less bottlenecked, as
220 evidenced by there being at least three distinct mtCOI haplotypes.

221 What are some possible pathways of introduction for *Bootanomyia* parasites of oak galls?
222 *B. dorsalis* has only been recorded as a parasite of oak galls (Askew et al., 2013; Doğanlar,
223 2011), so one possibility is that they were introduced along with Palearctic oak trees infested
224 with galls. Nicholls et al., 2010 and 2018 record *B. dorsalis* emerging from 23 oak gall hosts on
225 oak species including *Quercus robur* L. (sect. *Quercus*) and *Q. cerris* L. (sect. *Cerris*). Both trees
226 have been introduced to North America and *Q. robur* has been widely planted since the 17th
227 century and has been documented in the Canadian provinces of British Columbia, Ontario, and
228 New Brunswick along with the U.S. states Ohio, Pennsylvania, New York, Massachusetts, New
229 Hampshire, and Maine (*USDA Plants Database Plant Profile General*, 2024). *Q. cerris* has been
230 documented on iNaturalist across the east coast, including one within 13 kilometers of a location

231 where a *B. dorsalis* was collected. We also find one iNaturalist observation of *Neuroterus*
232 *quercusbaccarum* (L.) (a Palearctic gall wasp host of *B. dorsalis*) in Papineauville, QC, Canada
233 (approx. 525 kilometers from the NY discovery). *Neuroterus quercusbaccarum* is one of just two
234 Palearctic oak gall hosts of *B. dorsalis* known to have been introduced to North America (Table
235 S3). Alternatively, perhaps these wasps were introduced separately from their hosts or host trees.
236 Adult females of other megastigmid species have a maximum longevity of 27 days (Bouaziz &
237 Roques, 2006), ample time for an inconspicuous insect to hitchhike in cargo or on a passenger
238 plane across the Atlantic.

239 Are the introduced North American *Bootanomyia* parasites of cynipid oak galls also
240 invasive? The ecological effects of parasitoid introductions are not well documented but could be
241 substantial. The eupelmid wasp *Eupelmus messene* Walker may be the earliest documented
242 parasitoid introduction (Burks 1979). Now suspected to be a complex of two or more species
243 (Fusu, 2017), *E. messene* wasps attack and kill immature insects across hundreds of hosts from at
244 least six orders in their introduced range (Gibson, 1990). The braconid wasp *Cotesia glomerata*
245 (L.), accidentally introduced to the Canary Islands, threatens the endemic butterfly *Pieris*
246 *cheiranthi* (Hübner) (Lepidoptera: Pieridae; Lozan et al., 2008). Introduced parasitoids can also
247 affect interactions other parasites have with their hosts. The introduction of *Heterospilus*
248 *prosopidis* Viereck (Hymenoptera: Braconidae) as a secondary biological control agent for the
249 azuki bean beetle, *Callosobruchus chinensis* (L.) (Coleoptera: Chrysomelidae), disrupts the
250 efficacy of the native parasitoid *Anisopteromalus calandrae* (Howard) (Hymenoptera:
251 Pteromalidae) (Nakamichi et al., 2020; Tuda & Shimada, 2005).

252 It remains to be seen whether these two specific introductions of *Bootanomyia* wasps to
253 North America present an existential threat to native gall wasps or other gall-associated insects.

254 The Pacific coast introduction is already geographically widespread, and a bugguide.net report of
255 *Bootanomyia* that appears to be *B. dorsalis* was recorded in Groton, MA on August 10th, 2021
256 (roughly 240 kilometers from the discovery in NY), suggesting the east coast introduction also
257 has a wider distribution. Regardless of the extent of introduction, the primary relevance of
258 documenting these introductions is in what they portend for the myriad other parasitoid
259 communities that are not well-studied or regularly sampled. This finding of multiple
260 introductions into the North American oak gall system – one of them apparently quite well-
261 established across the Pacific coast – makes it reasonable to presume that the portcullis has been
262 raised for parasitic wasp invasions, as it has been for so many other insects (Hokkanen & Lynch,
263 2003). Efforts towards detection and identification of newly introduced species should consider
264 investing in methods and resources that are inclusive of parasitic wasps.

265

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276

277 **Data Accessibility**

278 mtCOI sequences are available on GenBank (accessions PV174173–PV174193). Script for
279 demultiplexing sequence data, developing distance matrices, and stylizing the gene tree is
280 available on Zenodo (<https://zenodo.org/records/15555447>).

281

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