Investigation of Possible Cryptic Species within Orchestes mixtus Blatchley and O. pallicornis Say (Coleoptera: Curculionidae: Curculioninae: Rhamphini) Using DNA Barcoding

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NOTE

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Orchestes mixtus Blatchley is a widespread, North American leaf-mining weevil with larval hosts in Betulaceae (*Alnus* Mill., *Betula* L., *Carpinus* L., *Corylus* L., *Ostrya* Scop.) and Ulmaceae (*Ulmus* L.), as summarized by Eiseman (2022). Eiseman (2022) suggested that a cryptic species may be involved, since Betulaceae and Ulmaceae belong to two different plant orders (Fagales and Rosales, respectively), and since records of *O. mixtus* from *Ulmus* are restricted to Ontario and the central and southeastern USA—conspicuously absent from the northeastern USA where this species is common on Betulaceae.

On 29 April 2023, TSF found an *Orchestes* leaf mine on winged elm (*Ulmus alata* Michx.) in North Carolina (Durham Co., Durham, Leigh Farm Park, 35.916694, -78.979833). An adult male resembling *O. mixtus* emerged from this mine on 16 May 2023 (specimen #CSE8201). Seeing an opportunity to compare DNA barcodes of *Orchestes* reared from different host plants, on 27 May 2023 CSE collected mines of *O. mixtus* on black birch (*Betula lenta* L.) and of *O. pallicornis* Say on pin cherry (Rosaceae: *Prunus pensylvanica* L.f.) in Massachusetts (Franklin Co., Northfield, 42.653711, -72.429192). One adult female *O. mixtus* (CSE8257) emerged from a mine on black birch and one adult male *O. pallicornis* (CSE8256) emerged from a mine on pin cherry on 12–13 June 2023. In addition, a parasitoid wasp (Hymenoptera: Eulophidae: *Pnigalio* sp.; CSE8249) emerged from one of the pin cherry mines on 8 June, and four parasitoids (Eulophidae: *Elachertus* sp.; CSE8268) emerged from one of the black birch mines on 16 June; these are deposited in the Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa.

Molecular methods and analyses were performed by SM and MAB. DNA was extracted from whole specimens using a leaching method whereby they were placed in tubes containing 180 µL ATL buffer (Qiagen, Hilden, Germany) and 20 µL of Protienase K on a heat block at 55°C overnight. One extraction failed (CSE8257) and, subsequently, the entire insect (minus the abdomen) was crushed and extraction followed the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) protocol. PCR was carried out using LCO1490F and HCO2198R primers (Folmer et al. 1994) that amplify a segment of the mitochondrial cytochrome c oxidase subunit I (COI) gene. The amplified PCR products were purified with Exo-SAP-IT (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Sequencing in both directions was performed by the NC State University Genomic Sciences Laboratory, using the same primers employed in the amplification process. Existing COI sequences from other *Orchestes* specimens were accessed and downloaded from BOLD: The Barcode

of Life Data System (https://www.boldsystems.org/; Ratnasingham and Hebert 2007) and NCBI GenBank (Clark et al. 2016). Sequences generated during this study were submitted to GenBank and are as follows: OR769071 (*Orchestes mixtus* CSE8201); OR769072 (*O. pallicornis* CSE8256); and OR769073 (*O. mixtus* CSE8257). Alignments and analyses of COI sequences were performed using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura et al. 2021). Neighbor-joining analysis was performed using the default setting in MEGA11 with the addition of 1000 bootstrap replicates. For genitalia preparation and observation, the whole abdomen of each specimen was removed after extraction, and further cleared in 10% KOH. Structures of interest were then carefully dissected out of the abdomen, and stored and imaged in glycerin. Specimens are deposited in the North Carolina State University Insect Museum.

We amplified approximately 686 bp of the COI gene. Neighbor-joining analysis of aligned sequences from *O. mixtus*, *O. pallicornis*, and an outgroup species (*O. fagi* (L.); native to the Palearctic) show three well-supported clades representing the three putative species (Fig. 1). The specimen in question from *Ulmus* is nested well within the *O. mixtus* clade, supporting Anderson's (1989) hypothesis of a single species with a broad host range. Furthermore the male genitalia of

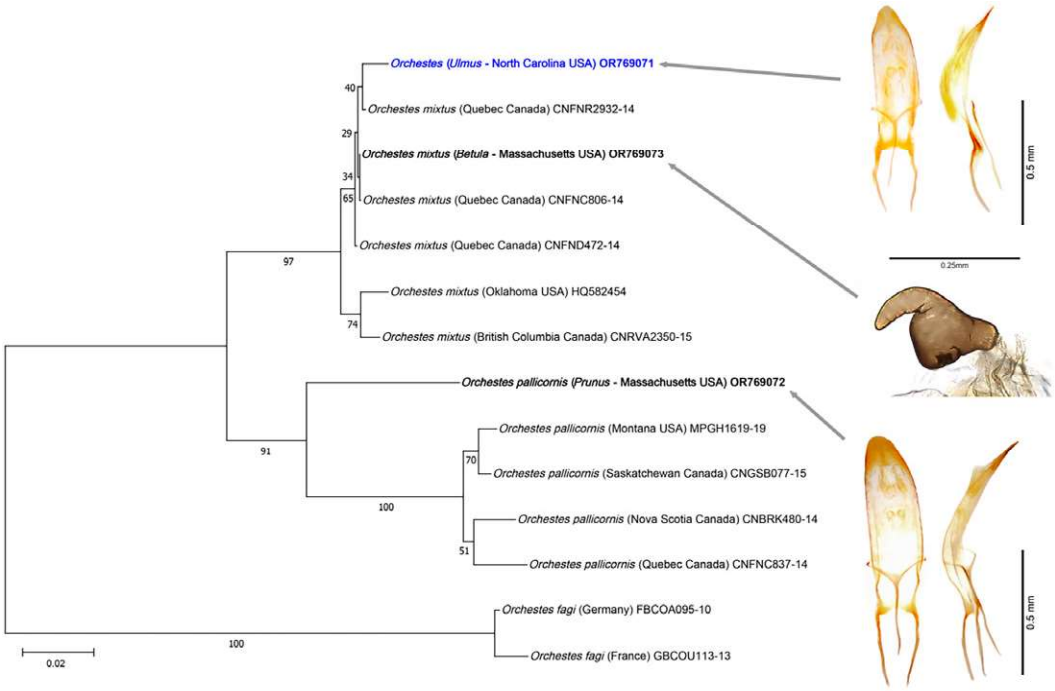

Fig. 1. Tree generated using Neighbor-joining method based on COI sequences from specimens in this study (labels in bold typeface; blue label represents specimen of interest from *Ulmus*) and representative sequences accessed from BOLD (Ratnasingham and Hebert 2007) and NCBI GenBank (Clark et al. 2016) (accession numbers shown after taxon name and geographic location). Numbers on nodes were generated from 1000 bootstrap replicates. Images of the genitalia correspond to the three specimens included in the study, i.e, aedeagi of the male *Orchestes mixtus* that emerged from *Ulmus* and the male *O. pallicornis* that emerged from *Prunus,* and the spermatheca from the female *O. mixtus* that emerged from *Betula*.

the specimen in question match the drawings by Anderson (1989) for *O. mixtus*.

Although it is possible for two species to share a DNA barcode (Tavares et al. 2011, Liu et al. 2017 and references cited therein, Lopez-Vaamonde et al. 2021, Doorenweerd et al. 2024, C. Eiseman and B. Rulik unpubl.), Anderson (1989) found no morphological differences among *O. mixtus* specimens reared from *Ulmus* spp. and Betulaceae. Thus, apart from the curious lack of observations of *O. mixtus* using elm in the USA northeast of West Virginia, there is no evidence that this name represents more than one biological species. A comparable geographic difference in host range has been observed in the leaf-mining midge *Metriocnemus erythranthei* Namayandeh, Eiseman, van der Linden, and Palmer (Diptera: Chironomidae), which is apparently restricted to *Erythranthe* Spach (Phrymaceae) and *Veronica* L. (Plantaginaceae) (both Lamiales) across most of the USA, but is broadly polyphagous in the Pacific Northwest (Eiseman et al. 2023). The phenomenon of insect host range varying by geography was discussed at length by Fox and Morrow (1981).

One noteworthy result of this molecular analysis, however, is that the *O. pallicornis* reared from *Prunus* in Massachusetts appears to be distinct from other specimens of this species in the tree, with good support. Comparing pairwise distances between all of the included *O. pallicornis* sequences reveals that the Massachusetts specimen, despite the relatively close geographic proximity to the Canadian specimens, has an average of 9.5% sequence difference from them, as well as from the Montana specimen (total range = 8.7–10.7%). This suggests that there may be cryptic species within *O. pallicornis* as presently defined, though resolving these cryptic species is beyond the scope of this paper.

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