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A TaqMan Assay for the Detection and Monitoring of Potentially Invasive Lasiocampids, With Particular Attention to the Siberian Silk Moth, *Dendrolimus sibiricus* (Lepidoptera: Lasiocampidae)

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Abstract

The Siberian silk moth, *Dendrolimus sibiricus* Tschetverikov, is a very serious pest of conifers in Russia and is an emerging threat in North America where an accidental introduction could have devastating impacts on native forest resources. Other *Dendrolimus* Germar species and related Eurasian lasiocampids in the genus *Malacosoma* (Hubner) could also present a risk to North America's forests. Foreign vessels entering Canadian and U.S. ports are regularly inspected for *Lymantria dispar* (Linnaeus) and for the presence of other potentially invasive insects, including suspicious lasiocampid eggs. However, eggs are difficult to identify based on morphological features alone. Here, we report on the development of two TaqMan (Roche Molecular Systems, Inc., Rotkreuz, Switzerland) assays designed to assist regulatory agencies in their identification of these insects. Developed using the barcode region of the *cytochrome c oxidase I* (COI) gene and run in triplex format, the first assay can detect *Dendrolimus* and *Malacosoma* DNA, and can distinguish North American from Eurasian *Malacosoma* species. The second assay is based on markers identified within the internal transcribed spacer 2 (ITS2) region and was designed to specifically identify *D. sibiricus*, while discriminating closely related *Dendrolimus* taxa. In addition to providing direct species identification in the context of its use in North America, the *D. sibiricus* assay should prove useful for monitoring the spread of this pest in Eurasia, where its range overlaps with those of the morphologically identical *D. superans* (Butler) and similar *D. pini* (Linnaeus). The assays described here can be performed either in the lab on a benchtop instrument, or on-site using a portable machine.

Key words: *Dendrolimus*, *Malacosoma*, Lasiocampidae, molecular identification, TaqMan

Forest invasive alien species pose a major risk to ecosystem stability and can have substantial economic and social impacts. For example, since its accidental introduction in Massachusetts in the late 1860's, the spongy moth, *Lymantria dispar* (Linnaeus) (previously known as the gypsy moth), has caused billions of dollars in losses for the forest industry and urban communities (Bradshaw et al. 2016). As a result, North American plant protection regulatory agencies invest considerable resources in monitoring alien pests whose biological attributes suggest they could easily become established on this continent and cause significant disruption. The family Lasiocampidae features several Eurasian species that pose an invasive threat to North American forest resources, and prominent on that list is the Siberian silk moth, *Dendrolimus sibiricus* Tschetverikov (Hardin and Suazo 2012). In its natural range, which covers the eastern portion of European Russia, all of Asian Russia in moderate latitudes, and parts of Kazakhstan, Mongolia, China, and the Korean peninsula, this species is considered the most serious pest of conifers, where it can develop on the majority of native coniferous species (EPPO 2005). Because the potential for *D. sibiricus* to develop on Pinaceae species not found in its native range is considered high (Kirichenko

et al. 2006, Hardin and Suazo 2012), the North American boreal and neighboring coniferous forests are likely to offer both adequate host species and suitable climatic conditions for the Siberian silk moth to thrive.

The Siberian silk moth is an irruptive species whose populations, in different parts of its range, reach outbreak levels every 10–16 yr (Soulchovolsky et al. 2020), typically covering areas of thousands of hectares and often causing extensive tree mortality (EPPO 2005, Averensky et al. 2010). For example, the nine outbreaks reported during the 20th century in Central Siberia have covered ~10 million ha cumulatively (Baranchikov and Kondakov 1997). In addition to the physical damage they cause, *D. sibiricus* outbreaks promote wildfires that end up destroying vast areas of forest ecosystem and endangering nearby human communities (Kharuk and Antamoshkina 2017). *Dendrolimus sibiricus* typically completes its life cycle in two years, with a flight period occurring between late May/early June and the first half of August (Rozhkov 1963). Eggs are laid on the foliage of host trees (pines, spruces, larches, and firs), and early instars feed on the needles for a period 3–4 wk. In September, newly molted third instars descend to the ground to overwinter under moss. They resume feeding on foliage in the following spring and continue feeding and molting throughout the summer, but return to the ground in the fall for a second overwintering period. It is in the following spring that the most important defoliation occurs, just before larvae undergo pupation (EPPO 2005).

Dendrolimus Germar is a Palearctic genus comprising ~15 species (Jeong et al. 2018), some of which have a controversial taxonomic status (Kononov et al. 2016). The close relative of *D. sibiricus*, *D. superans* (Buttler) (Sakhalin silk moth), as well as the similar *D. pini* (Linnaeus) (pine-tree lappet), are also of biosecurity concern to North America (Hardin and Suazo 2012); both are Eurasian conifer feeders (but displaying different host preferences) with a biology similar to that of *D. sibiricus* and each species' range overlaps with that of *sibiricus* (Fig. 1). Attesting to the high degree of similarity between *D. sibiricus* and *D. superans*, the former is still considered a subspecies of the latter (*D. superans sibiricus*) by many specialists of the genus *Dendrolimus* (e.g., Zolotuhin 2015). Not only are adult moths of these two taxa very similar morphologically, but males can respond to the same pheromone lure. Nonetheless, some authors contend that they are distinct species based on both molecular and morphological evidence (Mikkola and Ståhls 2008, Kononov et al. 2016, Jeong et al. 2018). Recognizing that this taxonomic issue has not been settled yet, we here treat these two entities as distinct 'taxa', for practical purposes only.

The lasiocampid genus *Malacosoma* (Hubner) also features some Eurasian species of biosecurity concern to North America. Unlike the genus *Dendrolimus*, however, it has a Holarctic distribution, with some well-known North American species (tent caterpillars) that are native to the New World and considered important forest pests in their range (*M. disstria* Hubner, *M. americanum* (Fabricius), *M. californica* (Packard); Lait and Hebert 2018). Some Old World pest species, however, could become invasive if accidentally introduced in the western hemisphere. Such is the case, for example, of the European tent caterpillar, *M. neustria* (Linnaeus), which feeds on a wide range of hosts and whose eggs are very similar to those of North American *Malacosoma* species (Kimoto and Duthie-Holt 2006).

The most likely pathway of introduction for the above lasiocampid pests is by plant material infested with eggs, larvae, or pupae, which could easily survive transport from Europe or Asia to North America. Overwintering larvae present in the soil of host plants must also be considered (Hardin and Suazo 2012).

To support North American plant regulatory agencies' mandate to detect and monitor the Siberian silk moth and allied pests, we designed a rapid qPCR-based TaqMan (Roche Molecular Systems, Inc., Rotkreuz, Switzerland) assay capable of detecting *Dendrolimus* or *Malacosoma* species using DNA extracted from intercepted specimens. This COI-based triplex assay will also enable

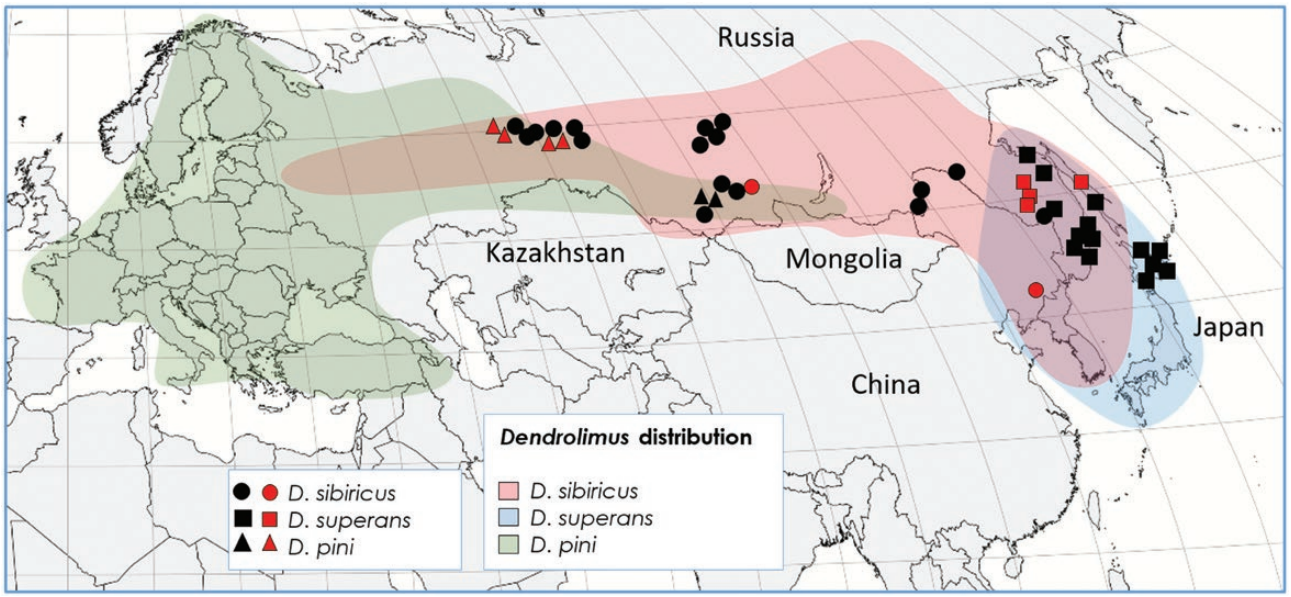

Fig. 1. Distribution of *Dendrolimus sibiricus, D. superans* and *D. pini*. Known ranges of *Dendrolimus sibiricus* (central shaded area), *D. superans* (right-most shaded area), and *D. pini* (left-most shaded area) based on data from Zolotuhin (2015), Kononov et al. (2016), and the EPPO Global database (https://gd.eppo.int/). Symbols (circles, squares, and triangles) show the approximate positions of samples used to generate Table 4, plus some older specimens whose sample location was known (see Supp File S1, tab 6 [online only] for details). Black symbols (dark-colored) denote specimens that had been correctly identified using morphological criteria, as subsequently verified through ITS2 amplicon sequencing; conversely, red symbols (light-colored) denote specimens that were initially misidentified using morphological criteria. Misidentified specimens were collected in zones of range overlap and visually mistaken for the species with which they share the range. Background map from MapChart (https://mapchart.net/).

discrimination between Eurasian and North American *Malacosoma* species. Finally, we describe a second, ITS2-based assay that can verify whether a sample flagged as *Dendrolimus* in the above assay belongs to the species *sibiricus*. Design of the assays presented here is based on the 'molecular key' approach that we developed in earlier studies (Stewart et al. 2016, 2018; Nisole et al. 2020), which has been shown to provide sensitive and accurate taxon identification.

Materials and Methods

Sources of Biological Material and Collection of Data From Databases

As a first step towards developing our own marker database, we retrieved relevant COI and ITS2 sequences from GenBank and BOLD. For taxa where either no or too few sequences were available, markers were PCR-amplified and sequenced (see details below) using biological material obtained from different sources (see Supp File S1, tabs 3 and 4 [online only]). Specimen identification was validated through COI and ITS2 amplification and sequencing. DNA from sequence-validated specimens was used for the validation of TaqMan assays. The sequences we gleaned from public databases, along with their descriptors, and sequences we generated ourselves are provided in Supp File S1, tabs 1–4 (online only).

Phylogenetic Tree Construction

COI and ITS2 sequences were aligned using MUSCLE (Edgar 2004a,b). Phylogenetic trees were constructed using the Maximum Likelihood method with the General Time Reversible model for COI and the Jukes-Cantor model for ITS2, and 500 bootstrap replicates. Analyses were conducted in MEGA X (Kumar et al. 2018).

gBlocks Gene Fragments

For taxa where biological material was not available, gBlocks gene fragments were designed and synthesized using sequences available from GenBank and BOLD (Integrated DNA Technologies, Coralville, IA). The gBlocks gene fragments (125–3000 bp dsDNA fragments) were used for TaqMan assay validation. While the *Malacosoma*, *Tolype*, and *Trabala* gBlocks fragments comprised the 658 bp COI-5′ barcode sequence of each taxon, the *Dendrolimus* fragments were designed as a 1,000 bp concatemer comprising the COI 5' barcode and ITS2, allowing them to be used for the validation of both COI- and ITS2-based assays. gBlocks sequences are provided in Supp File S1, tab 5 (online only).

DNA Extraction

For DNA extraction, we used two different approaches: (1) a standard column-based extraction protocol during assay development and (2) a rapid procedure to generate a crude extract suitable for on-site use with a portable PCR instrument.

For assay development, DNA was extracted from *Dendrolimus* and *Malacosoma* moth legs (immature stages were not available for this work) using the DNeasy Blood and Tissue Kit (Qiagen, cat. No. 69504) and a modified supplementary protocol ('Purification of total DNA from insects using the DNeasy Blood and Tissue Kit'). Two moth legs were placed in a 2-ml Eppendorf Safe-Lock tube with a 5 mm stainless steel bead. A volume of 180 µl PBS (pH 7.2) was added to the tube, and the sample was homogenized at 25 Hz for 60 s using a Qiagen Tissuelyser. This step was repeated if the sample was not completely homogenized after the first 60 s. After homogenization, we followed the manufacturer's instructions for the purification of total DNA from insects. The sample was eluted in a volume of 200 µl buffer AE.

For rapid DNA extraction, we used the ARCIS DNA sample preparation kit (ARCIS Biotechnology, cat. no. UFL002; approximate cost: US$10/sample), which can be used to process either eggs or moth legs. For the latter, two legs were transferred to a 1.5 ml tube (micro tube homogenizer system, Fisher Scientific, cat. no. 03-421-227) and homogenized in 100 µl of Reagent 1 using the kit's micro pestle (note: perfect fit of the tube/micro pestle combination is necessary to ensure proper homogenization of the sample). After a 1-min incubation at room temperature, 20 µl of the homogenate was transferred to a separate 1.5 ml tube containing 20 µl of Reagent 2 (1:1 ratio mixture of the two reagents). The tube was flicked to mix the sample after which the mixture was diluted 20× with 760 µl H_2O for use in qPCR. DNA from egg masses can be extracted using the same method; eggs are simply crushed with the micro pestle to release their nucleic acids.

Marker Amplification and Sequencing

To sequence the 5' region of the COI gene, we used a set of universal COI primers, modified from Folmer et al. (1994). For the ITS2 region, we used the primers of Jeong et al. (2018). The primer sequences can be found in Table 1. DNA (2 µl; ~1–2 ng, in a final volume of 20 µl containing 500 nM primers) was amplified on an Applied Biosystems 7500 Fast real-time PCR machine using the Qiagen Quantitect SYBR Green PCR Kit (Qiagen cat. no. 204145). PCR conditions were as follows: an initial denaturation step at 95°C for 15 min and 45 cycles of 95°C for 15 s, 49°C for 30 s, and 65°C for 90 s. PCR products were visualized using the Applied Biosystems 7500 software (version 2.06) and were used for direct Sanger sequencing; none of the chromatograms yielded double peaks.

SYBR Green-Based qPCR Quantification of DNA

DNA concentration of all samples (biological material and synthesized gBlocks gene fragments) was standardized by qPCR quantification using the lasiocampid general primers (Table 1). Standardization of DNA quantities before running the TaqMan assays allowed us to confirm that DNA was present at a high enough concentration to ensure discrimination of all closely related species in the assays. Using the LRE method of Rutledge (2011), DNA quantity was standardized to ~5000 COI copies, which corresponds to a Ct value of 20. Lasiocampid general primers were designed in a conserved region of the 5' end of the COI gene (Supp File S1 [online only]) and generated a 139 bp amplicon. qPCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR machine (Thermofisher, Waltham MA). 96-well MicropAmp Fast Optical real-time PCR plates were used with MicroAmp Optical Adhesive Film (Thermofisher, Waltham, MA). The 10 µl amplification reactions contained 2 µl DNA and 500 nM of primers. PCR conditions were as follows: a 15 min initial denaturation step at 95°C for enzyme activation, followed by 45 cycles of 95°C for 15 s, 50°C for 30 s, and 65°C for 90 s. PCR was performed using the Qiagen Quantitect SYBR Green PCR Kit (Qiagen, Venlo, Netherlands).

Design of Primers and Probes for the TaqMan Assays

Primers were designed using the Oligo Explorer program (GeneLink, Orlando, FL). Locked nucleic acid (LNA) probes were designed using the IDT Biophysics design tool (available through IDT). LNA probes were used to increase sensitivity and specificity of the assays. We also used an 'Amplification Refractory Mutation

Table 1. Primers and probes used for the detection of *Dendrolimus* and *Malacasoma*. DNA sequencing and DNA quantification primers are also included in this table

Primer/probe use	Primer/probe name	Primer/probe sequence	Amplicon size (bp)
DNA sequencing	LCO1490F mod	AYCAACAAATCATAAAGATATYGG	658
	HCO2198R mod	TAAACTTCAGGRTGMCCA	
DNA sequencing	ITS2A	TGTGAACTGCAGGACACAT	
	ITS2B	TATGCTTAAATTCAGGGGGT	607
DNA quantification	Las Gen COI F60-81[a]	TAATTCGIGCWGAATTAGGIAC	139
	Las Gen COI R173-99[a]	TAATCAATTWCCAAAYCCICCAATTAT	
DNA quantification	Dend ITS2 GEN F220-35[b]	CGCTCGCGGTCCCTCG	197
	Dend ITS2 GEN R399-416[b]	CGACGCGCCTCTTCGGAC	
Detection of *Dendrolimus*	Dend COI F429-54	TAGGIGCAATTAAYTTTATTACAACA	195
	Dend COI R604-24	CCAGCAGGRTCAAARAATGAT	
	Dend COI T555-69 (FAM-LNA)[d]	T+TGCTCCRGC+AA+G+AA[e]	
Detection of *Dendrolimus sibiricus*	DS ITS2 F102-27	GATCGTCCGTTTAAATATAAAAAATA	257
	DS ITS2 2A/T R339-58[f]	TCGAACAGTAYTAGTCTCTT	
	DS ITS2 R339-58[g]	TCGAACAGTAYTAGTCTCAT	
	DS ITS2 T293-304 (Cy5-LNA)	CGGC+C+A+CAGAAC	
Detection of *Malacasoma*	Malc COI F247-71	TATAAGATTTTGATTATTACCYCCA	95
	Malc COI R322-41	GRTAAAYTGTTCATCCTGTA	
	Malc COI T296-314 (Tex 615-LNA)	A+G+T+AGAA+T+A+GTAGAAAATG	
Detection of Eur. *Malacasoma*	Malc COI F247-71	TATAAGATTTTGATTATTACCYCCA	95
	Malc COI R322-41	GRTAAAYTGTTCATCCTGTA	
	Malc Eur COI A/G T312-21 (Cy5-LNA)	C+C+A+GC+T+CCGT[c]	

[a]These primers will amplify *Dendrolimus*, *Malacasoma*, *Tolype* and *Trabala*. They are used to quantify DNA samples for the COI-based TaqMan assays.
[b]These primers will amplify *Dendrolimus*. They are used to quantify DNA samples for use in the *Dendrolimus sibiricus* ITS2 TaqMan assay.
[c]Sequences containing an underlined character are ARMS bases, where the underlined letter represents the introduced mismatch.
[d]In brackets: probe type.
[e]In probe sequences, bases preceded by a '+' sign are LNA bases.
[f]This reverse ARMS primer is used with the SensiFast Probe mastermix.
[g]This reverse primer is used with the SensiFast Lyo-Ready mastermix.

System' (ARMS) base in the design of a primer (DS ITS2 R339-58) and an LNA probe (*Malacosoma* Eurasian COI assay) to increase the assay's discrimination power. For more information on LNA probe and ARMS design, see Stewart et al. (2016, 2018) and Nisole et al. (2020). Primer and probe sequences are provided in Table 1.

TaqMan Conditions

During development, TaqMan assays were tested in both simplex and triplex formats on the 7500 Fast Real-Time PCR System (Thermofisher, Waltham, MA), using the Sensifast probe No-Rox 2X Mastermix (Meridian Biosciences, Cincinnati, OH, USA). Final primer and probe concentrations were set at 500 nM and 100 nM, respectively, in a 10 µl reaction volume.

For the TaqMan COI-based triplex assay and the *Dendrolimus sibiricus* ITS2 assay, all probes were Affinity Plus LNA probes (Integrated DNA Technologies, Coralville, IA). The *Dendrolimus* COI probe had a fluorescein (6-FAM) label at the 5' end and an Iowa Black FQ quencher at the 3' end. The *Malacosoma* COI probe had a TEX 615 label at the 5' end and an Iowa Black RQ quencher at the 3' end, while the Eurasian *Malacosoma* COI probe had a Cy5 label at the 5' end and an Iowa Black RQ quencher at the 3' end. The *Dendrolimus sibiricus* ITS2 probe had a Cy5 label at the 5' end and an Iowa Black RQ quencher at the 3' end. 2 µl of column-extracted DNA, equivalent to approximately 5,000 COI copies, was used for each reaction. PCR conditions for the TaqMan reactions were as follows: a 5-min initial denaturation step at 95°C for enzyme activation, followed by 45 cycles of 95°C for 10 s and 60°C for 45 s. Two technical replicates were run for each sample.

For the portable assay, amplifications were tested in triplex format on the Franklin portable real-time PCR thermocycler (Biomeme, Philadelphia, PA) using the same conditions as above, except for the final volume (20 µl instead of 10 µl). Reactions were run in 100 µl MicroAmp Fast 8-tube strips (Thermofisher, Waltham, MA). A single drop of mineral oil was added to the reaction to prevent evaporation of the sample. A field-ready master mix was also prepared for use on the Franklin. The Lyo-Ready™ qPCR Mix (Meridian Bioscience, Cincinnati, OH, USA) was used to prepare the master mix. This is a glycerol-free formulation that contains lyo-excipients. The master mix was lyophilized using the protocol described in Capron et al. (2020). Briefly, for each reaction, 1 µl of 20× primer/probe mix for each assay (10 mM primers and 2 mM probe) was added to 10 µl of Lyo-Ready qPCR 2× Master mix. The reaction mix was aliquoted to 100 µl MicroAmp Fast 8-tube strips (Thermofisher, Waltham, MA), frozen at –20°C and then lyophilized for 90 min in a Freezone 2.5 liter freeze dryer (Labconco, Kansas City, MO). After lyophilization, the strips were stored at room temperature in sealable foil pouches, with each pouch containing a desiccation packet. To test the TaqMan assays, the lyophilized reagents were resuspended in a final volume of 20 µl. For reactions run with column-extracted DNA, 2 µl of DNA was diluted in 18 µl H_2O. For reactions run with DNA prepared from the rapid extraction method, we used 20 µl of DNA.

Results

Assay Development

Design of the *Dendrolimus*/*Malacosoma* triplex assay presented here (Fig. 2) was based on the observation that the COI barcode

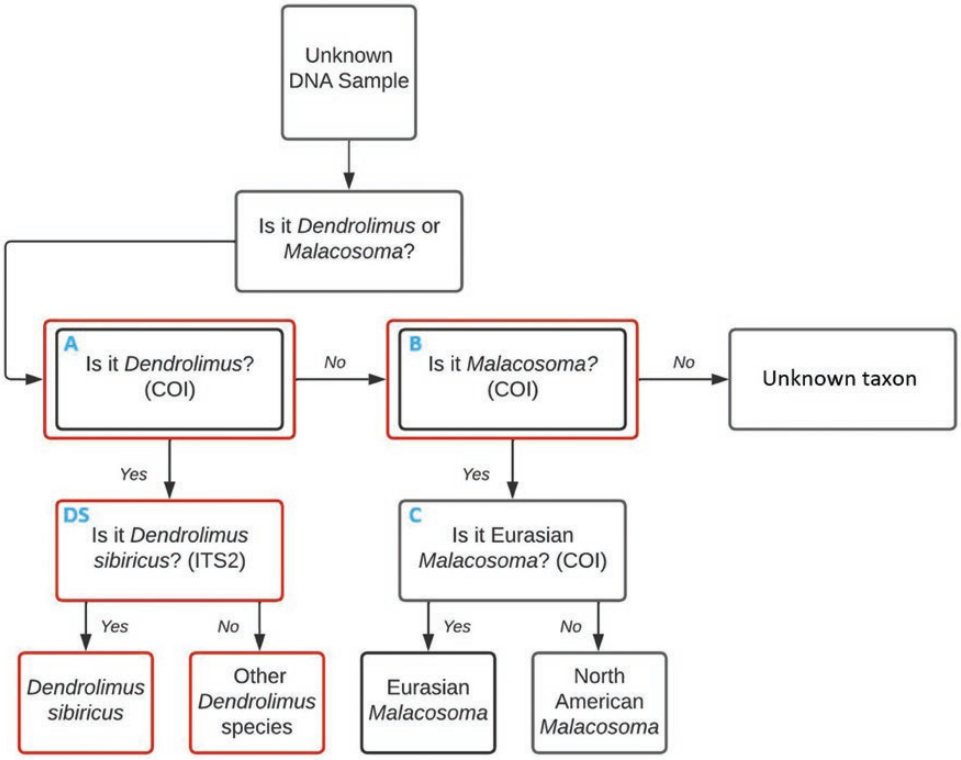

Fig. 2. Flowchart for the *Dendrolimus/Malacosoma* COI triplex assay (A, B, C) and alternative COI/ITS2 triplex assay (A, B, DS). DNA is extracted from an unknown egg sample, diluted, and passed through either the triplex assay (black [A, B, C] boxes) or the alternative triplex assay (red [A, B, DS] boxes). Sub-assays A and B are common to the two assays. They will identify *Dendrolimus* (A) and *Malacosoma* (B) species. For all samples that test positive for the *Malacosoma* sub-assay B, sub-assay C will determine if the sample is of North American or Eurasian origin. Sub-assay DS from the alternative triplex assay will identify *Dendrolimus sibiricus* from all positive *Dendrolimus* results (sub-assay A).

provides resolution between the genera *Dendrolimus* and *Malacosoma*, as well as between Eurasian and North American *Malacosoma* species, as illustrated by the ML tree shown in Fig. 3A. However, as reported earlier (Kononov et al. 2016), the *Dendrolimus* COI barcode provides inadequate resolution among the *sibiricus, superans* and *pini* taxa (Fig. 3A), whereas ITS2 resolves them well (Fig. 3B). For this reason, we chose the ITS2 marker to design a TaqMan assay aimed at detecting *D. sibiricus* specifically (Fig. 2). A close examination of the tree presented in Fig. 3A shows that bootstrap support for the Eurasian versus North American *Malacosoma* dichotomy was low (35), pointing to a limited number of substitutions available for discriminating between these two groups. This, however, proved not to be an obstacle to assay development (see below).

As a first step towards designing the above assays, we generated multiple alignments of marker sequences (i.e., COI or ITS2) obtained from geographically relevant species, including both the target species (i.e., species meant to produce a positive amplification) and those meant to be discriminated against (i.e., species that should *not* yield a positive amplification). An example of such an alignment is provided in Fig. 4, where the aim was to design primers and a probe that would successfully amplify and detect COI DNA from any *Malacosoma* species (Fig. 2: Triplex B sub-assay) while discriminating against *Dendrolimus, Tolype* and *Trabala* species (which this assay successfully did; see validation results below). Alignments used to develop the other assays presented in this paper are provided in Supp File S2 (online only), along with examples of amplification curves.

Description of Assays

Dendrolimus/Malacosoma COI Triplex Assay

The *Dendrolimus* COI sub-assay (A, Fig. 2) was designed to determine whether an unknown sample contains *Dendrolimus* DNA; it will amplify DNA from any *Dendrolimus* species while discriminating against *Malacosoma*, *Tolype*, and *Trabala* species. Similarly, the *Malacosoma* COI sub-assay (B, Fig. 2) was designed to determine if an unidentified specimen is a member of the genus *Malacosoma*; it will amplify DNA from all *Malacosoma* species while discriminating against *Dendrolimus*, *Tolype*, and *Trabala* species. Finally, the Eurasian *Malacosoma* COI sub-assay (C; Fig. 2) was designed to flag members of the genus *Malacosoma* that are found in Eurasia while being absent in North America, thus potentially posing an invasive threat in North America. Here, discrimination is provided by a single substitution targeted by the probe (see Supp File S2 [online only]). For this assay, a positive result will be considered reliable only if the sample also yielded a positive result for the *Malacosoma* COI sub-assay (B).

Dendrolimus sibiricus ITS2 'DS' Assay

The DS assay was originally designed to provide direct identification of *D. sibiricus* when the above triplex assay indicates *Dendrolimus* DNA is present in a sample. It will amplify *D. sibiricus* DNA while discriminating against closely related *Dendrolimus* species/subspecies, including *superans* and *pini*. The DS assay can also be used as part of an alternative triplex assay in place of the Eurasian *Malacosoma* COI sub-assay (see 'A, B, DS' in Fig. 2). Whether used alone or as part of a triplex assay, the DS assay should prove useful to monitor the spread of *D. sibiricus*

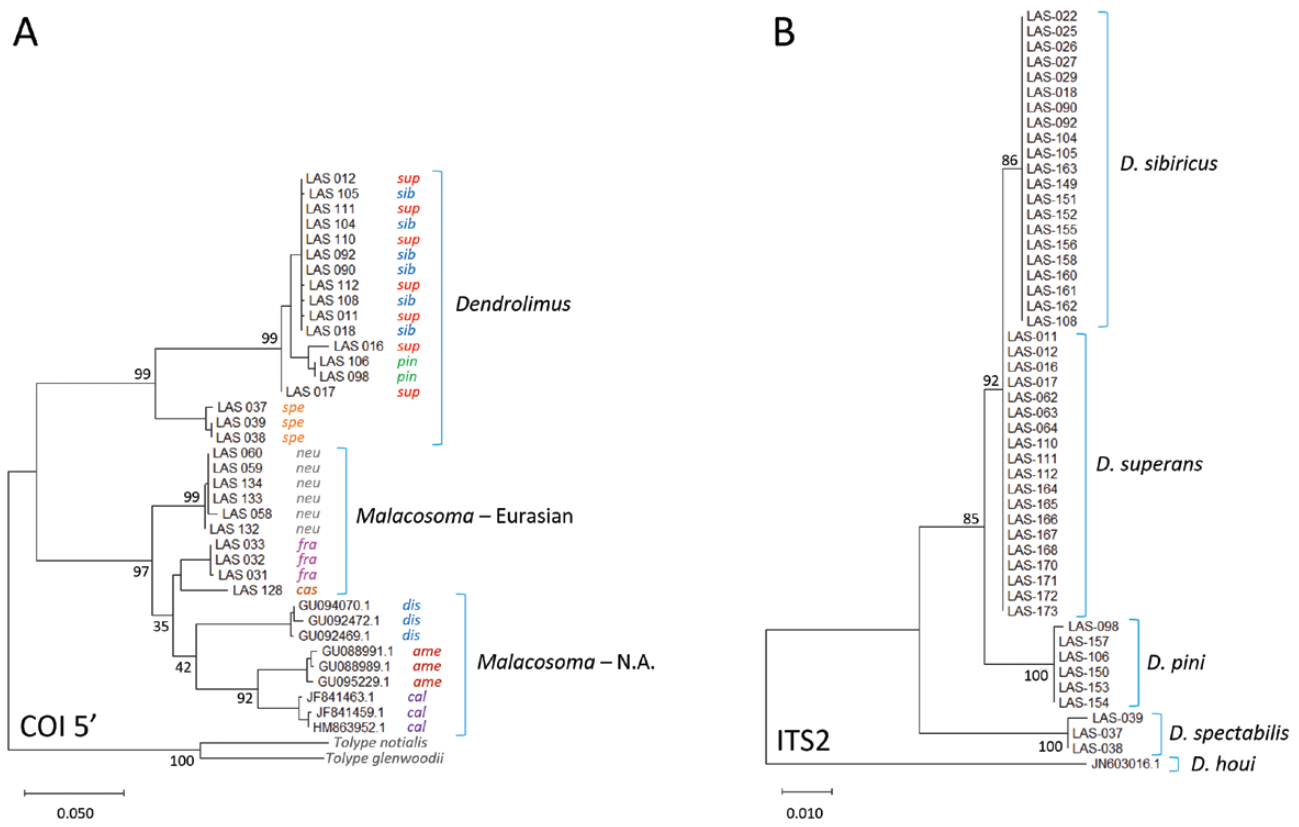

Fig. 3. Maximum likelihood phylogenetic trees for *Dendrolimus* and *Malacosoma* species considered in the design of the triplex assays presented here. (A) Tree constructed using COI 5′ sequences. Colored three-letter species abbreviations: *sib = sibiricus; pin = pini; sup = superans; spe = spectabilis; neu = neustria; fra = franconica; cas = castrensis; dis = distria; ame = americanum; cal = californicum. Tolype* species were used as outgroups (selection based on Regier et al. 2000). **(B)** Tree constructed using ITS2 sequences. *Dendrolimus houi* was used as outgroup (selection based on Kononov et al. 2016). 'LAS' labels refer to sample names (see Supp File S1 [online only] for details) while other labels are GenBank accession numbers (North American *Malacosoma* species and *Dendrolimus* outgroup).

in Europe, Siberia, and the Far East. Since *D. sibiricus* and *D. superans* are still considered by some taxonomists to form a single species (see Introduction), we also developed a variant of the DS assay that will produce a positive amplification in the presence of DNA from either taxon while discriminating against other *Dendrolimus* species (Files S4).

Validation of Assay Specificity

The Triplex assay (A, B, C) and the alternative 'A, B, DS' assay were initially tested on our Applied Biosystems 7500 Fast Real-Time PCR instrument using column-extracted DNA or gBlocks gene fragments and the SensiFast Probe No-Rox mastermix. A total of 44 specimens and 17 gBlocks gene fragments were tested in both simplex and triplex formats. The results can be seen in Table 2 and Figs. 5A and 6A, as well as in the first tab of Supp File S3 (online only). All identifications provided by the molecular assays matched those made by taxonomists, except for six *Dendrolimus* specimens originally identified as belonging to *pini*, *sibiricus*, or *superans*, but for which the DS assay results did not match these provisional identifications (LAS-018, -094, -108, -110, -111, -112; see Supp File S1, tab 4 [online only]). However, the DS assay results were subsequently deemed accurate, based on an analysis of the ITS2 sequence data for the six specimens.

A subsample of 22 specimens/gBlocks gene fragments was used to assess the specificity of the Triplex assay on a Biomeme Franklin portable real-time PCR thermocycler, using the SensiFast Probe No-Rox mastermix and the lyophilized SensiFast Lyo-ready No-Rox mastermix. All assays produced results identical to those obtained with the Applied Biosystems 7500 Fast PCR machine (Table 3; Fig. 5B; Supp File S3, tabs 2 and 3 [online only]). The alternative triplex assay was also validated on the Biomeme Franklin using a subsample of 12 *Dendrolimus* specimens/gBlocks gene fragments. All molecular assays produced the expected results (Fig. 6B; Supp File S3, tabs 2 and 3 [online only]).

Proof of Concept Using Freshly Collected Russian *Dendrolimus* Specimens

To assess the robustness of the modified triplex assay (A, B, DS; Fig. 2), DNA was extracted from the legs of 24 *Dendrolimus* moths provisionally identified as *sibiricus* or *superans* and collected in 2021 in Siberia and the Russian Far East (Supp File S1, Tab 4 [online only]; LAS149–LAS173). Two legs were collected from each specimen. One leg was used for column extraction of DNA while the other was used for rapid DNA extraction. As an example of a bench-top analysis, column-extracted DNA was run on the 7500 Fast Real-Time PCR instrument, using the SensiFast Probe No-Rox mastermix, while for a simulation of an on-site analysis, DNA from the rapid extraction method was run on a Biomeme Franklin portable real-time PCR thermocycler, using lyophilized strips of the Sensifast Lyo-Ready No-Rox mastermix (including assay primers and probes). Both the laboratory and field-based simulations correctly identified the specimens (Table 4), except for four *D. sibiricus* specimens (LAS-150, -153, -154, -157; Supp File S1, tab 4 [online only]) for which

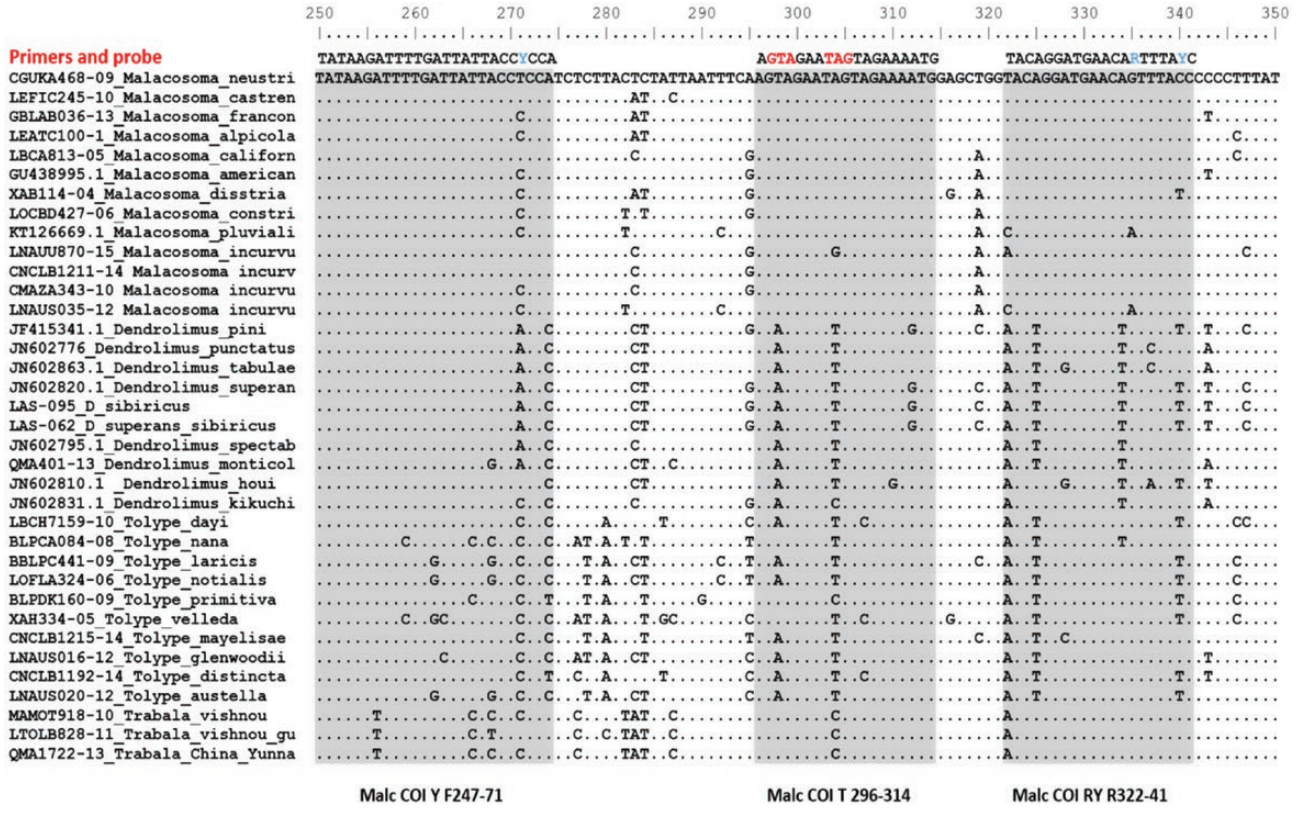

Fig. 4. Sequence alignment for the *Malacosoma* COI TaqMan sub-assay. This multiple sequence alignment shows how the *Malacosoma* COI sub-assay was designed to amplify DNA from both North American and Eurasian *Malacosoma* specimens while discriminating *Dendrolimus, Tolype*, and *Trabala* species. The *Malacosoma* and Eurasian *Malacosoma* COI sub-assays (see Fig. 1) share the same primer pair, but use different probes; the probes have a 3 bp overlap, but are on different DNA strands, thus preventing cross-detection. LNA bases (probe) and degenerate bases (primers) are shown in contrasting colors. Alignments used for the development of the other sub-assays are provided in Supp File S2 (online only).

the DS assay output did not agree with the taxonomic identifications provided to us. However, results of the DS assay later proved to be correct, as an analysis of the ITS2 sequence data for the four contentious specimens indicated that they were *D. pini* and not *D. sibiricus* (Supp File S3, tab 4 [online only]; see also Fig. 1).

Discussion

The *Dendrolimus/Malacosoma* COI triplex assay described here was designed as a tool for the early detection of potentially invasive lasiocampids whose immature stages, especially eggs, are difficult to identify using morphological characteristics. The assay can be used either on a bench-top instrument or on-site using a portable qPCR thermocycler. While this assay does not provide identification at the species level, it can accurately flag any intercepted *Dendrolimus* or Eurasian *Malacosoma* specimen, two groups that feature species that are of biosecurity concern to North America. In cases where the suspicious specimen is identified as *Dendrolimus* sp. by this assay, analysis of its DNA using the ITS2-based DS assay can establish whether it belongs to the *sibiricus* taxon. If operationally desirable, species-level identification of other *Dendrolimus* and *Malacosoma* specimens can be obtained through sequencing of their ITS2 or COI amplicons, respectively, followed by sequence comparisons against public databases or against the sequence collection provided here in Supp File S1 (online only).

All assays reported here were initially validated on a bench-top qPCR instrument, in both simplex and triplex formats, using either column-extracted DNA or gBlocks gene fragments. Assays run in both simplex and triplex formats yielded correct identification of all specimens/gBlocks tested and produced similar Ct values (Table 2; Supp File S3 [online only]). Although the *Dendrolimus* COI sub-assay typically produced a curve with a baseline drift, this phenomenon did not affect our ability to ascertain a positive amplification. In the case of the alternative triplex assay, where the DS sub-assay substitutes for the Eurasian *Malacosoma* sub-assay, we observed some interaction between the *Dendrolimus* COI and *D. sibiricus* ITS2 sub-assays, resulting in a reduction of the F_{max} value for the latter sub-assay. This, however, had no impact on the conclusiveness of the assay.

Validation tests of the field-ready versions of the assays were initially conducted on the Biomeme Franklin portable qPCR machine using column-extracted DNA and the same mastermix used for analyses conducted on the bench-top instrument. This approach enabled a direct comparison of results obtained with the two instruments under similar analytical conditions. The lyophilized mastermix (i.e., the one meant to be used with the portable machine) was subsequently tested using both column-extracted DNA and DNA extracted from moth legs using the rapid extraction method (i.e., the method meant to be used on-site). Whereas results generated by the portable machine for the *Dendrolimus/Malacosoma* COI triplex assay were similar to those obtained using the bench-top instrument for all DNA/mastermix combinations tested, use of the lyophilized mastermix in the alternative triplex assay resulted in a substantially lower F_{max} value for the *D. sibiricus* ITS2 sub-assay, combined with a Ct value about three cycles later than that observed on the bench-top instrument, a phenomenon that was not observed with

Table 2. Specificity validation results for *Dendrolimus/Malacosoma* COI and alternative COI/ITS2 assays. Results obtained using column-extracted DNA of known species. Samples were run on an Applied Biosystems Real-Time PCR system, using the SensiFAST probe No-Rox 2X master mix. Both simplex and triplex results, including Ct values, are provided in Supp File S3 (online only)

			Triplex Assay			Alternative Triplex Assay		
Specimen ID	Species	Expected positive result	*Dendrolimus* COI (A)	*Malacosoma* COI (B)	Eur. *Malacosoma* COI (C)	*Dendrolimus* COI (A)	*Malacosoma* COI (B)	*D. sibiricus* ITS2 (DS)
LAS-098	*Dendrolimus pini*	A	+	–	n/a	+	–	–
LAS-099	*D. pini*	A	+	–	n/a	+	–	–
LAS-106	*D. pini*	A	+	–	n/a	+	–	–
LAS-117	*D. pini*	A	+	–	n/a	+	–	–
LAS-118	*D. pini*	A	+	–	n/a	+	–	–
LAS-119	*D. pini*	A	+	–	n/a	+	–	–
LAS-037	*D. spectabilis*	A	+	–	n/a	+	–	–
LAS-038	*D. spectabilis*	A	+	–	n/a	+	–	–
LAS-039	*D. spectabilis*	A	+	–	n/a	+	–	–
LAS-011	*D. superans*	A	+	–	n/a	+	–	–
LAS-012	*D. superans*	A	+	–	n/a	+	–	–
LAS-016	*D. superans*	A	+	–	n/a	+	–	–
LAS-017	*D. superans*	A	+	–	n/a	+	–	–
LAS-019	*D. superans*	A	+	–	n/a	+	–	–
LAS-062	*D. superans*	A	+	–	n/a	+	–	–
LAS-063	*D. superans*	A	+	–	n/a	+	–	–
LAS-064	*D. superans*	A	+	–	n/a	+	–	–
LAS-094	*D. superans*	A	+	–	n/a	+	–	–
LAS-110	*D. superans*	A	+	–	n/a	+	–	–
LAS-111	*D. superans*	A	+	–	n/a	+	–	–
LAS-112	*D. superans*	A	+	–	n/a	+	–	–
LAS-018	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-022	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-025	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-026	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-027	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-029	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-090	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-092	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-103	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-104	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-105	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
LAS-108	*D. sibiricus*	A;DS	+	–	n/a	+	–	+
gblk-Dhoui	*D. houi*	A	+	–	n/a	+	–	–
gblk-Dkik	*D. kikuchii*	A	+	–	n/a	+	–	–
gblk-Dpunc	*D. punctatus*	A	+	–	n/a	+	–	–
gblk-Dtab	*D. tabulaeformis*	A	+	–	n/a	+	–	–
LAS-142-2	*Malacosoma alpicola*	B;C	–	+	+	–	+	n/a
LAS-143-2	*M. alpicola*	B;C	–	+	+	–	+	n/a
LAS-129-2	*M. castrensis*	B;C	–	+	+	–	+	n/a
LAS-031-2	*M. franconica*	B;C	–	+	+	–	+	n/a
LAS-032-2	*M. franconica*	B;C	–	+	+	–	+	n/a
LAS-033-2	*M. franconica*	B;C	–	+	+	–	+	n/a
LAS-058-2	*M. neustria*	B;C	–	+	+	–	+	n/a
LAS-059-2	*M. neustria*	B;C	–	+	+	–	+	n/a
LAS-060-2	*M. neustria*	B;C	–	+	+	–	+	n/a
LAS-132	*M. neustria*	B;C	–	+	+	–	+	n/a
LAS-133	*M. neustria*	B;C	–	+	+	–	+	n/a
LAS-134	*M. neustria*	B;C	–	+	+	–	+	n/a
LAS-146	*M. parallela*	B;C	–	+	+	–	+	n/a
gblk-Malp	*M. alpicola*	B;C	–	+	+	–	+	n/a
gblk-Mcas	*M. castrensis*	B;C	–	+	+	–	+	n/a
gblk-Mfra	*M. franconica*	B;C	–	+	+	–	+	n/a
gblk-Mneu	*M. neustria*	B;C	–	+	+	–	+	n/a
gblk-Mam	*M. americanum*	B	–	+	–	–	+	n/a
gblk-Mcal	*M. californicum*	B	–	+	–	–	+	n/a
gblk-Mcon	*M. constrictum*	B	–	+	–	–	+	n/a
gblk-Mdis	*M. disstria*	B	–	+	–	–	+	n/a

Table 2. Continued

Specimen ID	Species	Expected positive result	Triplex Assay			Alternative Triplex Assay		
			Dendrolimus COI (A)	*Malacosoma* COI (B)	Eur. *Malacosoma* COI (C)	*Dendrolimus* COI (A)	*Malacosoma* COI (B)	*D. sibiricus* ITS2 (DS)
gblk-Minc	*M. incurvum*	B	–	+	–	–	+	n/a
gblk-Mplu	*M. pluvialis*	B	–	+	–	–	+	n/a
gblk-Tday	*Tolype dayi*	–	–	–	n/a	–	–	n/a
gblk-Tglen	*T. glenwoodii*	–	–	–	n/a	–	–	n/a
gblk-Tnot	*T. notialis*	–	–	–	n/a	–	–	n/a

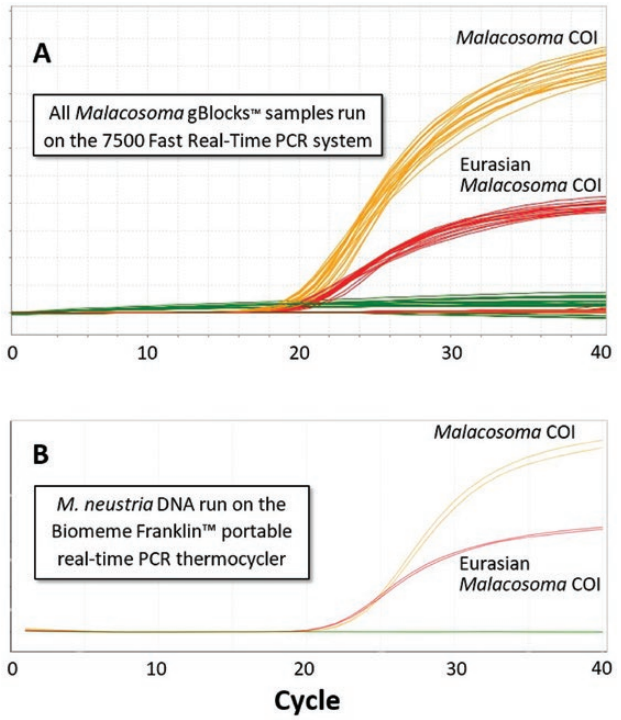

Fig. 5. Example of validation results for the *Dendrolimus*/*Malacosoma* COI triplex assay (A, B, C). Amplification curves for the *Malacosoma* COI and Eurasian *Malacosoma* COI TaqMan assays with North American and Eurasian *Malacosoma* DNA samples. The *Malacosoma* COI assay amplifies all *Malacosoma* samples (orange curve) while the Eurasian *Malacosoma* COI assay amplifies only the Eurasian *Malacosoma* samples (red curve). The green lines represent negative results for the *Dendrolimus* COI assay. **(A)** Result for all *Malacosoma* gBlocks samples listed in Table 3, run on the 7500 Fast Real-Time PCR system (SensiFAST Probe No-Rox 2X master mix). Red lines along the baseline show absence of detection of North American *Malacosoma* samples by the Eurasian *Malacosoma* sub-assay. **(B)** Result for *M. neustria* on the Biomeme Franklin portable real-time PCR thermocycler, using the field-ready mix (SensiFAST Lyo-Ready No-Rox 2X master mix).

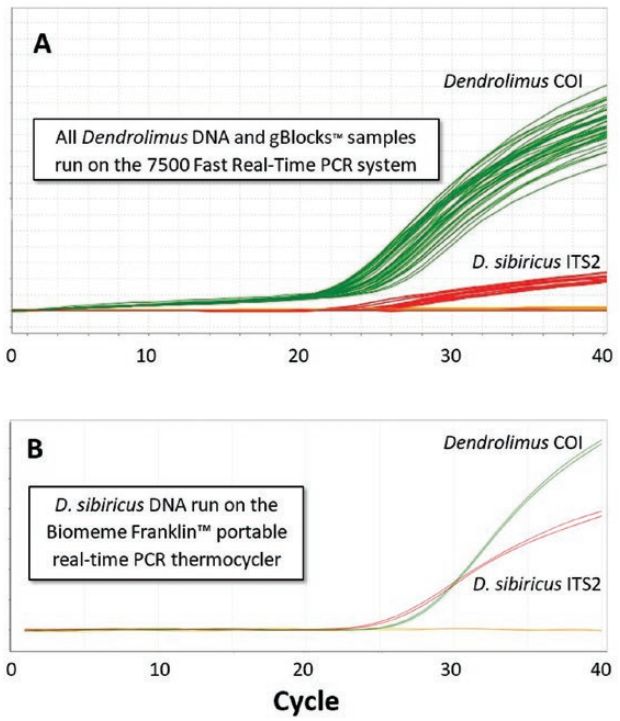

Fig. 6. Example of validation results for the alternative COI/ITS2 triplex assay (A, B, DS). Amplification curves presented for *Dendrolimus sp.* samples. **(A)** Positive amplification curves for the *Dendrolimus* COI assay (green) and the *D. sibiricus* ITS2 assay (red) on the 7500 Fast Real-Time PCR system (SensiFAST Probe No-Rox 2X master mix). While all *Dendrolimus* samples amplify with the COI assay (green curves), only the *D. sibiricus* samples amplify with the *D. sibiricus* ITS2 assay (red curves). **(B)** A triplex result for a *D. sibiricus* DNA sample run on the Biomeme Franklin portable real-time PCR thermocycler (SensiFAST Probe No-Rox 2X master mix). Both the *Dendrolimus* COI assay (green curve) and *D. sibiricus* ITS2 assay (red curve) produce a positive result, with the *Malacosoma* COI assay (orange curve) presenting a negative result.

the SensiFAST Probe No-ROX 2X mastermix. This phenomenon was not completely unexpected as different mastermixes can behave differently in the presence of a 5' SNP. To remedy this problem, the reverse ARMS primer used for the bench-top version of the assay was replaced with a more permissive non-ARMS primer featuring a higher Tm (Table 1). Although this alternative primer provided suboptimal discrimination during assay development, it worked well in combination with the lyophilized mastermix used on the portable instrument. These observations suggest that the lyophilized mastermix confers greater discriminatory power to the assay than the mastermix used for analysis on the bench-top instrument.

As a result of constraints on material availability, the assays described here were developed using moth legs as opposed to eggs, i.e., the life stage viewed as the primary target for molecular identification of intercepted lasiocampids in North America. While the accuracy of neither the bench-top nor the portable assay reported here was assessed using eggs as starting material, extensive testing of a similar assay targeting *L. dispar*, where eggs were employed for DNA extraction, showed that eggs provide good DNA yields, leading to efficient and accurate TaqMan species discrimination (Stewart et al. 2016, Stewart 2022). Based on these observations, the present assays are expected to work as well (if not better) with eggs as with moth legs.

Table 3. Specificity validation results for the field-ready COI triplex assay. Assay results using the Biomeme Franklin portable real-time PCR thermocycler. The SensiFAST Probe No-Rox 2X mastermix and the lyophilized SensiFAST Lyo-Ready No-ROX 2X mastermix were compared using column extracted DNA. Triplex results, including Ct values, are provided in Supp File S3 (online only)

			Biomeme			Biomeme Lyo Mix		
Specimen ID	Species/subspecies	Expected positive result	Dend COI (A)	Malac COI (B)	Eur Malac COI (C)	Dend COI (A)	Malac COI (B)	Eur Malac COI (C)
LAS-099	*Dendrolimus pini*	A	+	-	n/a	+	–	n/a
LAS-037	*D. spectabilis*	A	+	-	n/a	+	–	n/a
LAS-110	*D. superans*	A	+	-	n/a	+	–	n/a
LAS-104	*D. sibiricus*	A	+	-	n/a	+	–	n/a
gblk-Dhoui	*D. houi*	A	+	-	n/a	+	–	n/a
gblk-Dkik	*D. kikuchii*	A	+	-	n/a	+	–	n/a
gblk-Dpunc	*D. punctatus*	A	+	-	n/a	+	–	n/a
gblk-Dtab	*D. tabulaeformis*	A	+	-	n/a	+	–	n/a
gblk-Malp	*Malacosoma alpicola*	B;C	–	+	+	–	+	+
gblk-Mcas	*M. castrensis*	B;C	–	+	+	–	+	+
gblk-Mfra	*M. franconica*	B;C	–	+	+	–	+	+
gblk-Mneu	*M. neustria*	B;C	–	+	+	–	+	+
gblk-Mam	*M. americanum*	B	–	+	–	–	+	–
gblk-Mcal	*M. californicum*	B	–	+	–	–	+	–
gblk-Mcon	*M. constrictum*	B	–	+	–	–	+	–
gblk-Mdis	*M. disstria*	B	–	+	–	–	+	–
gblk-Minc	*M. incurvum*	B	–	+	–	–	+	–
gblk-Mplu	*M. pluvialis*	B	–	+	–	–	+	–
gblk-Tday	*Tolype dayi*	-	–	-	n/a	–	–	n/a
gblk-Tglen	*T. glenwoodii*	-	–	-	n/a	–	–	n/a
gblk-Tnot	*T. notialis*	-	–	-	n/a	–	–	n/a

Table 4. Specificity validation results for the alternative triplex assay using Russian field samples. Results of alternative triplex assay (*Dendrolimus sibiricus* ITS2 'DS' assay replacing Eurasian *Malacosoma* COI assay; see Fig. 2). DNA was extracted from specimens collected in 2021 using both column-based and rapid extraction methods. Column-extracted DNA was analyzed with the SensiFast Probe No-Rox mix on the Applied Biosystems 7500 Fast Real-Time PCR system as an example of a laboratory bench-top analysis and DNA extracted using the rapid method was analyzed with the SensiFast Lyo-Ready No-Rox mix on the Biomeme Franklin portable real-time PCR thermocycler to simulate an on-site analysis. Triplex results, including Ct values, are provided in Supp File S3 (online only)

				7500 Fast, Column-ext. DNA			Biomeme Lyo Mix, Rapid DNA ext.		
Specimen ID	Species	Sample location	Expected positive result	*Dend* COI (A)	*Malac* COI (B)	*Dend sib* ITS2 (DS)	*Dend* COI (A)	*Malac* COI (B)	*Dend sib* ITS2 (DS)
LAS 149	*D. sibiricus*	Northern Nizhnevartovsk	A;DS	+	–	+	+	–	+
LAS 150	*D. pini*	Northern Nizhnevartovsk	A	+	–	–	+	–	–
LAS 151	*D. sibiricus*	Northern Nizhnevartovsk	A;DS	+	–	+	+	–	+
LAS 152	*D. sibiricus*	Northern Nizhnevartovsk	A;DS	+	–	+	+	–	+
LAS 153	*D. pini*	Northern Nizhnevartovsk	A	+	–	–	+	–	–
LAS 154	*D. pini*	North of Khanty-Mansiysk	A	+	–	–	+	–	–
LAS 155	*D. sibiricus*	North of Khanty-Mansiysk	A;DS	+	–	+	+	–	+
LAS 156	*D. sibiricus*	North of Khanty-Mansiysk	A;DS	+	–	+	+	–	+
LAS 157	*D. pini*	North of Khanty-Mansiysk	A	+	–	–	+	–	–
LAS 158	*D. sibiricus*	North of Khanty-Mansiysk	A;DS	+	–	+	+	–	+
LAS 160	*D. sibiricus*	Yeniseysk, Krasnoyarsk kray	A;DS	+	–	+	+	–	+
LAS 161	*D. sibiricus*	Yeniseysk, Krasnoyarsk kray	A;DS	+	–	+	+	–	+
LAS 162	*D. sibiricus*	Yeniseysk, Krasnoyarsk kray	A;DS	+	–	+	+	–	+
LAS 163	*D. sibiricus*	Yeniseysk, Krasnoyarsk kray	A;DS	+	–	+	+	–	+
LAS 164	*D. superans*	Yuzhno-Kurilsk, Kurile Islands	A	+	–	–	+	–	–
LAS 165	*D. superans*	Yuzhno-Kurilsk, Kurile Islands	A	+	–	–	+	–	–
LAS 166	*D. superans*	Yuzhno-Kurilsk, Kurile Islands	A	+	–	–	+	–	–
LAS 167	*D. superans*	Yuzhno-Kurilsk, Kurile Islands	A	+	–	–	+	–	–
LAS 168	*D. superans*	Yuzhno-Kurilsk, Kurile Islands	A	+	–	–	+	–	–
LAS 169	*D. superans*	Bychiha, Khabarovsk kray	A	+	–	–	+	–	–
LAS 170	*D. superans*	Bychiha, Khabarovsk kray	A	+	–	–	+	–	–
LAS 171	*D. superans*	Bychiha, Khabarovsk kray	A	+	–	–	+	–	–
LAS 172	*D. superans*	Bychiha, Khabarovsk kray	A	+	–	–	+	–	–
LAS 173	*D. superans*	Bychiha, Khabarovsk kray	A	+	–	–	+	–	–

Some authors have suggested that the range of *D. sibiricus* may be expanding in Eurasia, particularly towards Europe (Gninenko and Orlinskii 2002; Kirichenko et al. 2006, 2009, 2011; Kononov et al. 2016), although there is no general agreement about whether such spread is actually occurring (Baranchikov et al. 2007, Mikkola and Ståhls 2008, Flø et al. 2020). Monitoring of this species is typically conducted using pheromone traps baited with a *D. sibiricus* pheromone lure. However, other *Dendrolimus* species/subspecies, including *pini* and *superans*, can respond to this lure (Baranchikov et al. 2007, Mikkola and Ståhls 2008). It follows that in regions where the ranges of these species overlap, captured moths can easily be misidentified, particularly in view of the limited number of morphological criteria available to tell them apart (Baranchikov et al. 2007, Mikkola and Ståhls 2008). Results of the survey presented in Table 4 (see also Fig. 1) tend to confirm this view: use of the DS assay to identify *D. sibiricus* specimens among moths collected over the known range of this species indicated that some moths collected in regions of range overlap had been given inaccurate provisional identifications; this conclusion was based on ITS2 sequences obtained for specimens showing discrepancies between the provisional identifications and the DS assay results. Thus, the DS assay reported here should prove useful in surveys investigating shifts in the range of *D. sibiricus* in Eurasia, including its potential spread towards Europe.

As pointed out in the Introduction, some authors consider *D. sibiricus* to be a continental subspecies of *D. superans* (Zolotuhin 2015). Whether or not this is the case, the present work indicates that the *superans* genotype is now present in continental Russia (Fig. 1), having presumably crossed the Nevelskoi Strait separating Sakhalin from the continent. While the two taxa are known to display different host preferences (Hardin and Suazo 2012), it is unclear whether *superans* x *sibiricus* crosses occur in nature and whether they yield reproductively viable hybrids. If confirmed, the latter condition would provide support for the one-species hypothesis. Should North American plant regulatory authorities choose to treat these two taxa as a single species, thereby considering both subspecies as representing a similar invasive risk, a variant of the DS assay presented here could be used to flag both taxa or potential hybrids simultaneously (see Supp File S4 [online only]).

Whether *D. pini* and *D. sibiricus* can hybridize in regions where their ranges overlap has not been investigated. However, in view of the conspicuous differences observed in the male genitalia between these two species (see Fig. 7 in Mikkola and Ståhls 2008), and the observation that ITS2 sequences obtained for the *D. pini* specimens used in the present study formed a clade clearly distinct from those of *D. sibiricus* and *D. superans* (Fig. 3), the likelihood that such hybrids can form seems low.

Conclusion

We developed bench-top and field-ready versions of two TaqMan assays that can be used for the identification of suspicious lasiocampid eggs intercepted during vessel inspections in North American ports. In US jurisdictions where pheromone traps are used to detect potential incursions of *Dendrolimus* moths near ports, the DS assay can also be used to determine whether any captured specimen belongs to *D. sibiricus*. Finally, the latter assay should prove useful for monitoring the spread of *D. sibiricus* in Eurasia.

Acknowledgments

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Author Contributions

DS: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing—original draft; Writing—review & editing. AD: Formal analysis; Visualization; Writing—review & editing. DH: Conceptualization; Resources; Writing—review & editing. TK: Conceptualization; Resources; Writing—review & editing. AC: Methodology; Writing—review & editing. VVD: Data curation; Funding acquisition; Resources; Visualization; Writing—review & editing. YBA: Resources; Writing—review & editing. MEY: Resources; Writing—review & editing. VVM: Project administration; Funding acquisition; Resources; Writing—review & editing. MC: Conceptualization; Data curation; Funding acquisition; Project administration; Supervision; Visualization; Writing—original draft; Writing—review & editing.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Supp File 1 – GenBank and BOLD accession numbers for the COI 5' and ITS2 sequences used in this study; COI 5' and ITS2 marker sequences generated in the course of this study, along with information about specimen sources and collectors; gBlocks sequences.

Supp File 2 – Sequence alignments used for (i) the design of primers and probes for each sub-assay and (ii) the design of primers for quantification of *Malacosoma* and *Dendrolimus* DNA.

Supp File 3 – Full assay validation results, including Ct values.

Supp File 4 – Alternative assay for simultaneous detection of *D. sibiricus* and *D. superans*: primers, probe, and examples of amplification curves.

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