Real-time PCR identification of the ambrosia beetles, Trypodendron domesticum (L.) and Trypodendron lineatum (Olivier) (Coleoptera: Scolytidae)

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Abstract
The European hardwood ambrosia beetle (Trypodendron domesticum) and the striped ambrosia beetle (Trypodendron lineatum) are wood-boring pests that can cause serious damage to lumber, resulting in a need for management of these pests in logging and lumber industries. Natural populations of ambrosia beetles exist throughout the world, but movement of ambrosia beetles into new habitats, particularly via international trade, can result in the establishment of invasive species that have the potential to spread into new territory. Efforts to monitor ambrosia beetle populations are time-consuming and could be greatly enhanced by the use of molecular methods, which would provide accurate and rapid identification of potentially problematic species. Here, we present new real-time PCR assays for the detection and identification of T. domesticum and T. lineatum. The methods described herein can be used with a variety of sampling strategies to enable timely and well-informed decision-making in efforts to control these ambrosia beetles.

Introduction
Ambrosia beetles are persistent forest pests that cause serious damage to cut wood, resulting in significant loss of quality due to dark-stained pin holes in lumber or veneer processed from infested logs. Wood degradation and dark staining arise from the growth of fungi that are carried into the sapwood by parental adult beetles and serve as the source of nutrition for the larval brood (Massoumi Alamouti et al. 2009; De Fine Licht and Biedermann 2012). Species of ambrosia beetles are problematic both in confiers and in deciduous trees, typically after trees have been cut or weakened by disease, but some taxa are also known to attack apparently healthy trees (Kühnholz et al. 2001). In addition to the loss in value of wood products resulting from the staining and pinhole damage, serious indirect impacts have been observed when the fungi vectored by attacking beetles prove to be highly pathogenic to living trees attacked by ambrosia beetles. Such impacts have recently been observed in forest and agricultural ecosystems as well as in urban environments (Hulcr and Dunn 2011; Suh et al. 2011; Evans et al. 2013; Freeman et al. 2013; Carrillo et al. 2014).

As many as 3400 xylomycetophagous species have been described from widely diverse lineages of Scolytidae and Platypodidae globally (Farrell et al. 2001; Hulcr 2013; Hulcr et al. 2015). The vast majority of ambrosia beetle species indigenous to forested habitats throughout the world are generally not problematic. Problems with invasive exotic species can arise as a consequence of international trade, especially through the movement of wood packaging (Humble 2010; Marini et al. 2011). Exotic species, once introduced to a new area, can quickly establish and extend their range (Humble 2001). Ambrosia beetles serve a vital function in forest ecosystems as initiators of wood decomposition; therefore, they are not considered a
threat to the environment, but from an economic standpoint, ambrosia beetles can severely impact the value of wood products or the health of tree plantations, and as such, there is a need for effective control strategies. Control strategies in forestry include both insect trapping and cultural control such as timing of harvest (Shore 1998; Livingston 2010), while chemical control by insecticide spraying is routinely used in nurseries and plantations (Peña et al. 2011; Reding et al. 2013).

The genus *Trypodendron* consists of fourteen described species that differ in the degree of damage that they cause to host trees, as well as in their geographic distribution and host range. Two of the most economically important species are *T. domesticum* (L.) and *T. lineatum* (Olivier). *Trypodendron domesticum* is found on a wide range of hardwood trees, but not on conifers, and it is distributed throughout Europe and western Russia (Dobesberger 2004). It is considered an invasive exotic species in Canada where it has recently become established (Humble 2001). It has also been detected in the USA, and monitoring is in place to prevent its spread (Rabaglia et al. 2008). *Trypodendron lineatum*, in contrast to *T. domesticum*, is a significant pest on conifers and does not attack hardwoods (Borden et al. 1997). It is native to Europe, Siberia, Turkey, North Africa and North America (Oranen 2013) and is considered a potential invasive species in Japan, where it has been detected on imported wood (Okabe et al. 2008).

Both *T. domesticum* and *T. lineatum* are commonly found in coniferous, deciduous and mixed forests, have overlapping flight times and respond to the same aggregation pheromone and host volatiles (Salom and McLean 1990; Petercord 2006; Humble 2009). Consequently, both species are easily pulled into the same surveillance trapping systems, and accurate identification becomes crucial due to the fact that they are morphologically similar (Carlson 2011; Jackson 2011). Two host-produced volatiles, ethanol (EtOH) and alpha-pinene (α-pinene), are commonly employed as attractants in surveillance programmes for the detection of invasive bark and ambrosia beetles. While effective in attracting ambrosia beetles for which pheromones are not known, host volatiles do not attract the large numbers of individuals of *Trypodendron* spp. drawn in by the use of aggregation pheromones which rapidly attract large numbers of conspecifics to susceptible hosts. Both *T. lineatum* and *T. domesticum* utilize lineatin as an aggregation pheromone (Nijholt and Shonherr 1976; Borden et al. 1982; Byers 1992). In areas where *T. lineatum* is established, lineatin lures attract hundreds to thousands of adult *T. lineatum* beetles, making detection of other species difficult due to the time required to examine and identify each beetle (Sweeney et al. 2007). For example, the relative abundance of *T. lineatum* and *T. domesticum* in trap captures in south-western British Columbia can vary both across stand type and across the flight season (Fig. S1). Detection of invasive species such as *T. domesticum* from traps in North America is therefore a time-consuming process that requires expertise in morphological identification. Such expertise is also required at international trade ports during inspection of imported materials for invasive species. To speed up the process of detection, morphological identification could easily be supplemented with molecular identification tools such as quantitative (real-time) polymerase chain reaction (qPCR), which relies on DNA differences to distinguish separate species. Molecular methods for insect identification have been bolstered recently by large-scale biodiversity DNA sequencing projects such as the Barcode of Life Project (Ratnasingham and Hebert 2007), which has produced unique DNA barcode sequences for thousands of insect species, including many species of ambrosia beetles (Jordal and Kambestad 2014). The goal of the current study was to develop a practical tool for ambrosia beetle identification using DNA barcodes as a design template. Here, we present the first molecular diagnostic tool for species identification in the genus *Trypodendron*, using species-specific qPCR assays which amplify the barcode region of the mitochondrial cytochrome c oxidase I (COI) gene to accurately identify *T. domesticum* and *T. lineatum*.

**Material and Methods**

**Specimen collection**

Detailed collection information for ambrosia beetle specimens is shown in Table S1. Adult beetles were collected in the spring and summer of 2012 and 2013. Twelve-unit multiple funnel traps (Lindgren 1983) with the wet-cup option (Contech Enterprises Inc., Delta, British Columbia) were hung from metal stakes with collecting cups located 90 cm above the ground. All release devices were secured to the outside of the top funnel arm on the traps, and the collection cups were filled with 125 ml of propylene glycol to ensure retention and preservation of trap captures. In 2012, *Trypodendron* spp. were lured with a commercial lineatin flexlure (Contech Enterprises Inc.) used in combination with an ethanol low-release pouch and an α-pinene low-release bottle. In 2013, other
Scolytidae were lured with an ultrahigh release (UHR) ethanol pouch used alone or combined with α-pinene, conophorin, hexanediol or hexanone. Trap contents were collected into Whirl-Pac bags (Nasco, Fort Atkinson, Wisconsin) and transported to the laboratory where they were washed, preserved in 95% ethanol and identified. Several species of Scolytidae were collected by rearing. Portions of infested trees were cut, and the ends of each log were sealed with paraffin wax to reduce desiccation. The logs were placed in individual rearing cages to allow resident beetles to complete their life cycle. Emergent beetles were collected and stored in ethanol. Voucher specimens of ambrosia beetles used in the current study have been deposited in the Canadian Forest Service-Pacific Insect Reference Collection (Victoria, British Columbia). Mixed beetle samples of 100 individuals containing varying species compositions were prepared and stored in ethanol prior to DNA extraction (table 1). Beetles in mixed samples were identified to species but were not vouched. As the entire contents of each mixed sample were used for DNA extraction, beetles were obtained either by trapping or by rearing. Trapped beetles were captured between the 19th of May and 27th of June 2012 in 12-unit multiple funnel traps, using UHR EtOH, retusol and sulcatol at the UBC Malcolm Knapp Research Forest, Maple Ridge, BC. Reared beetles emerged in the laboratory from cut wood collected on the 16th of July 2012 from the UBC Malcolm Knapp Research Forest, Maple Ridge, BC, and on the 1st of October 2012 from Richmond Nature Park, Richmond, BC.

**Trypodendron domesticum**, **Xyleborinus attenuatus** and **Xyloterinus politus** were reared from birch. **Dryocoetes sp.**, **Gnathotrichus sulcatus** and **Trypodendron lineatum** were reared from Douglas-fir, and **Alniphagus aspericollis** was reared from red alder.

**DNA extraction**

*From individual beetles*

DNA extraction from individual ambrosia beetles was performed using a CTAB method where either a single leg crushed in liquid nitrogen or a whole intact body was soaked overnight (8 h) at 55°C in 700 μl of 2X CTAB solution (100 mM Tris pH 8.0, 1.4 mM NaCl, 20 mM EDTA, 55 mM CTAB, 0.2% β-mercaptoethanol, 0.2 μg/ml proteinase K). Soaked samples were then extracted with 650 μl chloroform, followed by 650 μl phenol:chloroform (1:1) and then again with 650 μl chloroform. For each extraction, tubes were inverted 20 times to mix and centrifuged at 16 000 g for 15 min to collect aqueous phase. DNA was then

| Source | Sample size | Trypodendron domesticum | Trypodendron lineatum | Xyleborinus saxeseni | Xyleborinus politus | Xyleborinus attenuatus | Xyloterinus politus |
|--------|-------------|-------------------------|-----------------------|---------------------|-------------------|-----------------------|-------------------|
| Trap   | NGS-1       | 100                     | 75                    | 5                   | 5                 | 5                     | 5                 |
| Trap   | NGS-2       | 100                     | 100                   | 90                  | 2                 | 2                     | 2                 |
| Trap   | NGS-3       | 100                     | 2                     | 95                  | 1                 | 1                     | 1                 |
| Trap   | NGS-4       | 100                     | 24                    | 50                  | 5                 | 5                     | 5                 |
| Rearing | NGS-5       | 100                     | 2                     | 75                  | 2                 | 2                     | 2                 |
| Rearing | NGS-6       | 100                     | 100                   | 95                  | 1                 | 1                     | 1                 |
| Rearing | NGS-7       | 100                     | 24                    | 50                  | 5                 | 5                     | 5                 |
| Rearing | NGS-8       | 100                     | 24                    | 50                  | 5                 | 5                     | 5                 |

The *Xyleborinus saxeseni* beetles in sample NGS-8 were obtained from trap captures rather than rearing.

**Table 1 Species composition of mixed bark and ambrosia beetle samples.** Each sample contained 100 beetles artificially prepared from either trap captures or rearing in the laboratory.
precipitated from chloroform extract by adding two volumes of ice-cold 100% EtOH and incubating at −20°C overnight (8+ h). DNA was pelleted by centrifugation at 16 000 g for 30 min, and pellet was washed with ice-cold 80% EtOH and allowed to dry. Dried DNA pellet was resuspended in TE buffer and stored at −20°C.

**From mixed beetles**

DNA extraction from mixed beetle samples was performed using a commercial kit for tissue extraction (Nucleospin Tissue Kit, Macherey-Nagel, Bethlehem, PA). A modified version of the manufacturer’s standard extraction protocol was followed as per user manual version 06/2012, Rev.12, including the support protocol for purification of genomic DNA from insects. Mixed beetle samples were removed from ethanol and air-dried overnight. Dried beetles were ground in liquid nitrogen with a mortar and pestle, and 50 mg of the powder was transferred to a 1.5-ml microtube. Buffer T1 and proteinase K were added, and samples were incubated at 56°C for 2 h with occasional vortexing. Samples were centrifuged at 11 000 g for 5 min to pellet debris, and supernatant was centrifuged again in a new 1.5-ml tube to remove as much debris as possible. After transferring supernatant of the second spin to a new 1.5-ml tube, buffer B3 was added, resuming the manufacturer’s standard protocol at step 3. After the final elution of DNA from the spin column, eluant was re-applied to the column for an additional incubation and spin to maximize recovery of DNA from the column. Eluted DNA was stored at −20°C.

**Endpoint PCR amplification of COI region**

Amplification of the barcode region of COI was performed using the forward primer S1718, 5′-GGA GGATTGGGAATGTAGATTCC-3′ (Normark 1996) and the reverse primer LepR, 5′-TAAACTTCTGG ATGTCAAAAATCA-3′ (Hajibabaei et al. 2006), which amplify an approximately 500-bp fragment of COI. PCR volume was 25 μl, containing 1 ng of template DNA and final concentrations of 1X PCR Buffer (-MgCl₂), 2 mM MgCl₂, 0.2 mM dNTPs, 0.4 μM of each primer and 0.04 U/μl Platinum® Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). Thermocycling profile was 95°C for 3 min, 2 cycles of 95°C for 35 s, 55°C for 35 s and 72°C for 1 min; 2 cycles of 95°C for 35 s, 53°C for 35 s and 72°C for 1 min; 2 cycles of 95°C for 35 s, 51°C for 35 s and 72°C for 1 min; and 32 cycles of 95°C for 35 s, 53°C for 35 s and 72°C for 1 min, followed by a final extension at 72°C for 8 min and a hold at 10°C. PCR products were visualized on a 110 ml 1.5% agarose gel by electrophoresis at 130V for 1.5 h, followed by staining with ethidium bromide (0.5 μg/ml).

**Real-time PCR primer design**

Real-time PCR detection of *T. domesticum* and *T. lineatum* was performed using newly designed primers, targeting a portion of the barcode region of the COI gene. Primers were specific to each species and targeted the same 200-bp region of COI in each species (table 2). Primers were designed based on an alignment of publicly available Scolytidae COI sequences, including sequences from BOLD (SCOL project) and GenBank. The alignment contained 105 species, and *Trypodendron* species represented in the alignment were *T. domesticum* (5 sequences), *T. lineatum* (23 sequences), *T. retusum* (1 sequence), *T. scabricollis* (1 sequence) and *T. signatum* (2 sequences). When possible, degenerate bases were avoided near the 3′ end of the primers. Length of each primer was optimized to enable the use of a single melting temperature for all primers. Primers were purchased from IDT (Coralville, Iowa).

**Real-time PCR**

Real-time PCR volume was 25 μl, containing 1 ng of template DNA and final concentrations of 1X GoTaq® qPCR Master Mix (Promega, Madison, WI) and 0.3 μM each of forward and reverse primers. Thermocycling profile was 95°C for 13.5 min, followed by 45 cycles of 94°C for 15 s, 62°C for 30 s and 68°C for 30 s. Fluorescence readings were taken at the extension step (68°C) of each cycle. The melt curve, after an initial denaturation at 95°C for 15 s, was run from 55°C to 95°C, increasing by 1°C every 6 s. Real-time PCR amplification was performed on a Rotor-Gene Q 5-Plex HRM cycler (Qiagen, Hilden, Germany). The number of cycles required for fluorescence to exceed the threshold (cycle threshold (Ct)) was recorded for each sample. If no signal was observed, a Ct of 45 was recorded. Samples were run in duplicate, and the
average Ct was calculated for each sample. Signal detection from both replicates was considered necessary for reporting a positive detection. Therefore, an average Ct of 45 was recorded for samples where one replicate did not amplify.

**Sensitivity and linearity**

A standard curve was generated from 10-fold serial dilutions of *T. domesticum* and *T. lineatum* DNA from 2 ng to 0.2 fg per reaction, to determine the range, detection limits and efficiency of the assays.

**Results**

In the *T. domesticum* assay, signals were observed for *T. domesticum* between Ct = 22.4–26.5, and the melt temperatures were between 79.5 and 79.7°C. None of the non-target species produced a signal in the *T. domesticum* assay. In the *T. lineatum* assay, signals were observed for *T. lineatum* between Ct = 29.6–34.2, and the melt temperatures were between 80.5 and 81.1°C. One non-target species (*Pseudohylesinus sericus*) produced a signal in the *T. lineatum* assay with Ct = 38.3 and a melt temperature of 81.5°C (fig. 1). Six non-target species produced a signal in one of the two *T. lineatum* assay replicates, but Ct values in all six cases were above 38 and melt temperatures were 81.3°C or higher, so these species were considered to be negative detections and were recorded as Ct = 45 due to lack of detection replication. No false negatives were observed in this study, and no signal was observed for any no-template ‘blank’ controls. The endpoint PCR using primers

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**Fig. 1** Species specificity of *T. domesticum* and *T. lineatum* qPCR assays. Melt temperatures of positive detections are shown at the top of the graph.
S1718 and LepR amplified COI from all of the non-target species, showing that the negative qPCR detection of non-targets was related to assay specificity and not due to lack of amplifiable template DNA (Fig. S2).

The standard curve for the *T. domesticum* assay ($R^2 = 0.998$) showed a linear range of 2 ng–2 pg, a detection limit of 0.2 pg and efficiency of 93%. The standard curve for the *T. lineatum* assay ($R^2 = 0.996$) showed a linear range of 2 ng–2 pg, a detection limit of 2 pg and efficiency of 65% (Fig. S3).

From the mixed beetle samples, *T. domesticum* and *T. lineatum* were both detected from all samples using their respective qPCR assays. In neither assay was a strong quantitative relationship evident between beetle quantity and qPCR-estimated DNA quantity, although the *T. domesticum* assay did show a stronger relationship ($R^2 = 0.81$) than the *T. lineatum* assay ($R^2 = 0.24$) (fig. 2).

**Discussion**

Accurate identification of invasive ambrosia beetles is essential to prevent unwanted establishment and spread of exotic species. *Trypodendron domesticum* and *T. lineatum* can be destructive pests, and as such, their introduction into new habitats should be mitigated. The real-time PCR assays described here provide an accurate and sensitive new molecular tool to identify these problematic ambrosia beetles, enabling strong validation of putative *T. domesticum* and *T. lineatum* specimen identifications. These assays also enable testing for *T. domesticum* or *T. lineatum* in bulk DNA extracts from trap captures, which provides an alternative to the time-consuming sorting and visual inspection that is traditionally required for detection of species of interest.

The assay for *T. domesticum* was very specific, as demonstrated by the complete lack of false-positive amplification from the non-target species tested. It is also sensitive, allowing detection of as little as 0.2 picograms of DNA. The power of this assay to quantify *T. domesticum* beetles in a sample is reasonable yet limited ($R^2 = 0.81$), but this is not unexpected due to the fact that mitochondrial DNA is multicopy, and variable numbers of COI copies will be present in any given DNA extract. The size of individual beetles is another variable that would affect the COI copy number and therefore confound beetle quantification. The *T. lineatum* assay was specific except for one false-positive detection from *Pseudohylesinus sericeus*, but the Ct value of this detection was quite late (Ct = 38.3), and the melt temperature was outside the range of melt temperatures for *T. lineatum* (81.5°C for *P. sericeus* versus a maximum of 81.1°C for *T. lineatum*). As such, we can recommend a melt temperature cut-off of 81.1°C for positive *T. lineatum* detection with this assay. The sensitivity of the assay is good, providing detection of as little as 2 picograms of DNA. The ability to quantify *T. lineatum* beetles is poor, however, ($R^2 = 0.24$), which may be due to low PCR efficiency caused by the degeneracy of the primer sequences. Degenerate primers were a necessity in this case.

![Fig. 2](image-url) Quantitative capacity of qPCR assays using mixed beetle samples. DNA quantity was estimated from qPCR Ct values by comparison with the standard curves for each target species. Relationship between number of target beetles and qPCR-estimated DNA quantity is shown, including $R^2$ values.
because *T. lineatum* is a diverse species with a broad geographic range, and our intention was to develop an assay that would be able to detect COI from all *T. lineatum* genotypes.

The practicality of these *Trypodendron* qPCR assays stems from the low cost of double-stranded DNA-binding fluorescence chemistry compared to probe-based fluorescence. Probe-based fluorescence generally provides greater sensitivity, but the sensitivity of the assays described here was more than adequate when testing low-concentration DNA produced by non-destructive extraction from single legs or body soaking. Mixed beetle samples of one hundred individuals showed that the *T. domesticum* assay was easily able to detect a single *T. domesticum* beetle in this mixture (Ct = 19.3 and 20.6 from mixed samples containing one *T. domesticum*). This level of sensitivity would enable detection of *T. domesticum* from a mixed sample in which it is far outnumbered by other beetles, which is commonly the case in trap captures where the lures attract multitudes of *T. lineatum* beetles. In regions with endemic populations of *T. lineatum*, the ability to quickly and easily detect *T. domesticum* from trap captures without the need for visual inspection of each beetle could greatly facilitate monitoring efforts and reduce the requirement for expert attention to morphological features (Humble 2009; Carlson 2011). Due to the ability of *T. domesticum* to attack a wide variety of hardwood trees, a rapid response to *T. domesticum* detection could be critical, for instance when trees are more susceptible to attack, such as after a severe frost (La Spina et al. 2013).

The qPCR assays presented here represent the first molecular detection assays for the genus *Trypodendron* and for ambrosia beetles in general. These assays can significantly augment traditional morphological identifications of *Trypodendron*, which require adult specimens to be slide-mounted for microscopic examination by an expert taxonomist. Rapid identification with qPCR can now be performed by non-specialists to manage *T. domesticum* and *T. lineatum* populations and to limit the unwanted spread of these pests into new habitats. These qPCR assays use a cost-effective fluorescence chemistry to provide sensitive and specific detection in a short time frame. In this way, the use of qPCR can now be considered a viable method to aid in the detection and identification of two important *Trypodendron* species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pooled captures of adult *T. lineatum* (solid line) and *T. domesticum* (dashed line) from five 12-funnel Lindgren multiple funnels baited with lineatin flex lure, α-pinene and ethanol, (a) in sawmill in the Malcolm Knapp Research Forest, Haney, British Columbia and (b) in the east block of the Richmond Nature Park, Richmond, British Columbia.

Figure S2. Gel electrophoresis result of COI endpoint PCR from 20 non-target species.

Figure S3. Standard curve of each qPCR assay using serial dilutions of target DNA ranging from 2 ng to 200 fg per reaction. Each DNA quantity was tested in triplicate.

Table S1. Specimen collection info for all bark and ambrosia beetles used in this study.