Insect science

Insect Science (2016) 23, 728–738, DOI 10.1111/1744-7917.12221

ORIGINAL ARTICLE

Population divergence of aggregation pheromone responses in *Ips subelongatus* in northeastern China

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Abstract The Asian larch bark beetle, *Ips subelongatus*, is considered to be the major pest of larch within its natural range. We investigated the electrophysiological and behavioral characteristics as well as mitochondrial DNA cytochrome oxidase subunit I sequences of *I. subelongatus* from 13 geographic populations throughout northeastern China in order to explore population divergence of aggregation pheromone responses and the extent of potential genetic divergence. Electrophysiological analyses showed that antennae of *I. subelongatus* from all the six tested populations responded strongly to (*S*)-(−)-ipsenol (100% detection; 0.35–0.73 mV) in gas chromatography (GC)–electroantennographic detection (EAD) analyses, while its antipode, (*R*)-(+)-ipsenol was antennally inactive. *I. subelongatus* populations varied in their responses to (*R*)-(−)- and (*S*)-(+)-ipsdienol in GC-EAD analyses. Behavioral bioassays demonstrated that (*S*)-(−)-ipsenol alone was significantly attractive at all the tested sites, supporting its status as a key pheromone component of *I. subelongatus*, whereas (*S*)-(+)-ipsdienol was inactive alone. Adding (*S*)-(+)-ipsdienol to (*S*)-(−)-ipsenol did not have any effect on the trap catches from some populations in Inner Mongolia. However, (*S*)-(+)-ipsdienol showed a strong synergistic effect on (*S*)-(−)-ipsenol from several populations in Jilin and Liaoning Provinces, and a weak synergistic effect from some transition populations in Heilongjiang Province. Furthermore, 27 mitochondrial haplotypes were found among the 13 populations (intraspecific nucleotide divergence, 0.1%–1.1%). Analyses of molecular variance and haplotype networks indicated that different geographic populations have developed some genetic variation but did not form completely independent groups. From an applied point of view, a universal synthetic binary blend of racemic ipsenol and (*S*)-(+)-ipsdienol might have a potential for monitoring or even mass-trapping of *I. subelongatus* across northeastern China, even though some populations only use (*S*)-(−)-ipsenol alone as their active pheromone component.

Key words aggregation pheromone; GC-EAD; field trapping; *Ips subelongatus*; mtDNA-COI; population divergence

Introduction

Northeastern China has large areas of conifer habitats and extensive mountain ranges with distinct distributions of *Larix* spp. For example, Dahurian larch (*Larix gmelinii* Rupr.) is mainly distributed in the Greater Khingan and Lesser Khingan mountains, Prince Rupprecht larch (*Larix principis-rupprechtii* Mayr.) is in the Yanshan

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mountains, and Korean larch (*Larix olgensis* Henry) and Japanese larch (*Larix kaempferi* Carr.) are in the Baekdu mountains. The Asian larch bark beetle, *Ips subelongatus* Motschulsky (Coleoptera: Curculionidae: Scolytinae), is a severe pest of larches in northeastern China, where it is capable of killing relatively healthy trees during outbreaks (Zhang & Niemeyer, 1992; Yu, 1992). Host-associated preferences in geographically isolated populations of *I. subelongatus* might lead to differentiation in pheromone phenotypes and nucleotide sequences.

Ips subelongatus is closely related to the European larch bark beetle, *Ips cembrae* (Heer), which occurs throughout Europe, and it has long been considered as a synonym of the latter by European colleagues. However, more recent phylogenetic studies have shown that *I. subelongatus* and *I. cembrae* are distinct species (Stauffer *et al*., 2001). The aggregation pheromone system of *I. cembrae* consists of (*S*)-(−)-ipsenol, (*S*)-(+)-ipsdienol and 3-methyl-3-buten-1-ol (331-MB) (Stoakley *et al*., 1978; Francke & Vité, 1983) plus amitinol (Kohnle *et al*., 1988). The first three components were also identified in male hindgut extracts of *I. subelongatus* in northeastern China (Zhang *et al*., 2000). Ipsenol, ipsdienol and 331-MB were attractive to *I. cembrae* (Stoakley *et al*., 1978), while racemic ipsenol alone was as attractive as ipsenol-based binary or ternary blends to *I. subelongatus* in Keshiketeng Qi, Inner Mongolia, China (Zhang *et al*., 2007). Song *et al*. (2011) further confirmed that ipsenol, either racemic or 97%-(*S*)-(−)-enantiomer, was the only individual compound that significantly attracted both sexes of *I. subelongatus* in a *L. principis-rupprechtii* plantation of Keshiketeng Qi. However, they also reported that in a *L. gmelinii* plantation of Jingyuetan, Jilin Province, both ipsenol and ipsdienol (racemates or enantiomerically pure compounds) are needed to significantly attract *I. subelongatus*. This demonstrates that there is a strong geographical variation in aggregation pheromone responses by *I. subelongatus* populations in northeastern China. Similar geographic variations in pheromone composition were reported in *Ips typographus* (L.) (Bakke, 1989), *Ips pini* (Say) (Miller *et al*., 1997; Cognato *et al*., 1999) and *Dendroctonus rufipennis* (Kirby) (Borden *et al*., 1996). In particular, the pine engraver, *I. pini*, is known to have three pheromone races in North America that are generally associated with distinct mitochondrial DNA (mtDNA) haplotype lineages (Cognato *et al*., 1999).

Behavioral characteristics like pheromone responses often correlate with a population's genetic structure. To ascertain phylogeographic patterns, mitochondrial cytochrome oxidase subunit I (mtDNA COI) sequences have been successfully used at the inter- and intraspecific level in beetles (Funk *et al*., 1995; Langor & Sperling, 1997; Volger & Welsh, 1997; Cognato *et al*., 1999, 2005, 2007; Hunt *et al*., 2007). For example, mtDNA COI sequences were used to infer the phylogeny and genetic diversity of *Ips* species (Cognato & Sperling, 2000; Cognato & Sun, 2007). High interspecific (up to 21.4%) and intraspecific (up to 3.8%) COI sequence divergences have been observed in the Chrysomelid leaf beetle from genus *Ophraella* (Funk *et al*., 1995). Therefore, species boundaries may be identifiable by a predetermined, generally applied number of nucleotide differences between COI haplotypes (Hebert *et al*., 2004; Cognato & Sun, 2007).

The objectives of this study were: (i) to explore whether there is a significant geographical variation of *I. subelongatus* populations in electrophysiological responses to and behavioral functionality of two key potential aggregation pheromone components, (*S*)-(−)-ipsenol and (*S*)-(+)-ipsdienol in northeastern China, and to identify the extent of population divergence; 331-MB was left out of this study because it was behaviorally inactive in studies by Zhang *et al*. (2007) and Song *et al*. (2011); and (ii) to evaluate potential genetic variations among these populations via mtDNA COI sequence analysis and its correlation, if any, with pheromone response divergences and host tree species.

Materials and methods

Insects

In this study *I. subelongatus* adults were sampled from their host trees in 13 locations belonging to four subdivisions: Subdiv. A: GXIM, PQHB; Subdiv. B: HGIM, WCIM and GHIM; Subdiv. C: YCHL and MJHL; and Subdiv. D: LKHL, WQJL, HSJL, EDJL, JYJL and WDLN (see Table 1 and Fig. 3 for details). Multiple individuals (17–23) per population were collected and stored in absolute alcohol at -20°C for mtDNA COI analyses. Live beetles were maintained on newly cut host bark (*Larix* spp.) in 15 mL tubes at 4°C until used for antennal recordings.

Gas chromatography–electroantennographic detection (GC-EAD) analyses

A synthetic mixture (1 μL) of (-50/+50)-ipsenol and (-50/+50)-ipsdienol (100 ng/μL each) was injected splitlessly into an HP-6890 gas chromatograph with flame ionization detector (GC-FID; Agilent Technologies, Palo Alto, CA, USA) equipped with a CycloSil-B column (30 m × 0.25 mm × 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA) and a 1 : 1 effluent splitter

Table 1 Locality, host data and cytochrome *c* oxidase I haplotypes of *Ips subelongatus* collections from northeastern China.

Population symbol	Locality	Latitude, longitude, and altitude	Collection date	Host plant	*n*	Haplotype (individuals)
PQHB	Pingquan city, Hebei Province	N41°20′, E118°43′, 520 m	June 2011	*Larix principis-rupprechtii*	23	1, 2(15), 4, 5(3), 6(3)
GXIM	Guangxing Forest Farm†, Keshiketeng Qi, Inner Mongolia	N42°35′, E117°34′, 1267 m	July 2012		20	2(12), 5(2), 6(4), 17, 19
HGIM	Huanggangliang Forest Farm‡, Keshiketeng Qi, Inner Mongolia	N43°50′, E117°31′, 1447 m	August 2011		20	1(2), 2(9), 5(2), 6(4), 19, 20, 21
WCIM	Wuchagou town†,‡, Aershan city, Inner Mongolia	N46°46′, E120°18′, 783 m	July 2011, July 2012	*L. gmelinii*	18	2(13), 6, 7, 12(2), 13
GHIM	Ecological Research Station†,‡, Genhe city, Inner Mongolia	N50°54′, E121°31′, 870 m	July 2011, July 2012		17	2(15), 6(2)
YCHL	Youhao Forest Farm‡, Yichun city, Heilongjiang Province	N47°49′, E128°48′, 319 m	August 2011		23	2(15), 6(6), 8, 11
MJHL	Mengjiagang Forest Farm†, Jiamusi city, Heilongjiang Province	N46°25′, E130°39′, 185 m	July 2012	*L. olgensis*	20	1(2), 2(11), 3, 6, 11, 12, 16, 17, 18
LKHL	Linkou county, Mudanjiang city, Heilongjiang Province	N45°15′, E130°16′, 287 m	August 2012		19	1(2), 2(8), 5(3), 6, 19, 23(2), 24, 25
WQJL	Wangqing county, Yanji city, Jilin Province	N43°19′, E129°44′, 231 m	July 2012		21	1(8), 2(7), 5(4), 14, 15
JYJL	Jingyuetan Forest Farm, Changchun city, Jilin Province	N43°47′, E125°27′, 260 m	August 2011		19	1(4), 2(10), 5(4), 18
HSJL	Huangsongpu Forest Farm‡, Antu county, Jilin Province	N42°15′, E128°10′, 1001 m	August 2011		18	1(3), 5(12), 6, 9, 10
EDJL	Erdaobaihe town†, Antu county, Jilin Province	N42°26′, E128°7′, 695 m	July 2012		18	1(4), 5(10), 6, 19(2), 27
WDLN	Wendao Forest Farm†,‡, Funshun city, Liaoning Province	N41°46′, E124°9′, 209 m	August 2011		19	1(4), 2(6), 5(5), 6, 7, 22, 26

n – number of individuals used for DNA sequence analyses.

†Field trapping experiments conducted in this site.

‡Individuals from this site were tested with gas chromatography electroantennographic detection.

Table 2 Electroantennographic detection (EAD) responses (mV) of *Ips subelongatus* antennae (mean ± SE) and percentage of the beetle antennae responding to chiral pheromone candidates from six geographic populations across northeastern China.

Population†	*n*	(*S*)-(−)-ipsenol		(*R*)-(+)-ipsenol		(*R*)-(−)-ipsdienol		(*S*)-(+)-ipsdienol	
		EAD response (mV)	% responded	EAD response (mV)	% responded	EAD response (mV)	% responded	EAD response (mV)	% responded
HGIM	36	0.46 ± 0.04	100	0	0	0.23 ± 0.03	58	0.11 ± 0.03	11
WCIM	16	0.35 ± 0.04	100	0	0	0.15 ± 0.02	44	0.15 ± 0.04	31
GHIM	29	0.39 ± 0.03	100	0	0	0.18 ± 0.02	68	0.10 ± 0.01	39
YCHL	23	0.66 ± 0.13	100	0	0	0.26 ± 0.03	85	0.15 ± 0.02	69
HSJL	37	0.73 ± 0.07	100	0	0	0.34 ± 0.03	89	0.17 ± 0.01	84
WDLN	26	0.64 ± 0.05	100	0	0	0.27 ± 0.02	96	0.13 ± 0.01	92

n – number of individuals used for GC-EAD analyses.
†Population abbreviations are defined in Table 1.

(SGE, part # OSS-2) that allowed simultaneous recording of FID and EAD of the separated volatile compounds. The oven temperature was 50°C held for 1 min before increasing to 80°C at 6°C/min and then to 210°C at 2°C/min (isothermal for 10 min). Nitrogen was used as a carrier gas at 10 psi column head pressure. The outlet for the EAD was inserted into a humidified air-stream (500 mL/min) over an *I. subelongatus* antennal preparation. A detached antenna from a male or female was mounted directly between two electroantennographic (EAG) probes (PRG-2, Syntech, Kirchzarten, Germany) with electrically conductive gel (Spectra 360 electrode gel, Parker Laboratories, Orange, NJ, USA). EAD responses were amplified, recorded, and processed with an EAD amplifier and software from Ockenfels Syntech GmbH (Kirchzarten, Germany). The numbers of tested individuals per population and the population information are listed in Tables 1 and 2.

Field behavioral assays of enantiomeric aggregation pheromone candidates

A field trapping experiment with three synthetic pheromone candidate treatments [97%-(*S*)-(−)-ipsenol alone; a binary blend of 97%-(*S*)-(−)-ipsenol and 97%-(*R*)-(−)-ipsdienol; a binary blend of 97%-(*S*)-(−)-ipsenol and 97%-(*S*)-(+)-ipsdienol], plus an unbaited blank control, was conducted during June–August 2012 at six sites (locations) in northeastern China. Site 1 was a 30-year-old larch plantation stand of *L. principis-rupprechtii* at Guangxing Forest Farm, Keshiketeng Qi, Inner Mongolia (GXIM; test period, June 27–July 27). Site 2 was in a log yard (*L. gmelinii*) in Wuchagou, Aershan, Inner Mongolia (WCIM; test period, June 14–August 3). Site 3 was a 40–50-year-old larch plantation stand of *L. gmelinii* at the Ecological Research Station, Genhe, Inner Mongolia (GHIM; test period, June 17–August 6). Site 4 was located in a log yard (*L. olgensis*) at Mengjiagang Forest Farm, Jiamusi, Heilongjiang Province (MJHL; test period, July 6–August 11). Site 5 was in a log yard (*L. olgensis*) in Erdaobaihe, Antu County, Jilin Province (EDJL; test period, July 14–August 10). Site 6 was in a log yard (*L. olgensis*) at Wendao Forest Farm, Fushun, Liaoning Province (WDLN; test period, July 4–21) (see Table 1 for details). Another field trapping experiment was also conducted from August 12–21, 2013, at Mengjiagang Forest Farm (MJHL) to test the behavioral functionality of individual enantiomers of ipsenol and ipsdienol, and a binary blend of 97%-(*S*)-(−)-ipsenol and 97%-(*S*)-(+)-ipsdienol.

Enantiospecific 97%-(*S*)-(−)-ipsenol, 97%-(*R*)-(+)-ipsenol, 97%-(*S*)-(+)-ipsdienol and 97%-(*R*)-(−)-ipsdienol were dispensed from separate bubble-caps

(Contech Enterprises, Delta, BC, Canada) with release rates of 0.4 mg/day (40 mg loads) for ipsenol and 0.2 mg/day for ipsdienol (40 mg loads) at 25°C (Contech Ent.). Black cross-barrier traps (Pherobio Technology Co., Ltd., Beijing, China) were suspended from wire with the collecting cups 0.8–1 m above ground. Traps at each site were deployed in four randomized complete blocks. Traps were separated by at least 15 m within each set (block/line) and >30 m between sets. Trap captures were recorded and emptied every two days. To minimize positional effects, dispensers within each set were rotated after each observation. An unbaited blank control trap was included in each set at all sites.

DNA extraction, polymerase chain reaction (PCR), and sequencing

Whole DNA was extracted from the head and thorax of each specimen using the TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). PCR reactions were carried out in 18-μL volumes containing 13.3 μL dH_2O, 2 μL 10× buffer (Mg^{2+}), 2 μL deoxynucleotide triphosphate (dNTP: 20 mmol/L), 0.5 μL primers (10 μmol/L; "Dick" 5′-TTCTTCCTGGGTTTGGT-3′ and "Pat" 5′-TTTCTTACTGCTGGTAGTTCA-3′) and 0.2 μL *Taq* (Promega, Beijing, China). PCR was performed in a MasterCycler (Eppendorf, Hamburg, Germany) in 200 μL tubes with an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 94°C (30 sec), 51°C (60 sec) and 72°C (90 sec), and a final extension step at 72°C (10 min). Amplification was confirmed on 2% agarose gels. Sequencing reactions were performed in both directions on an ABI 3730XL automated sequencer (Applied Biosystems, Foster city, CA, USA) at Majorbio Company in Beijing, China. All *I. subelongatus* haplotype sequences were submitted to GenBank. *I. cembrae* (accession no. AF113338) and *I. typographus* (L.) (AF113385) were used as outgroups to polarize the *I. subelongatus* haplotype phylogeny.

Statistical analyses

Trap catch data were converted to proportion (P) of total captured beetles within each replicate. Data were then transformed by arcsin $\sqrt{p}$ to meet the assumptions of normality and homogeneity of variances for analysis of variance (ANOVA). The means among different treatments were compared by ANOVA followed by the Least Significant Difference (LSD) test at $\alpha = 0.05$ (SPSS 18.0, IBM, Chicago, IL, USA).

Sequences of mtDNA COI were edited and assembled by BioEdit (Ibis Biosciences, Carlsbad, CA, USA) and DNAman 6 (Lynnon Biosoft, Vandrenil, Quebec, Canada) according to sequence peak then aligned and edited to a standardized sequence length in MEGA5 (Tamura *et al*., 2011). The numbers of indels, transitions and transversions were counted using DnaSP 5.10 (Librado & Rozas, 2009). Haplotypes were analyzed for population level genealogies with TCS v. 1.21 (Clement *et al*., 2000). MEGA5 was used to determine the mean pair-wise distances between haplotypes and populations (Tamura & Nei, 1993). We also tested the population structure among all populations and conducted the analyses of molecular variance (AMOVA) using F statistics in Arlequin 3.1 (Excoffier *et al*., 2005). Nucleotide and haplotype diversity were estimated in DnaSP 5.10. Tajima's test of selection (Tajima, 1989) was also conducted in DnaSP 5.10.

Results

GC-EAD recordings

Antennae of all individuals from all six tested geographic populations strongly and consistently responded to (*S*)-(−)-ipsenol (0.35–0.73 mV). In contrast, its enantiomer, (*R*)-(+)-ipsenol, elicited no electrophysiological activity from any *I. subelongatus* population (Table 2, Fig. 1). Reproducible EAD responses were also recorded to (*R*)-(−)-ipsdienol (0.15–0.34 mV) and (*S*)-(+)-ipsdienol (0.10–0.17 mV). The ratio of the EAD amplitudes of (*S*)-(−)-ipsenol, (*R*)-(−)-ipsdienol, and (*S*)-(+)-ipsdienol was estimated as ca. 4 : 2 : 1 among beetle populations from all the sites (Table 2). Over 69% of individuals from YCHL, HSJL and WDLN showed significant antennal activities, whereas only < 40% of beetles from HGIM, WCIM and GHIM gave weak EAD responses to (*S*)-(+)-ipsdienol. No differences in antennal responses between males and females were found.

Field behavioral assays

Traps baited with 97%-(*S*)-(−)-ipsenol alone captured significantly more *I. subelongatus* beetles than did the blank control traps for all the six sites in 2012 (Table 3). Addition of 97%-(*S*)-(+)-ipsdienol to 97%-(*S*)-(−)-ipsenol did not increase trap catches in WCIM, but significantly increased trap catches by 103%, 134% and 220% in GHIM, MJHL and EDJL, respectively; and drastically increased trap catches by 1063% and 700% in GXIM and WDLN, respectively (Table 3). Addition

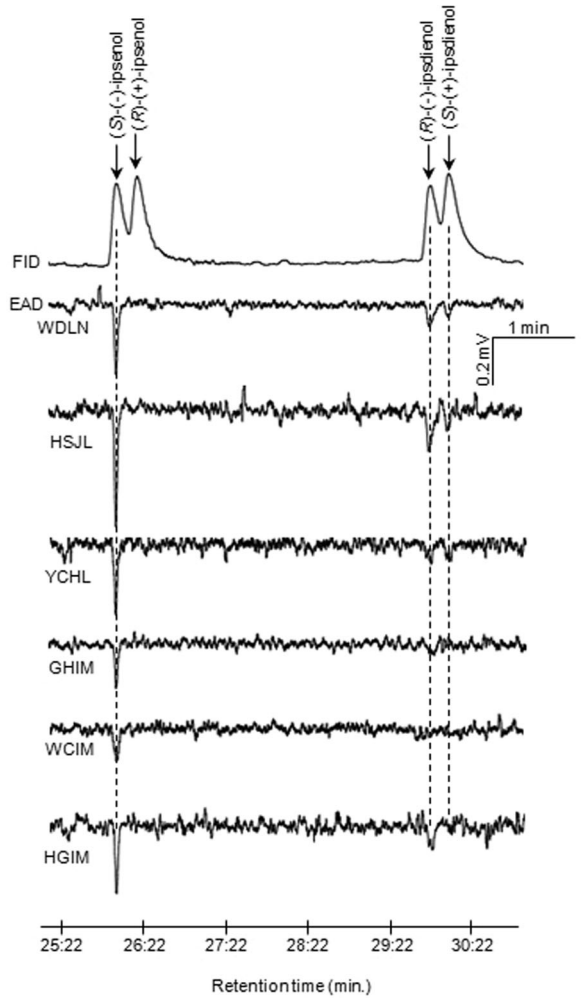

Fig. 1 Simultaneously recorded flame ionization detection (FID) and electroantennographic detection (EAD) responses of *Ips subelongatus* antennae from six different geographical locations (populations) in northeastern China to the separated enantiomers of (±)-ipsenol and (±)-ipsdienol via chiral gas chromatography column. Population abbreviations are defined in Table 1.

of (*R*)-(−)-ipsdienol, not produced by male hindguts, to 97%-(*S*)-(−)-ipsenol significantly reduced trap catches in GXIM, GHIM and EDJL, and showed no effects in WCIM, WDLN and MJHL. A further field trapping experiment in 2013 in MJHL showed that 97%-(*S*)-(−)-ipsenol alone was again significantly attractive to *I. subelongatus* beetles, whereas 97%-(*S*)-(+)-ipsdienol, 97%-(*R*)-(−)-ipsdienol and 97%-(*R*)-(+)-ipsenol were inactive when individually tested. However, the binary blend of 97%-(*S*)-(−)-ipsenol and 97%-(*S*)-(+)-ipsdienol

Table 3 Mean catches (± SE) of *Ips subelongatus* in cross-barrier traps baited with 97%-(*S*)-(−)-ipsenol alone and its combinations with 97%-(*S*)-(+)-ipsdienol or 97%-(*S*)-(−)-ipsdienol from six sites in northeastern China.

Treatments	Trap baits	GXIM[†]	WCIM	GHIM	WDLN	EDJL	MJHL
1	97%-(-)-ipsenol	96.50 ± 13.91 b[‡]	246.50 ± 76.15 ab	176.00 ± 33.34 b	276.50 ± 31.68 b	37.50 ± 7.58 c	163.00 ± 10.67 b
2	97%-(-)-ipsenol + 97%-(-)-ipsdienol	36.50 ± 4.87 c	156.75 ± 31.93 b	6.00 ± 2.27 c	119.25 ± 34.64 b	56.25 ± 11.82 b	168.00 ± 15.08 b
3	97%-(-)-ipsenol + 97%-(+)-ipsdienol	1122.5 ± 44.07 a	391.25 ± 98.82 a	357.50 ± 47.08 a	2212.75 ± 235.10 a	120.00 ± 10.89 a	382.00 ± 153.87 a
4	Control	38.25 ± 9.28 c	19.75 ± 1.44 c	0.75 ± 0.25 c	34.50 ± 5.27 c	3.00 ± 1.78 d	41.25 ± 6.88 c

[†]Population abbreviations are defined in Table 1. Traps at each site were deployed in four replicates.

[‡]Mean captures per trap at the same location (site) followed by the same lowercase letter are not significantly different ($P > 0.05$) by Least Significant Difference test after analysis of variance on the arcsin $\sqrt{p}$ transformed data of the relative catches, that is, proportion (P) of total captured beetles within each replicate (GXIM, $F_{3,12} = 286.73$, $P < 0.001$; WCIM, $F_{3,12} = 12.33$, $P < 0.001$; GHIM, $F_{3,12} = 178.87$, $P < 0.001$; WDLN, $F_{3,12} = 89.06$, $P < 0.001$; EDJL, $F_{3,12} = 35.90$, $P < 0.001$; MJHL, $F_{3,12} = 25.64$, $P < 0.001$).

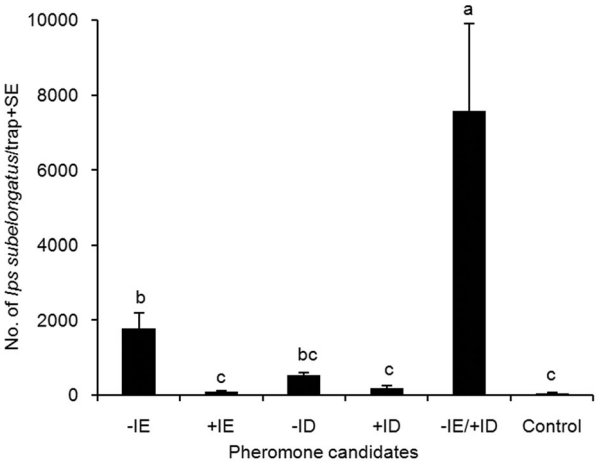

Fig. 2 Mean catches of *Ips subelongatus* in cross-barrier traps baited with 97%-(*S*)-(−)-ipsenol (−IE), 97%-(*R*)-(+)-ipsenol (+IE), 97%-(*R*)-(−)-ipsdienol (−ID) or 97%-(*S*)-(+)-ipsdienol (+ID) individually, plus a binary blend of the two major pheromone components (−IE/+ID), during the flight period at MJHL, 2013. Bars with the same letter are not significantly different ($P > 0.05$) by Least Significant Difference test after analysis of variance on the arcsin $\sqrt{p}$ transformed data of the relative catches, that is, proportion (P) of total captured beetles within each replicate ($F_{5,18} = 16.30$, $P < 0.001$).

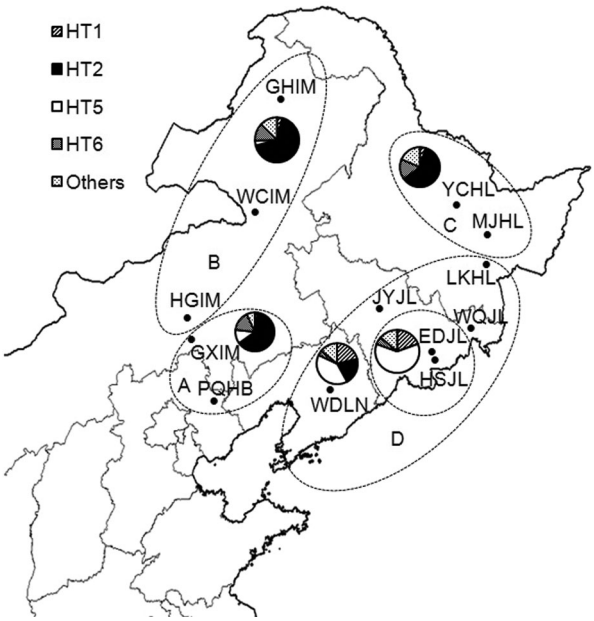

Fig. 3 Geographical distribution of *Ips subelongatus* haplotypes in northeastern China [GS2015(465)]. The pie graphs show the proportions of individuals in each population with each haplotype. "Others" includes haplotypes HT3, HT4 and HT7–HT27. The four regional subdivisions A, B, C and D are categorized according to the topography of northeastern China. Information on collection sites and population abbreviations are listed in Table 1.

was synergistically attractive, showing much higher trap catches than did 97%-(*S*)-(−)-ipsenol alone (Fig. 2).

Intraspecific variation of mtDNA COI sequence

Mitochondrial DNA haplotypes comprising 760 bp of the COI gene were sampled from 255 *I. subelongatus* individuals (GenBank accession numbers KC411926–KC411952). We identified 27 mtDNA COI haplotypes, as well as 11 individually variable sites and 15 parsimony informative sites. Nineteen of 27 haplotypes were restricted to a single locality; four occurred in two localities, and the others were found in most localities. The HT2 haplotype occurred in 11 populations, but not in HSJL and EDJL in the Baekdu mountains. A total of 121 of 255 individuals carried this haplotype (Table 1; Fig. 3). In contrast, the HT5 haplotype (represented in nine populations) was the most common haplotype in HSJL and EDJL. In addition, 24 individuals from 11 populations had the HT6 haplotype and 29 individuals from nine populations had HT1 haplotype. The remaining haplotypes were found in 1–5 individuals from different populations.

The haplotype and nucleotide diversity of the 13 geographic populations were 0.722 ± 0.024 and 0.00376 ± 0.00017, respectively. Based on haplotype networks, all haplotypes evolved radially, centered on HT2 (47.45% of individuals) to form two large groups (Fig. 4). A single evolutionary step led to haplotype HT1 (11.76%), which then evolved radially and is associated with individuals from the Lesser Khingan and Baekdu mountains. Three evolutionary steps resulted in HT9 and HT10 and subsequently HT5 (17.65%), which was primarily shared among individuals from the Baekdu mountains. HT6 (9.80%) was mainly found in individuals from the Yanshan, Greater Khingan, and Lesser Khingan mountains (Table 1).

Genetic variance among individuals within the same population was low, as haplotypes differed by no more than 10 nucleotides. Most of the haplotypes and sequence types were restricted to one locality, usually a single individual (Table 1). Nucleotide sequence divergence among the 27 haplotypes ranged from 0.1%–1.1% (mean = 0.6%). Analyses of molecular variance (AMOVA) revealed that 13 populations exhibited low but significant genetic differentiation ($F_{CT} = 0.09603$, $P < 0.05$) among the four regional subdivisions. There were also significant differences ($F_{ST} = 0.15683$, $P < 0.001$) within populations from the four regions.

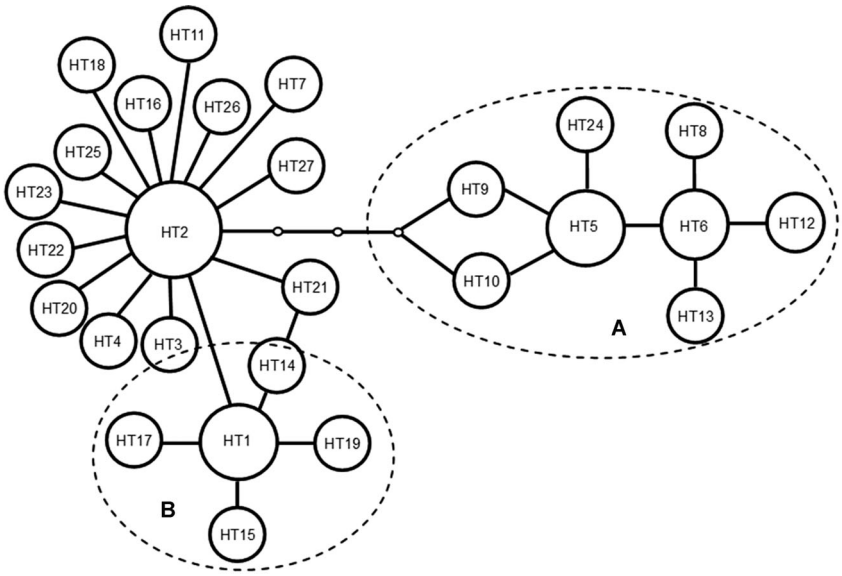

Fig. 4 Mitochondrial DNA haplotype networks for *Ips subelongatus* from northeastern China. Haplotypes HT1–HT27 correspond to those designated in Table 1. A single branch segment indicates one nucleotide difference and the size of the haplotype circle indicates the proportion of all individuals with that haplotype.

Discussion

(*S*)-(−)-ipsenol and (*S*)-(+)-ipsdienol are two major male-produced pheromone candidate compounds of *I. subelongatus* (Zhang *et al*., 2000, 2007). Despite the fact that the EAD activity of ipsenol and ipsdienol by *I. subelongatus* was well recorded (Zhang *et al*., 2007), nevertheless it was unknown which enantiomers of these two aggregation pheromone components evoked EAD responses. Our GC-EAD study on individuals from six geographically different populations clearly indicated that the strongest antennal response was to (*S*)-(−)-ipsenol, followed by (*R*)-(−)-ipsdienol and (*S*)-(+)-ipsdienol, whereas (*R*)-(+)-ipsenol elicited no antennal response. EAD response frequency to (*S*)-(+)-ipsdienol varied among populations with < 40% of individuals from western regions (HGIM, WCIM and GHIM) showing significant EAD activity. In contrast, > 70% of individuals from southern/eastern regions (YCHL, HSJL and WDLN) responded to this pheromone candidate. The differences in GC-EAD responses to the two major pheromone candidates, especially to the second major component (*S*)-(+)-ipsdienol, corresponded well with geographic Subdivisions B and C/D (see Materials and Methods) and their climatic conditions. Populations in Subdivision B (western region) seemed to be less EAD-responsive to (*S*)-(+)-ipsdienol than did the populations in the southern/eastern region (Subdivision C/D). In Subdivision B, the Greater Khingan mountains mainly have a temperate continental climate and in Subdivision D, the Baekdu mountains have predominately temperate monsoon climates. Antennal sensitivity to (*S*)-(−)-ipsenol and (*S*)-(+)-ipsdienol did not differ between males and females at any site. The inexpensive, racemic ipsenol is highly recommended as a part of the commercial *I. subelongatus* lure system for monitoring or mass-trapping applications, since the unnatural (*R*)-(+)-ipsenol was antennally inactive (meaning no antagonistic behavioral effect).

Our field trapping data provided strong support on geographical variation in pheromone responses among different populations of *I. subelongatus* in northeastern China. 97%-(*S*)-(−)-ipsenol was significantly attractive at all the six sites throughout northeastern China in our study, supporting its status as a key pheromone component of *I. subelongatus.* However, the behavioral role of the second component, (*S*)-(+)-ipsdienol varied geographically in a similar pattern as did the EAD responses (see below for details). Adding (*S*)-(+)-ipsdienol to the key pheromone component, (*S*)-(−)-ipsenol, did not have any effect on the trap catches at three locations in Inner Mongolia, WCIM (current study), Huanggangliang Forest Farm (HGIM) (Song *et al*., 2011) and Huamugou Forest Farm (about 120 km south-west of HGIM; about 60 km west of site GXIM) (Zhang *et al*., 2007), indicating that this compound is not a critical/active pheromone component for these populations that feed on *L. principis-rupprechtii* and *L. gmelinii* larch trees. However, (*S*)-(+)-ipsdienol showed a significant synergistic effect on the weakly

attractive (*S*)-(−)-ipsenol, resulting in 3–10 times more trap catches, in several southern populations from Jilin Province (EDJL: this study; Jingyuetan/Changchun [Song *et al*., 2011]), Liaoning Province (WDLN) and surprisingly in one population from Inner Mongolia (GXIM). (*S*)-(+)-ipsdienol or racemic ipsdienol alone was inactive in all the populations tested so far (Zhang *et al*., 2007; Song *et al*., 2011; and current study). Thus, the drastic trap catch increases at these locations should be the outcomes of a strong and significant synergistic interaction between these two critical pheromone components, (*S*)-(−)-ipsenol and (*S*)-(+)-ipsdienol. The strong disparity in pheromone responses between GXIM population (two-component synergistic blend) and its two neighboring populations – (*S*)-(−)-ipsenol as the single active component – in HGIM and Huamugou Forest Farm (only about 100 km or less apart) was totally unexpected, and deserves further testing in a comparative manner. On the other hands, (*S*)-(+)-ipsdienol showed a weaker synergistic effect when combining with the key pheromone component, (*S*)-(−)-ipsenol, in the two northern populations (GHIM and MJHL) than in the southern populations. Such behavioral response patterns to the two major male-produced pheromone candidate components are largely correspondent with EAD response patterns among the tested populations in northeastern China; such as the strong EAD-responses to (*S*)-(+)-ipsdienol from Liaoning and Jilin populations corresponding well with their strong synergistic behavioral attraction when combining with (*S*)-(−)-ipsenol. (*S*)-(+)-ipsdienol antipode, (*R*)-(−)-ipsdienol, not produced by male hindguts, showed either antagonistic or no significant effect on 97%-(*S*)-(−)-ipsenol, depending on the populations. Therefore, enantiomerically pure (*S*)-(+)-ipsdienol might have been used for some populations to achieve the maximum, but not for others. In addition, a low proportion of 331-MB in the three-component aggregation pheromone system of *I. cembrae* was critical for increased trap catches (Stoakley *et al*., 1978; Zhang & Niemeyer, 1995). This compound was also detected in minor amounts in the hindguts of male *I. subelongatus* (Zhang *et al*., 2000, 2007), but no GC-EAD activities have been observed so far (Zhang *et al*., 2007). This result, together with the fact that 331-MB did not have any effects on trap catches of *I. subelongatus* in China (Zhang *et al*., 2007; Song *et al*., 2011) indicated that 331-MB is not a part of the aggregation pheromone systems of the Asian larch beetles.

We also sampled mtDNA haplotypes of *I. subelongatus* from multiple locations and host-plant species to estimate population divergence in relation to geography and host specificity. We found a total of 27 haplotypes, with haplotypes HT2, HT5, HT1 and HT6 appearing with high frequency. HT2 could be characterized as the oldest *I. subelongatus* haplotype in northeastern China based on its frequency and wide geographic distribution. HSJL and EDJL mainly shared haplotype HT5; these populations were from the Baekdu mountains, where the conifer habitat is *L. olgensis* and the climate is temperate monsoon. Haplotype HT2 was not found in this area. The interspecific nucleotide divergence between *I. subelongatus* and *I. cembrae* ranged 13.7%–14.3% and between *I. subelongatus* and *I. typographus* 15.8%–16.6% (Stauffer *et al*., 2001). The mean intraspecific nucleotide difference among populations of *I. subelongatus* in northeastern China was 0.6%. Previous research has shown that intraspecific nucleotide divergence for mtDNA COI was ca. 0.3%–5.0% in some beetles (Funk *et al*., 1995; Cognato & Sperling, 2000). If we implement the 1.0% COI divergence level suggested by Cognato and Sun (2007) as a basis to identify *Ips* species boundaries, complete lineage divergence has not occurred among *I. subelongatus* populations in northeastern China, although different geographic populations have evolved some genetic variation.

Pheromone production and response specificity may be important to estimate *I. subelongatus* population divergence in relation to host specificity and distribution. So far, pheromone production data are available from only two populations in northeastern China: one from Fushun, Liaoning Province (Zhang *et al*., 2000) and one from Haumugou, Inner Mongolia (Zhang *et al*., 2007). No obvious variations in pheromone production (male-produced volatile compound compositions and ratios) between these two populations have been detected yet. More work is surely needed to further study the geographical variations of both the aggregation pheromone production and responses throughout its geographic range in China. From an applied point of view, a universal synthetic binary blend of racemic ipsenol and (*S*)-(+)-ipsdienol might have a potential for monitoring or even mass-trapping of *I. subelongatus* across northeastern China, even though some populations only use (*S*)-(−)-ipsenol alone as their active pheromone component.

Acknowledgments

We thank Yan-Jun Wang (Forestry Bureau of Keshiketeng Qi, Inner Mongolia, China), Ya-Dong Zhang (Forestry Bureau of Wuchagou, Inner Mongolia, China), Yong-Liang Zhang (Genhe Ecological Research Station, Inner Mongolia, China), Ji-Chun Liu (Mengjiagang Forest Farm, Heilongjiang Province, China), Li-Wen Song (Jilin

Provincial Academy of Forestry, China) and Jun Zhao (Wendao Forest Farm, Liaoning Province, China) for bark beetle collection and assistance with field work. Furthermore, we would like to thank Dr. Dariusz Czokajlo (president of Alpha Scent, Inc., USA) for reviewing an earlier version of this manuscript. This work was funded by grants from National Nonprofit Institute Research Grant of CAF (CAFRIFEEP201102) and '948' project (No. 2013–4–60) of the State Forestry Administration, China.

Disclosure

All authors declare no conflict of interest.

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Accepted November 23, 2014