Rapid Adaptive Evolution of Ophraella Communa in New Low Temperature Environment

Zhenqi Tian (tzq152519@163.com)
Chinese Academy of Agricultural Sciences Institute of Plant Protection https://orcid.org/0000-0001-6073-205X

Guangmei Chen
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Yan Zhang
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Chao Ma
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Zhenya Tian
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Xuyuan Gao
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Hongsong Chen
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Jianying Guo
Chinese Academy of Agricultural Sciences Institute of Plant Protection

Zhongshi Zhou
Chinese Academy of Agricultural Sciences Institute of Plant Protection

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Abstract

Low winter temperatures are a severe environmental pressure for newly arriving insect species. Adaptive evolutionary changes in cold tolerance facilitate establishment of these species in new environments. *Ambrosia artemisiifolia*, a noxious invasive plant, is distributed from southern to northern China. As an effective biological control agent of *A. artemisiifolia*, *Ophraella communa* is mainly spread in southern China. Previously, *O. communa* successfully established stable populations in Beijing (39.98°N, 115.97°E) following introduction from Laibin (23.62°N, 109.37°E) in 2012. This means that *O. communa* has adapted the cold winter temperatures in Beijing. However, the mechanisms underlying its rapid evolution of cold tolerance remain unknown. We investigated the levels of cryoprotectants and energy reserves in adult *O. communa* from two latitudes. We found that higher levels of trehalose, proline, glycerol, total sugar, and lipid were accumulated in *O. communa* from high latitudes. We also identified five potential genes (*Tret1a*, *Tret1b*, *Tret1-2*, *P5CS*, and *GST*) responsible for regulating cold tolerance that were highly expressed in high latitudes. These genes were involved in trehalose transport, proline biosynthesis, and glutathione S-transferase activation. These changes in hybridization indicated facilitating adaptation to cold temperatures. Our results demonstrate the genetic basis underlying the rapid adaptation of cold tolerance in *O. communa*, which indicates its extension in regions of *A. artemisiifolia* distribution at higher latitudes. This implies that the specialist herbivores can often adapt to a new low temperature environment where their host plant exist by rapid genetic evolution.

Key Message

Our results estimated the physiological and molecular mechanism under rapid adaptive evolution of cold tolerance in *Ophraella communa*.

Hybridization could enhance the evolutionary adaptation of cold tolerance.

Ophraella communa could be released to higher latitudes to manage *Ambrosia artemisiifolia*.

Introduction

When non-native insect species are introduced to new environments accidentally or intentionally, they often encounter a series of novel selection pressures. To adapt to the novel environmental conditions, their traits can shift through rapid evolution (Hoffmann and Sgrò 2011). As a severe environmental pressure for new arrivals, low winter temperatures are a limiting factor in the geographic distribution and abundance of organisms (Leather et al. 1993). Particularly, for insects from temperate regions, a cold winter often causes heavy mortality. Insects, as ectotherms, show positive shifts in biological and physiological traits with changes in temperature. They have evolved specific physiological strategies to respond to cold stress, such as the accumulation of low molecular cryoprotectants or energy reserves (Teets and Denlinger 2013; Toxopeus and Sinclair 2018). Rapid evolution of cold tolerance facilitates rapid adaptation to low temperatures and persistence in the novel environment.

The heritable evolutionary changes could enhance offspring fitness across diverse environments to maintain long-lasting establishment (Yin et al. 2019). Adaptive evolution is the process of evolution by natural selection, as introduced insects establish a population in a novel environment, the new environmental pressures would persist across generations. So a genetic change might rapidly occur enough to adapt to the new environment. This is particularly important for some introduced biological control agents because it could determine the success of application.

Rapid evolution in response to local adaptation has received much attention (Garnas 2018; Hoffmann 2017; Hoffmann and Sgrò 2011; Thompson 1998), but its genetic basis remains unclear and is gradually being elucidated. Recently, several studies have attempted to detect the genetic basis of rapid evolution, particularly thermal tolerance, under adaptation to local environmental conditions via genome or transcriptome sequencing (Dudaniec et al. 2018; Janes et al. 2014; Krehenwinkel et al. 2015; Lancaster et al. 2016). Nevertheless, whether these candidate genes improved the adaptation to the novel environment and whether these changes are heritable remain to be verified. Therefore, further empirical information is needed to evaluate the genetic basis in other insects.

Ambrosia artemisiifolia (Compositae) is a worldwide noxious invasive plant and is harmful to environmental or human health (Smith et al. 2013; Mazza et al. 2014). Its invasion always causes yield loss in agricultural system, and also reduces the biodiversity in field due to its strong competitive ability. *Ambrosia artemisiifolia* can release a plenty of pollen which is considered as a key cause of allergic rhinitis and asthma. It originates from North America, and now, has spread all around the world. In China, it is distributed from southern to northern, and has colonized Beijing for decades (Zhou et al. 2015). Its geographical distribution is continuously expanding, due to the climate warming and increasing of CO_2 (Rogers et al. 2006; Chapman et al. 2016). It is necessary to develop an effective management system.

Ophraella communa LeSage (Coleoptera: Chrysomelidae) is a crucial biological control agent of *A. artemisiifolia*, it has been widely applied in China and Europe (Guo et al. 2011; Schaffner et al. 2020; Zhou et al. 2015). As a specialist herbivore, *O. communa* prefers to feed on *A. artemisiifolia*, and it could not finish a complete generation on other similar Compositae plants, though fewer feeding behaviors could be observed in field (Zhou et al. 2011a; Augustinus et al. 2020). *Ophraella communa* originates from North America and was first recorded in mainland China in 2001, due to an accidental introduction (Meng and Li 2005). Subsequently, it distributed among south of the Yangtze River in China (Zhou et al. 2015). It could occur five generations each year in south China (Meng et al. 2007). In order to manage *A. artemisiifolia* in Beijing, *O. communa* was artificially introduced to Beijing (39.98°N, 115.97°E) from Laibin (23.62°N, 109.37°E) in 2012. Now, it can successfully survive the cold winter and has established a stable population in this area (Tian et al. 2020a). This suggests that *O. communa* has adapted to the local low temperatures. Prior quantitative genetic results have indicated that the cold tolerance of *O. communa* could be inheritable (Zhao et al. 2018). Meanwhile, a previous study documented that *O. communa* exhibits a geographic variation in cold tolerance across gradients (Zhou et al. 2011b), which also indirectly suggests that the evolutionary changes improved its capacity to cope with cold stress

(Garnas 2018; Hoffmann 2017; Hoffmann and Sgrò 2011). Therefore, we further verified the rapid evolution of *O. communa* in cold tolerance at the physiological and molecular levels.

Here, we compared the cold tolerant traits at the physiological level between two latitudes, and identified five potential key genes that exhibit differential expressions according to geographic variation in cold tolerance. We further confirmed that the physiological traits and gene expression were improved in hybrid offspring, suggesting that the evolutionary changes in cold tolerance were got promoted. These evolutionary changes involved the accumulation of low molecular weight cryoprotectants and energy reserves at physiological level, and trehalose transport, proline biosynthesis, and glutathione S-transferase (GST) activation at molecular level, all of which may contribute to the enhancement of cold tolerance and establishment in new low temperature environments. Due to the rapid evolution of cold tolerance in *O. communa*, we can release them to the northern region of *A. artemisiifolia* distribution for biological control. On the other hand, our study also revealed that the specialist herbivores may adapt a new environment where their host plant exist by rapid genetic evolution.

Materials And Methods

Host plants and insect source

Ambrosia artemisiifolia seeds were collected from fields at Langfang Experimental Station of CAAS during October 2018 and stored at 4 ℃. Two *A. artemisiifolia* plants were grown in each plastic pot (10 × 10 × 8 cm) without fertilizers.

Ophraella communa adults were collected from *A. artemisiifolia* plants in the fields in Laibin city, Guangxi Zhuang Autonomous Region, south China (23.62°N, 109.37°E) in late June 2019 and in Mentougou district, Beijing (39.98°N, 115.97°E) in mid July 2019. They were reared in cages (40 × 60 cm) at a laboratory at 26 ± 1 ℃ and 70 ± 5% relative humidity with a photoperiod of 14L:10D at Langfang Experimental Station of Chinese Academy of Agricultural Sciences (CAAS), Langfang, Hebei, North China (39°N, 116°E). Both populations were reared for one generation in laboratory, and their progenies (F0) were used for subsequent experiments.

Hybridization treatment

Forty adults (20 males and 20 females) were selected from both the Beijing (BJ) and Laibin (LB) populations and separated into two hybridization groups: BJ♀ × LB♂ and LB♀ × BJ♂. Their progenies (F1) were collected and used for measurement of physiological parameters and gene expression.

Measurement of total sugar, trehalose, glycerol, lipid, and proline contents

The total sugar, glycerol, and lipid contents were measured according to Zhou et al. (2011b) and Yue et al. (2014).

Trehalose content measurement

Individuals were weighed and homogenised with 1000 µL 10% trichloroacetic acid. The homogenate was centrifuged at 5,000 rpm for 10 min at 4 ℃. The supernate was transferred to a 1.5 mL Eppendorf tube, while the precipitate was mixed with 500 µL 10% trichloroacetic acid and centrifuged under the same conditions. The supernate was transferred and mixed with the above one. Then, 500 µL mixture was transferred to a new 1.5 mL Eppendorf tube, and 500 µL ethyl alcohol was added to this mixture. This mixture was refrigerated at 4 ℃ for 16 h and then centrifuged at 10,000 rpm for 20 min at 4 ℃, and the supernate was collected and transferred to a 10 mL centrifuge tube. Then, 1,000 µL 0.15 mol/L H_2SO_4 solution was added to the supernatant, heated, and hydrolysed in a boiling water bath for 10 min. After cooling, 1,000 µL 30% KOH solution was added to the mixture and heated again for 10 min. Then, 4 mL 0.20% anthrone-sulfuric acid reagent was added to the solutions and mixed, and the tube was heated for 10 min in the boiling water bath, cooled with running water, and equilibrated for 20 min. The zero point was set using a blank tube with solution, and the absorption at 520 nm was measured and recorded.

Proline content measurement

Individuals were weighed and homogenised with 500 mL sulfosalicylic acid in an ice bath, heated for 10 min at 100 ℃, and centrifuged at 10,000 ×g for 10 min at 25 ℃. After cooling, 0.25 mL supernatant, glacial acetic acid, and ninhydrin were individually added to a 2 mL Eppendorf tube. The mixture was incubated for 30 min at 100 ℃ with shaking every 10 min. After cooling, 0.5 mL toluene was added and shaken for 30 min for proline extraction. We selected 0.2 mL of the upper solution to detect the absorption using a quartz micro cuvette at 520 nm.

Three-day-old virgin adults were used in all above measurements, which were conducted using three replicates of 8 beetles each.

RNA extraction, cDNA synthesis, and gene cloning

Five differently expressed sequences were obtained from previous transcriptome data. Total RNA was extracted from 3-day-old adult females using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis and gene cloning were performed as previously described (Ma et al. 2020a; Tian et al. 2020b). The specific primers used to amplify the open reading frames (ORFs) of target genes were designed using Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA, USA) and are shown in Table 1. The amplification products were then purified using the Monarch Gel Extraction kit (NEB, Ipswitch, MA, USA) and cloned into a Trans1-T1 clone vector (TransGen Biotech) and sequenced (Ma et al. 2020a; Tian et al. 2020b).

Quantitative real-time PCR analysis

To determine the expression of cold tolerant genes in Beijing and Laibin, total RNA was extracted from 3-day-old adult females from Beijing and Laibin as described above. Primers for qPCR shown in Table 1 were designed using Beacon Designer 8.0 (Premier Biosoft International, Palo Alto, CA, USA). *Ribosomal protein L4* (RPL4) was used as the reference gene (Zhang et al. 2020; Tian et al. 2020b). Then, qPCR was

performed with SYBR Green Master Mix (Roche, Indianapolis, IN, USA) and the ABI 7500 Real-Time PCR System according to the manufacturer's instructions. The PCR conditions were as follows: 95 ℃ for 5 min, 40 cycles of 95 ℃ for 10 s, and finally, 60 ℃ for 30 s, followed by a melting curve analysis. The melting curves were checked to test the purity of qPCR reaction. Before gene expression analysis, the efficiency of the primers was also verified. Each sample was repeated in technical and biological triplicates. The data were expressed as relative mRNA levels normalised to the housekeeping reference gene *RPL4* in the same cDNA samples using the $2^{-\Delta\Delta Ct}$ [$\Delta\Delta CT = (Ct_{target} - Ct_{reference})_{treatment} - (Ct_{target} - Ct_{reference})_{control}$] (Livak and Schmittgen 2001; Pfaffl 2001).

RNA interference (RNAi)

Primers of double-strand RNA (dsRNA) of five target genes designed using Primer Premier 5.0 (Table 1). We also designed the primers of GFP-specific dsRNA (ds*GFP*) in order to verify that the injection and the response of immune system would not affect the expression of target genes and the results in study (Dai et al. 2017; Ma et al. 2020a; Tian et al. 2020b). They then were synthesized using the MEGAscript T7 High Yield Transcription Kit (Ambion, Austin, TX, USA). To explore the effect of dsRNA on the cold tolerance of *O. communa*, 1-day-old adult females were injected at the pronotum with 1 μg dsRNA using a PLI-100 Pico-Injector (Harvard Apparatus, Holliston, MA, USA), and manipulated with an MP-255 Micromanipulator (Sutter, Novato, CA, USA) under a stereomicroscope. Control groups were injected with an equal volume of ds*GFP*. Due to the high interference efficiency 48 h after dsRNA injection (Ma et al. 2020a; Tian et al. 2020b), their SCP, trehalose and proline contents, and GST enzyme activity were determined.

Measurement of SCP

Thermocouples were contacted with the cuticle of individual beetles. Then they were put into a -25 ℃ freezer and the cooling rate was approximately 1 ℃ per minute (Zhou et al. 2011b). The SCP was termed as the lowest temperature recorded before the suddenly increased in temperature, which was caused by the release of the latent heat of crystallization.

Trehalose content measurement

Detailed manipulation can be seen as above.

Proline content measurement

Detailed manipulation can be seen as above.

GST enzyme activity assays

The GST enzyme activity was detected with a GST Assay Kit (Solarbio, Beijing, China) according to the manufacturer's instructions. GST can catalyse the conjugation of glutathione and 1-chloro-2,4-dinitrobenzene. The product has an absorption peak at 340 nm. The GST enzyme activity was measured

by monitoring the rate of the increase in absorbance at 340 nm. One unit of enzyme activity was defined as 1 mg of enzyme protein that catalysed 1 nmol of product per minute in the reaction.

In all above bioassay experiments, ten individuals were measured as a replicate, and three replicates were conducted.

Statistical analysis

The differences in total sugar, trehalose, glycerol, lipid, and proline contents in F0 generation between the Beijing and Laibin populations or hybridization populations (F1 generation) were analysed using a *t*-test with SAS 8.1 (SAS Institute, Cary, NC, USA). Gene expression and bioassays were analysed using one-way analysis of variance followed by a least significant difference test with SAS 8.1 (SAS Institute, Cary, USA) to evaluate the significant differences among treatments.

Results

Total sugar, trehalose, glycerol, lipid, and proline contents

The content of trehalose, lipid, and proline in female *O. communa* from Beijing population were significantly higher than those from Laibin population (trehalose: $t = 4.92$, $P < 0.05$; lipid: $t = 2.96$, $P < 0.05$; proline: $t = 2.98$, $P < 0.05$) (Fig. 1b, d and e). However, the content of total sugar and glycerol in male *O. communa* from Beijing population were significantly higher than those from Laibin population (total sugar: $t = 6.09$, $P < 0.05$; glycerol: $t = 3.69$, $P < 0.05$) (Fig. 1a and e).

Total sugar contents in F1: BJ♀×LB♂ hybrids were intermediate between two parents. Due to a significant difference in cold tolerance between two genders (Zhou et al. 2011c), so we compared the significance between the hybridization and their parents with the same gender. We found that female hybrids significantly decreased compared to their female parents ($t = 6.81$, $P < 0.05$), but male hybrids slightly increased compared to their male parents ($t = 1.22$, $P = 0.29$) (Fig. 2a). Similarly, females in F1: LB♀×BJ♂ hybrids significantly decreased compared to their female parents ($t = 15.17$, $P < 0.05$), but male hybrids slightly decreased compared to their male parents ($t = 0.52$, $P = 0.63$) (Fig. 2b). Trehalose contents in F1: BJ♀×LB♂ hybrids showed intermediate levels between two parents, female hybrids slightly decreased compared to their female parents ($t = 0.87$, $P = 0.43$), and male hybrids slightly increased compared to their male parents ($t = 2.2$, $P = 0.09$) (Fig. 2c). Females F1: LB♀×BJ♂ hybrids had significantly higher trehalose contents than their female parents ($t = 3.45$, $P < 0.05$), but no difference existed between male hybrids and male parents ($t = 1.83$, $P = 0.14$) (Fig. 2d). Glycerol contents in F1: BJ♀×LB♂ hybrids showed intermediate levels between two parents, female hybrids slightly decreased compared to their female parents ($t = 1.45$, $P = 0.22$), and male hybrids slightly increased compared to their male parents ($t = 0.54$, $P = 0.62$) (Fig. 2e). However, glycerol contents in F1: LB♀×BJ♂ female hybrids slightly higher than those in their female parents, but there was no significant difference ($t = 0.93$, $P = 0.40$), and those in F1: LB♀×BJ♂ male hybrids were significantly lower than their male parents ($t = 3.36$, $P < 0.05$) (Fig. 2f). Lipid contents in females and males of two hybrids were higher than those in their female and male parents, respectively,

and males in F1: BJ♀×LB♂ hybrids (t = 13.87, P < 0.05) and F1: BJ♂×LB♀ hybrids (t = 6.62, P < 0.05) both increased significantly (Fig. 2g and h). Similarly, proline contents in females and males of two hybrids were higher than those in their female and male parents, respectively, and females in F1: BJ♀×LB♂ hybrids increased significantly (t = 15.82, P < 0.05) (Fig. 2i and j).

Geographic and hybrid expression profile

The mRNA levels of trehalose-specific transporters (*Trets*), delta 1-pyrroline-5-carboxylate synthetase (*P5CS*), and glutathione s-transferase (*GST*) in 3-day-old adult females from the Beijing population were all significantly higher than those from the Laibin population (*Tret1a*: P < 0.05; *Tret1b*: P < 0.05; *Tret1-2*: P < 0.05; *P5CS*: P < 0.05; *GST*: P < 0.05). *Tret1a*, *Tret1b*, *P5CS*, and *GST* mRNA levels in the Beijing population were nearly 2-fold higher than those of the Laibin population (Fig. 3).

Tret1a mRNA levels in F1: BJ♀×LB♂ hybrids were significantly reduced compared to those of their parents (females-female parents: P < 0.05; males-male parents: P < 0.05) (Fig. 4a). Meanwhile, F1: LB♀×BJ♂ hybrid females showed significantly reduced levels compared to their female parents (P < 0.05), whereas F1: LB♀×BJ♂ hybrid males had significantly increased levels compared to male parents (P < 0.05) (Fig. 4b).

Relative *Tret1b* expression in F1: BJ♀×LB♂ hybrid females was significantly reduced compared to that of their female parents (P < 0.05), males increased compared to their male parents, but without significance (P = 0.05) (Fig. 4c). *Tret1b* mRNA levels in F1: LB♀×BJ♂ hybrids were higher than those of their parents, but there were no significant differences (females-female parents: P = 0.45; males-male parents: P = 0.07) (Fig. 4d).

Tret1-2 mRNA levels in F1: BJ♀×LB♂ hybrid females were lower than those of their female parents while male hybrids showed higher levels than male parents, but there were no significant differences (females-female parents: P = 0.12; males-male parents: P = 0.16) (Fig. 4e). *Tret1-2* mRNA levels were significantly lower in F1: LB♀×BJ♂ hybrid females than in female parents (P < 0.05) and significantly higher in male hybrids than in male parents (P < 0.05) (Fig. 4f).

Relative *P5CS* expression in F1: BJ♀×LB♂ hybrid females was significantly higher than that in their female parents (P < 0.05); this was also lower in male hybrids than in male parents without significance (P = 0.07) (Fig. 4g). The relative expression of *P5CS* in females and males of F1: LB♀×BJ♂ hybrids was both lower than their parents, although this difference was only significant between male hybrids and parents (males-male parents: P < 0.05; females-female parents: P = 0.64) (Fig. 4h).

GST mRNA levels in F1: BJ♀×LB♂ hybrid females were higher than those in their female parents, but no significant difference was found (P = 0.13). Male hybrids showed significantly higher *GST* levels than male parents (P < 0.05) (Fig. 4i). *GST* mRNA levels in females and males of F1: LB♀×BJ♂ hybrids were significantly higher than their parental counterparts (females-female parents: P < 0.05; males-male parents: P < 0.05) (Fig. 4j).

Effect of dsRNA on Trets expression and trehalose content

After injection of ds*Tret1a*, ds*Tret1b*, and ds*Tret1-2* for 48 h, their mRNA levels were reduced by nearly 90.0%, 75.5%, and 87.9%, respectively, compared with *dsGFP* injection (Fig. 5a, b and c). Accordingly, the trehalose contents were 5.7, 5.6, and 5.9 μg/mg, respectively, which were significantly reduced compared with that after ds*GFP* injection (6.2 μg/mg) (Fig. 5f, g and h). To identify whether *Trets* could regulate cold tolerance in *O. communa*, their SCP were determined. The SCP of 3-day-old adult females injected with ds*Tret1a*, ds*Tret1b*, and ds*Tret1-2* were significantly increased compared with those following ds*GFP* injection (Fig. 5k, l, and m). This indicated that the cold tolerance of *O. communa* was reduced after silencing *Trets*.

Intriguingly, we found synergistic and complementary effects among the three *Trets*. The results indicated a complementary relationship between *Tret1-2*, and *Tret1a*, and *Tret1b*. When *Tret1-2* was silenced, the expression levels of *Tret1a* and *Tret1b* were increased, and vice versa (Fig. 5a, b and c). When either *Tret1a* or *Tret1b* was silenced, the other decreased (Fig. 5a and b). These findings suggested a synergistic relationship between them.

Effect of dsRNA on P5CS expression and proline content

Forty-eight hours after ds*P5CS* injection, *P5CS* mRNA levels were reduced by 88.2% compared with injection of ds*GFP* (Fig. 5d). To test whether the *P5CS* gene could influence the biosynthesis of proline, the proline contents were measured. In ds*P5CS*-injected beetles, proline levels were reduced by 36% compared with controls (Fig. 5i), which confirmed the function of *P5CS* in proline biosynthesis. The SCP of 3-day-old females injected with ds*P5CS* were significantly increased compared with those injected with ds*GFP* (Fig. 5n). This result demonstrated that the cold tolerance of *O. communa* was reduced after silencing *P5CS*.

Effect of dsRNA on GST expression and enzyme activity

Forty-eight hours after ds*GST* injection, *GST* mRNA levels were reduced by 75.5% compared with injection of ds*GFP* (Fig. 5e). This also significantly reduced the GST enzyme activity, which was 32.2 ± 0.4 and 42.0 ± 0.5 U/g wet weight with injection of ds*GST* and ds*GFP*, respectively (Fig. 5j). The SCP of 3-day-old females injected with ds*GST* were significantly increased compared with those injected with ds*GFP* (Fig. 5o). This indicated that cold tolerance was reduced following *GST* silencing.

Discussion

Ambrosia artemisiifolia is a severely invasive weed that spreads worldwide, and its distribution and influence may increase with climate change (Chapman et al. 2016). For instance, it has long colonised Russian Far East, where has a long, cold winter (Reznik 2009). Therefore, adaptability to new environments is important for using specific herbivorous natural enemies to control *A. artemisiifolia*. For instance, *Zygogramma suturalis* has been considered as an effective biological control agent of *A. artemisiifolia*, but its application in some areas has failed due to its weak adaptability to new environments (Wan et al. 1995). *Ophraella communa* has achieved positive effects on management of *A.*

artemisiifolia in China and Europe (Schaffner et al. 2020; Zhou et al. 2015). A previous study has demonstrated that *O. communa* has a high plasticity in cold tolerance based on the high heritability (Zhao et al. 2018). Here, it also shows strong rapid evolution of cold tolerance that may improve local adaptability, so it could be introduced to distribution ranges of *A. artemisiifolia* at higher latitudes and maintain long-term control.

A previous study examined the evolution of cold tolerance in *Drosophila melanogaster*, which revealed the genetic architecture underpinning rapid cold hardening and developmental acclimation (Gerken et al. 2015), but did not demonstrate adaptation to new low temperature environments. The present study demonstrated putative physiological and molecular mechanisms underpinning the rapid evolution of cold tolerance in *O. communa* following its release in Beijing. Thompson (1998) suggested that "rapid evolution" means genetic changes occurring over a century or less. Many insects showed rapid adaptation in a short timescale (Krehenwinkel et al. 2015; Lancaster et al. 2016; Hoffmann 2017), for example, *Aedes japonicus* adapted to changing environments within 7–10 years (Egizi et al. 2015). These findings likely suggest that insects have a strong capacity for rapid evolution to adapt to new temperature environments. However, the transgenerational effects (or parental effects) might often confound rapid adaptation, when establishing evolutionary shifts, it is necessary for populations to rear for one or two generations to remove parental effects (Hoffmann 2017). So the beetles used in our study were directly collected from filed and then reared for one generation under a common environment in a laboratory.

As a study previously described, a few physiological traits of *O. communa* apparently changed during cold adaption (Zhou et al. 2011b). Here, we found significant differences in content of trehalose, lipid, and proline in female adults between two latitudes, and significant differences in content of total sugar, and glycerol in male adults between two latitudes (Fig. 1). Thereby indicating main physiological mechanisms of rapid evolutionary changes in cold tolerance of *O. communa*. Trehalose, proline and glycerol are important low molecular weight cryoprotectants that are accumulated before overwintering to withstand the cold stress. This is a key physiological mechanism in insect cold tolerance (Denlinger and Lee 2010; Sinclair et al. 2003; Teets and Denlinger 2013; Toxopeus and Sinclair 2018). Trehalose, glycerol, and proline can stabilise the cell membrane and macromolecules or enhance the supercooling capability of insects to promote their cold tolerance (Teets and Denlinger 2013). Therefore, *O. communa* in Beijing accumulated more levels of cryoprotectants to against the cold injury in colder environments. Sugars and lipids are important energy reserves, scale studies have demonstrated that many overwintering insects would store more levels of them (Sinclair and Marshall 2018), including *O. communa* (Watanabe and Hirai 2004). There is a longer and colder winter in high latitudes, so *O. communa* may store more total sugars and lipids than in low latitudes.

Geographic variation in gene expression could suggest different adaptive responses to changing environmental conditions, and might indicate a genetic basis to local adaptation (Hoffmann 2017). At the genetic level, we found significantly higher transcript levels of *Tret1a*, *Tret1b*, and *Tret1-2*, *P5CS*, and *GST* in adult females of *O. communa* at the high latitude (Beijing) than at the low latitude (Laibin). These

genes are involved in trehalose transport, proline biosynthesis, and the oxidation-reduction process. Therefore, we suggest that high-latitude *O. communa* was likely to have a stronger capacity to transport trehalose, synthesise proline, and protect cells from oxidative damage than its low-latitude counterpart. Additionally, as an important antioxidant enzyme, GST can remove reactive oxygen species to decrease injuries caused by oxidative damage to cellular constituents or proteins (Lalouette et al. 2011; Storey and Storey 2013). Recently, a study demonstrated that *GST* expression in *Apis mellifera* was highly associated with local environmental conditions (Kojić et al. 2019). This may suggest that GST could be rapidly activated in response to environmental changes in insects.

Hybridization could increase the evolutionary potential and improve life history traits and fitness of hybrid offspring, however, it occasionally leads to neutral or negative effects (Bitume et al. 2017; Szűcs et al. 2012). Hybridization has been demonstrated to promote evolutionary adaptation to new environments (Hoffmann and Sgrò 2011). In our study, cold tolerance was improved after hybridization. At the physiological level, cryoprotectant or energy reserve contents in hybrids were mainly found in higher or intermediate levels compared to their parents (Fig. 2). At the molecular level, *Tret1a* expression mainly showed negative effects, whereas other genes largely exhibited positive or intermediate effects (Fig. 4). These results revealed that cold tolerance in *O. communa* is not inherited in a simple Mendelian manner, but under maternal or paternal effects (Bonduriansky et al. 2012). Furthermore, total sugar and lipid contents may have been inherited depending on paternal effects, but the trehalose, glycerol, and proline contents were likely influenced by maternal effects. The expression of *Trets* from each reciprocal hybrid seemed to be sex-linked. However, the *GST* expression in hybrids suggested overdominance. Moreover, direct allelic evidence was lacking, so the specific inheritance mode requires further study on alleles or chromosomes.

In our study, we found that transcription of TRET orthologs was important in the cold tolerance of *O. communa*. Ma et al. (2020b) also found that TRET orthologs were an important genetic basis of the cold-tolerance strategies of striped rice borer, *Chilo suppressalis*. Our study showed that after knockdown of *Trets*, trehalose levels in *O. communa* body were reduced (Fig. 5f-h). Trehalose is an appropriate stress protectant and preservative that can stabilise proteins and cells in invertebrates against damages caused by abiotic stresses, including coldness (Elbein et al. 2003; Thompson 2003). Accordingly, the SCP of *O. communa* was eventually reduced following knockdown of *Trets* (Fig. 5k-m), indicating a reduction in cold tolerance. The SCP (supercooling point) is the temperature that insect body fluid begins to spontaneously freeze. It might be an appropriate survival proxy for lethal temperature in many freeze intolerant insect species (Salt 1961; Ditrich 2018). It is a widely used metric to estimate the cold tolerance of insects (Zhou et al. 2011b; Van Damme et al. 2015; Feng et al. 2018; Li et al. 2020). This result suggests that *Trets* mediated trehalose levels to protect *O. communa* against the cold stress. This mechanism likely played a role in *Tret1*-mediated protection of *Anopheles gambiae* under desiccation and high temperatures (Liu et al. 2013). Additionally, studies have revealed that *Trets* could respond to other external environmental stresses, such as desiccation, salinity, low humidity, and high temperatures (Kikawada et al. 2007; Liu et al. 2013). These findings may suggest that *O. communa* could adapt to other new environmental pressures.

Proline is an important energy reserve for flight in insects. It also serves as an effective cryoprotectant, and its levels in insects have been widely reported to increase before overwintering or after rapid cold hardening and cold acclimation (Teets and Denlinger 2013; Toxopeus and Sinclair 2018). The *P5CS* gene has been well studied in plants, but little is known in insects. The putative *P5CS* gene encodes Δ^1-pyrroline-5-carboxylate synthetase, which catalyses the key step in proline biosynthesis in *Leptinotarsa decemlineata* (Wan et al. 2014). Here, the knockdown of *P5CS* reduced the proline contents in the *O. communa* body and increased the SCP (Fig. 5i). These findings confirm the pivotal role of *P5CS* in regulating proline biosynthesis. Meanwhile, this result indicated that *P5CS* could regulate cold tolerance-mediated changes in the proline level of *O. communa*.

Insects have evolved multiple strategies to respond to cold damage, including the accumulation of cryoprotectants as well as cytoprotectants, such as antioxidant defences (Toxopeus and Sinclair 2018). The up-regulation of antioxidant defences has been demonstrated under various stresses, including low temperatures (Storey and Storey 2013). In insects, studies have found that GST activity is associated with low temperatures (Grubor-Lajsic et al. 1997; Kojić et al. 2019). Although many studies have examined antioxidant enzymes, such as superoxide dismutase, catalase, and peroxidases, in invertebrates, little is known about the response of GST to cold tolerance in insects, however, this has been well studied in nematodes and vertebrates (Adhikari et al. 2010; Krivoruchko and Store 2010; Storey and Storey 2017). Various xenobiotics and aldehydic products of lipid peroxidation are neutralised by GST. These products could facilitate ROS formation by conjugating with reduced glutathione, followed by simultaneous excretion (Storey and Storey 2013). In our study, *GST* gene silencing reduced GST enzyme activity and increased the SCP of *O. communa* (Fig. 5j and o). This result indicated that the *GST* gene could regulate cold tolerance mediated by the GST enzyme activity of *O. communa*.

In our study, we find that *O. communa* strongly adapts to the new low temperature environment due to rapid evolution, which is reflected in changes at the physiological and molecular levels. These evolutionary changes mean that *O. communa* in high latitudes may have a stronger capability to accumulate more cryoprotectants, cytoprotectants, and energy reserves to respond to the cold winter temperatures. Importantly, we found that the physiological and molecular responses both involved the trehalose and proline, which probably reflects the essential role of them in insect cold tolerance. Further, we can expand its geographic distribution by artificial introduction to achieve better control efficiency for *A. artemisiifoli*, and reduce the harm to environment and human.

Declarations

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Conflicts of interest

The authors declare no conflict of interest.

Availability of data and material

All authors confirm that in the case that our manuscript is accepted, the supporting data will be archived and that a DOI to the archive will be supplied at the end of the article.

Authors' contributions

ZSZ conceived and designed research. ZQT, GMC and YZ conducted experiments. CM, ZYT, XYG, HSC, and JYG analyzed data. ZSZ and ZQT wrote the manuscript. All authors read and approved the manuscript.

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Tables

Table 1
Primers used in this study

Primer	Primer Sequence (5' to 3')
RT-PCR	
Tret1a-F	AACTTGTTTTTATACTTATC
Tret1a-R	CTGAATGCGAAGCAAGTA
Tret1b-F	AAGAAACTGACAACTCTA
Tret1b-R	ATTTGAATTTCCTCCAAAGT
Tret1-2-F	CATTTGCGGATCACTTTG
Tret1-2-R	AACGACACCGATTACGAC
P5CS-F	GTAGTTTTTTCGACAGGA
P5CS-R	CTTACTAATGATATTGCTT
GST-F	GTCCTGCTGTACGATCTA
GST-R	TTAAGCAAGGCGGCTTTTC
qPCR	
Tret1a-F	TGGACATCACCTGCTTTG
Tret1a-R	TCCTACGGTTGCTCCTAA
Tret1b-F	TCGGTGGAGGTGTCATAC
Tret1b-R	CCCAGAAACGACGATAATCC
Tret1-2-F	GCCGAACTATTTCCTACG
Tret1-2-R	CACTCCGAACCTTCCTAA
P5CS-F	GCCTTATGTCTCTGTACG
P5CS-R	GTTCCTTCTGGTCTCTTC
GST-F	AGCGTTGAATCGTTACCC
GST-R	GCGGCTTTTCATCATTCC
RPL4-F	TGTGGTAATGCTGTGGTAT
RPL4-R	TCTAGCACTGCATGAACA
dsRNA	
dsTret1a-F	TAATACGACTCACTATAGGGGCTGTGGTTCGCTATACTGT

Primer	Primer Sequence (5' to 3')
dsTret1a-R	TAATACGACTCACTATAGGGCTGTTGCCTTCTTCGTAA
dsTret1b-F	TAATACGACTCACTATAGGGAATATCTGTTTGGCCTCAA
dsTret1b-R	TAATACGACTCACTATAGGGAGCGGATTCTTGGTCTTG
dsTret1-2-F	TAATACGACTCACTATAGGGCATTTGCGGATCACTTTG
dsTret1-2-R	TAATACGACTCACTATAGGGCTTCCTTTTTATTTTTTCCTACC
dsP5CS-F	TAATACGACTCACTATAGGGCTTTGATGTCGGATGTGG
dsP5CS-R	TAATACGACTCACTATAGGGGATTTCTTAGTTTCGCTGGT
dsGST-F	TAATACGACTCACTATAGGGCCATCAAGGCATTAGGAG
dsGST-R	TAATACGACTCACTATAGGGAAACATTGGCAGAGGTCA
dsGFP-F	TAATACGACTCACTATAGGGTGAGCAAGGGCGAGGAG
dsGFP-R	TAATACGACTCACTATAGGGCGGCGGTCACGAACTCCAG

Figures

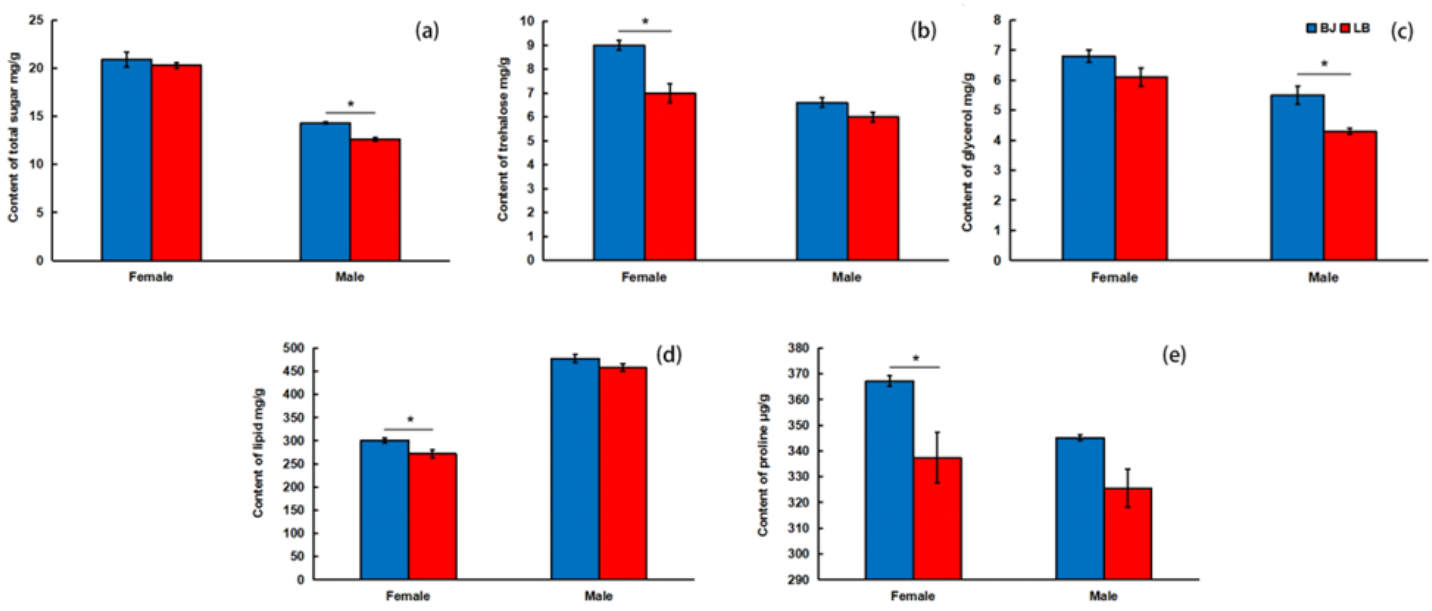

Figure 1

Contents of total sugar, trehalose, glycerol, lipid, and proline in adult Ophraella communa from two latitudes. (a) total sugar; (b) trehalose; (c) glycerol; (d) lipid; and (e) proline. Data are showed as the mean±SE. Bars marked with "*" are significantly different based on t-test (P<0.05).

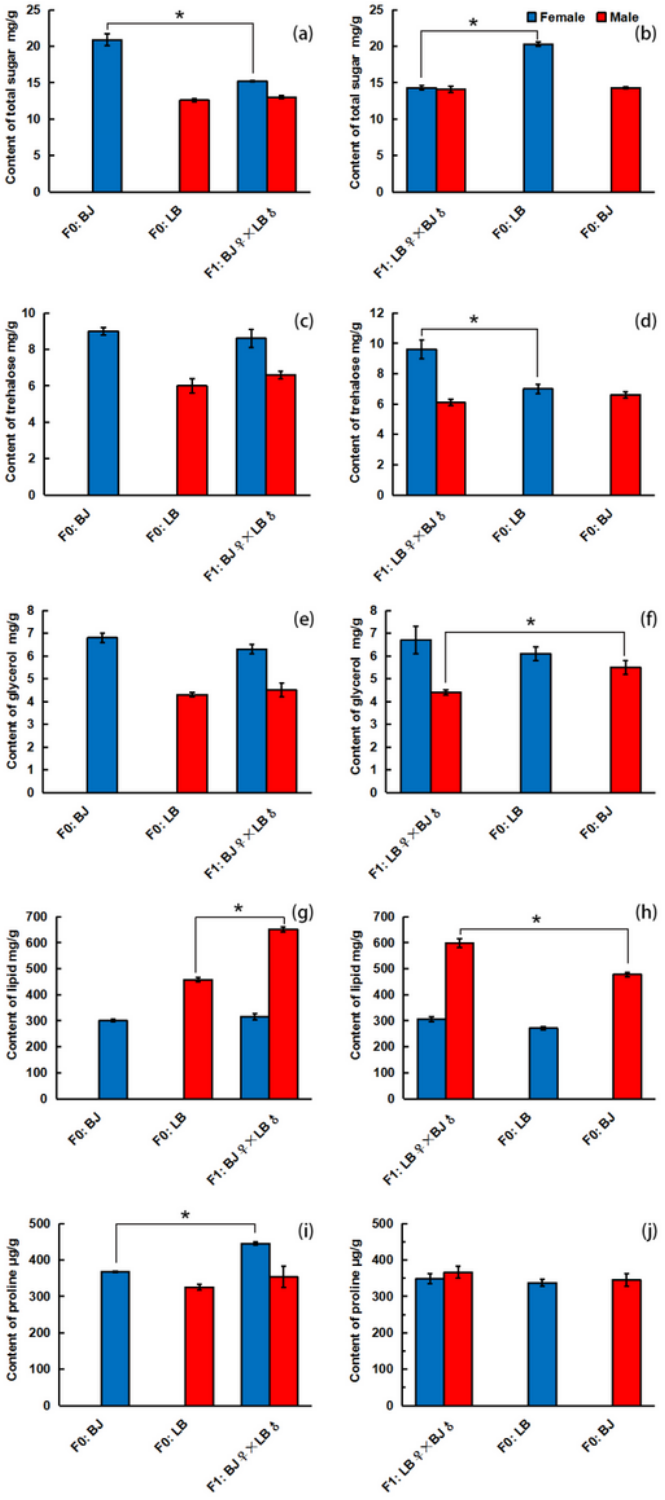

Figure 2

contents of total sugar, trehalose, glycerol, lipid, and proline in two hybridization populations of adult Ophraella communa. (a-b) total sugar; (c-d) trehalose; (e-f) glycerol; (g-h) lipid; and (i-j) proline. Data are showed as the mean±SE. Bars marked with "*" are significantly different based on t-test (P<0.05).

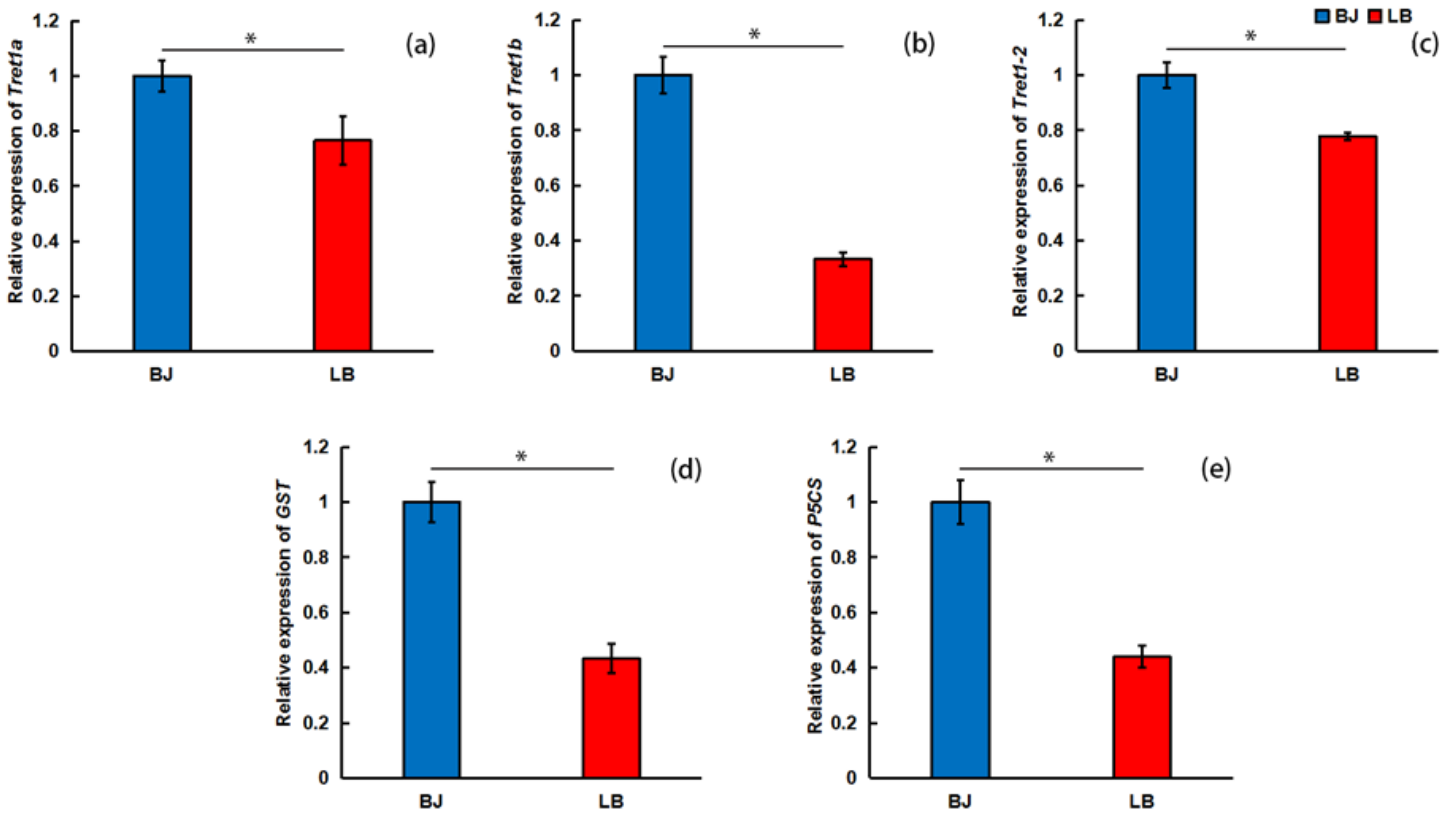

Figure 3

Relative expression of five identified genes responsible for cold tolerance in 3-day-old adult females of Ophraella communa. Panels depict the relative expression of: (a) Tret1a; (b) Tret1b; (c) Tret1-2; (d) P5CS; and (e) GST. The relative expression levels are showed as the mean±SE. Bars marked with “*” are significantly different based on one-way ANOVA followed by LSD test (P<0.05).

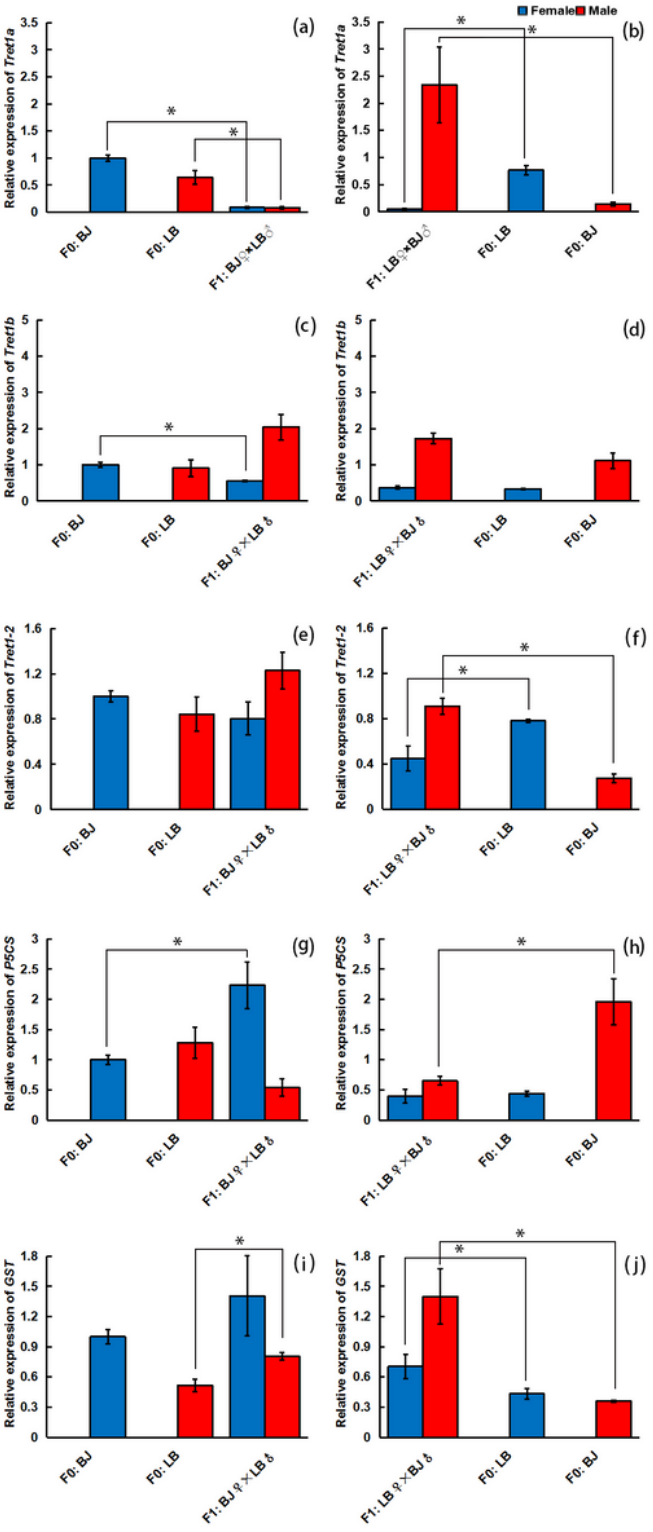

Figure 4

Relative expression of five identified genes responsible to cold tolerance in two hybridization populations of Ophraella communa. Panels depict the relative expression of: (a-b) Tret1a, (c-d) Tret1b, (e-f) Tret1-2, (g-h) P5CS, and (i-j) GST. The relative expression levels are showed as the mean±SE. Bars marked with "*" are significantly different based on one-way ANOVA followed by LSD test (P<0.05).

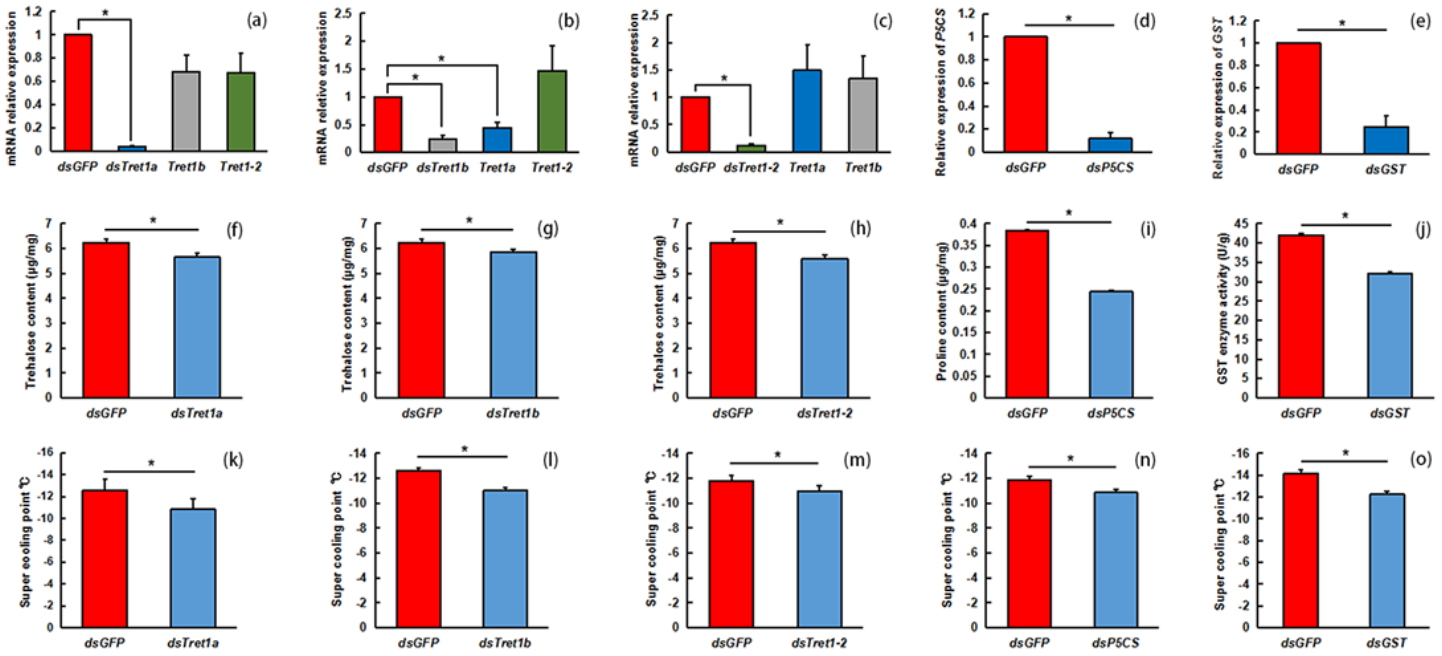

Figure 5

Effect of dsRNA on relative mRNA expression, bioassay results, and SCP. (a-e) relative mRNA expression levels; (f-h) trehalose contents; (i). proline contents; (j) GST enzyme activity; and (k-o) effect of dsRNA on SCPs. Data are showed as the mean±SE. Bars marked with "*" are significantly different based on one-way ANOVA followed by LSD test ($P<0.05$).