Insect Biochemistry and Molecular Biology 46 (2014) 31–42

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Comparative analysis of two phenologically divergent populations of the pine processionary moth (*Thaumetopoea pityocampa*) by *de novo* transcriptome sequencing

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ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form
11 January 2014
Accepted 13 January 2014

Keywords:
Pine processionary moth
Thaumetopoea pityocampa
de novo transcriptome assembly
Next Generation Sequencing
Population comparative analyses
SNP detection
Phenology

ABSTRACT

The pine processionary moth *Thaumetopoea pityocampa* is a Mediterranean lepidopteran defoliator that experiences a rapid range expansion towards higher latitudes and altitudes due to the current climate warming. Its phenology – the time of sexual reproduction – is certainly a key trait for the local adaptation of the processionary moth to climatic conditions. Moreover, an exceptional case of allochronic differentiation was discovered *ca.* 15 years ago in this species. A population with a shifted phenology (the summer population, SP) co-exists near Leiria, Portugal, with a population following the classical cycle (the winter population, WP). The existence of this population is an outstanding opportunity to decipher the genetic bases of phenology. No genomic resources were so far available for *T. pityocampa*. We developed a high-throughput sequencing approach to build a first reference transcriptome, and to proceed with comparative analyses of the sympatric SP and WP. We pooled RNA extracted from whole individuals of various developmental stages, and performed a transcriptome characterisation for both populations combining Roche 454-FLX and traditional Sanger data. The obtained sequences were clustered into *ca.* 12,000 transcripts corresponding to 9265 unigenes. The mean transcript coverage was 21.9 reads per bp. Almost 70% of the *de novo* assembled transcripts displayed significant similarity to previously published proteins and around 50% of the transcripts contained a full-length coding region. Comparative analyses of the population transcriptomes allowed to investigate genes specifically expressed in one of the studied populations only, and to identify the most divergent homologous SP/WP transcripts. The most divergent pairs of transcripts did not correspond to obvious phenology-related candidate genes, and 43% could not be functionally annotated. This study provides the first comprehensive genome-wide resource for the target species *T. pityocampa*. Many of the assembled genes are orthologs of published Lepidoptera genes, which allows carrying out gene-specific re-sequencing. Data mining has allowed the identification of SNP loci that will be useful for population genomic approaches and genome-wide scans of population differentiation to identify signatures of selection.

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1. Introduction

The pine processionary moth (hereafter, PPM) *Thaumetopoea pityocampa* (Lepidoptera, Notodontidae) is an insect pest occurring over the Mediterranean basin and the Atlantic coasts of France, Spain and Portugal (Kerdelhué et al., 2009). It causes considerable damage to pinewoods over its distribution range, and its gregarious, urticating larvae are responsible for severe public and animal health concern (Rodríguez-Mahillo et al., 2012; Vega et al., 2004). Its distribution range is in part driven by winter temperatures, as larval development mainly occurs during the coldest months.

Abbreviations: PPM, pine processionary moth; SP, summer population; WP, winter population; SNP, single nucleotide polymorphism; NGS, Next Generation Sequencing; SFF, standard flowgram file; NR, non-redundant NCBI protein database; GO, Gene Ontology; CDS, coding sequence; OHR, Ortholog Hit Ratio; UniProtKB, Uniprot knowledgebase; RBH, reciprocal best hit; Indel, insertion or deletion; UTR, untranslated region; N50, contig length for which half of the assembly is represented by contigs of this size or longer.

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0965-1748/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibmb.2014.01.005

Consistently, the PPM has been shown to expand northward and towards higher altitudes due to the current climate warming (Battisti et al., 2005).

This species typically has one generation per year, although prolonged pupal diapause can delay adult emergence by one to several years. Adults emerge in summer, mate and lay eggs in the following hours or days. After *ca.* 30 days of embryonic development, larvae hatch and develop in autumn and winter. The caterpillars spin characteristic silk nests where they gather during daytime, while they feed on pine needles at night. At the end of larval development, usually between January and March, the colony leaves the nest in a typical head-to-tail procession in search for an underground pupation site. After an obligate nymphal diapause, adults emerge the following summer. Local phenological variations are supposed to be adaptive responses in the moth populations and allow the species to occur under various environmental conditions: reproduction tends to take place in early summer in regions where winters are harsh, and the first, susceptible larval stages thus develop before the first frost; in contrast, reproduction takes place in late summer in regions with hot summers and mild winters, thereby avoiding larval mortality due to high temperatures (Huchon and Démolin, 1970). Phenology is thus a major trait involved in local adaptation of the PPM.

Interestingly, a population showing a shifted phenology was discovered in Portugal *ca.* 15 years ago in a coastal pine forest named Mata Nacional de Leiria. In this very peculiar population, reproduction occurs in spring and larvae develop all over the summer. Individuals with the classical life cycle co-occur in the same forest. Due to its summer larval development, the shifted population has been called the "summer population" (SP) while the sympatric population exhibiting a classical cycle is referred to as the "winter population" (WP) (Pimentel et al., 2006). The SP larvae never face winter conditions, and consequently do not spin nests, although they still show a gregarious behaviour. On the contrary, the most susceptible larval stages experience very high temperatures that are expected to be lethal in that species (Huchon and Démolin, 1970). Experimental approaches have shown that the first larval instars of the SP survive significantly better under high temperatures than the sympatric WP larvae (Santos et al., 2011b), suggesting a physiological adaptation. SP is phylogenetically very close to the sympatric WP according to mitochondrial and ITS sequences while microsatellite data suggest that current gene flow is very restricted between both populations (Santos et al., 2011a, 2007). This unique situation corresponds to a plausible recent allochronic differentiation, where gene flow is hampered by a shift in time of the reproductive period. The PPM found in the Leiria pine forest provides an unprecedented opportunity to study the genetic bases of phenology and adaptation to high temperatures.

We present here a *de novo* transcriptome sequencing approach to study and compare genes expressed in the SP and WP occurring in Leiria. We focussed on the late developmental stages (last larval instar, pupae and adults of both sexes) in which the genes involved in phenology (here, mostly the regulation of pupal diapause) and in reproduction are likely to be expressed. Data combine low- and high-throughput sequencing technologies (Sanger and 454 sequencing, respectively). The goal of the present study was fourfold: (i) build a *de novo* reference transcriptome for *T. pityocampa*, and significantly increase at the same time the genomic resources for this insect pest, which is phylogenetically distant from most studied Lepidoptera species (Mutanen et al., 2010); (ii) identify gene-targeted single nucleotide polymorphisms (SNP) for future genome wide analyses of diversity and differentiation; (iii) identify the sets of genes specifically expressed (or absent) in the shifted SP; (iv) identify the most divergent homologous genes between the SP and WP at the nucleotide level. These two latter points are the first steps towards the comprehension of the genetic architecture of phenology, *i.e.* of the trait responsible for the allochronic differentiation occurring in the Leiria forest and a major trait in PPM local adaptation.

2. Material and methods

2.1. Laboratory protocols

2.1.1. Sampling, RNA purification and isolation

All samples were initially collected in the field in the Mata Nacional de Leiria, Portugal (39°47′N 8°58′W). Larvae were sampled about one month after the L4 to L5 molt, *i.e.* at the end of the last larval instar, while still aggregated in the nest. Pupae were sampled about two months after the procession and about 4–5 months before adult emergence. Sampling of the adults took place one to two days after emergence; virgin females were collected, while the males had possibly mated. Concerning the SP, pupae were sampled between November 15th and December 17th 2007. Ten individuals were deep frozen at −80 °C immediately after field collection, while the other pupae were kept under laboratory conditions at the Instituto Superior de Agronomia of the University of Lisbon until adult emergence. Ten individuals were then frozen as adults in April 2008. L5 larvae were sampled in the field in September 2008, and immediately frozen at −80 °C. Concerning the WP, L5 larvae were collected in Leiria forest in January 2008. Ten of those were immediately frozen at −80 °C, while the others were kept in the laboratory at the Instituto Superior de Agronomia and fed with *Pinus pinaster* branches until pupation. Ten pupae were frozen from this rearing in July and ten adults in August 2008. Samples were sent in dry ice to the Max Planck Institute for Chemical Ecology (Jena, Germany) to proceed with RNA isolation and sequencing procedures. Total RNA was isolated from five L5 larvae (which could not be sexed), five pupae (males and females) and five adults (males and females) per population. The other individuals were kept frozen in Lisbon to ensure that the whole procedure could be repeated.

TRIzol Reagent (Invitrogen) was used to isolate the RNA according to the manufacturer's protocol. The RNA was precipitated overnight at −20 °C and the dried pellet was dissolved in 90 μl RNA Storage Solution (Ambion). An additional DNAse (Turbo DNAse, Ambion) treatment was then applied prior to the second purification step to eliminate any contaminating DNA. The DNAse enzyme was removed and the RNA was further purified by using the RNeasy MinElute Clean up Kit (Qiagen) following the manufacturer's protocol and eluted in 20 μl of RNA Storage Solution (Ambion). RNA integrity and quantity was verified on an Agilent 2100 Bioanalyser using the RNA Nano chips (Agilent Technologies, Palo Alto, CA), using both the high resolution gel and electropherogram views provided, even though the standard RIN method is not applicable to insect RNA because the 28S band tends to break (Winnebeck et al., 2010). RNA quantity was determined on a NanoDrop ND-1000 spectrophotometer. For each developmental stage of each *T. pityocampa* population two different high-quality RNA extractions were generated. Equal amounts of total RNA of all stages were subsequently pooled for each population (leading to one final RNA pool per population, including L5 larvae, pupae and adults).

2.1.2. Normalisation and construction of the cDNA library

For each of the RNA pools, a full-length enriched, normalised cDNA library was generated using a combination of the SMART cDNA library construction kit (Clontech) and the Trimmer Direct cDNA normalisation kit (Evrogen). We generally followed the manufacturer's protocol except for some important modifications, as described in Vogel et al. (2010). In brief, 2 μg of total RNA was

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