Molecular studies of psocids in China: Recent advances

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DOI: xx.xxxx/xxx.2014.xxx.xxx.xxx

Abstract

In the last two decades, as a new risk for global food security and safety, there is a growing awareness of the important pest status of psocids (Psocoptera: Liposcelididae) due to their feeding on stored grains, contaminating food, and agricultural commodities as well as transmitting harmful microorganisms. Totally, the genus *Liposcelis* includes 126 valid species worldwide, whereas, only 27 species were described in this genus from China. Recently in China, numerous studies have focused on this genus and a series of papers have been published about psocid bionomics, physiology and biochemistry, control measures, and molecular biology. In particular, research on psocids at the molecular levels, including molecular identification of similar species, mechanisms of resistance, population genetic structure, transcriptomics, and mitochondrial (mt) genomes, have had remarkable progress. In this review, we outline the contribution of molecular techniques to clarifying species identification and understanding genetic diversity. The features of the multipartite mt genomes observed in psocids were summarized and we introduced a new concept "mitochondrial chromosome karyotypes" to describe the complexity of animal mt genome. In addition, studies aimed at profiling the psocids transcriptome applied Illumina sequencing technology were also described. These comprehensive transcriptomic databases would further promote our understanding of the molecular mechanisms underlying insecticide resistance or environmental stress, and will facilitate studies on population genetics for psocids, as well as providing useful information for functional genomic research in the future. Meanwhile, we also summarize and discuss the current controversial views on the phylogenetic relationships between psocids and parasitic lice.

Keywords: booklice, resistance, DNA barcoding, transcriptome, mitochondrial genome

1. Molecular Identification

Psocids (especially *Liposcelis* species) have close morphological similarities and different species often occur in the same ecosystems. Therefore, accurate and timely identification to discriminate between different psocid pests is a necessary first step for effective integrated pest management. Molecular based methods provide a means for precise identification of difficult to identify species of both adult and immature stages, and requires less specialized knowledge and skills compared to traditional morphological identification methods. Currently, the mitochondrial DNA and the ribosomal internal transcribed spacers (ITS) region are often used as DNA barcoding for insect species discrimination (Hebert et al., 2003; Yao et al., 2010).

Based on the 16S rDNA gene, PCR-RFLP (Restriction Fragment Length Polymorphism) analysis was used to discriminate four *Liposcelis* species (Qin et al., 2008). The 16S rDNA sequences have been proven to be effective in defining intra-species diversity of *L. bostrychophila* (Li et al., 2011). The mitochondrial gene cytochrome c oxidase I (COI) gene could also be

used to discriminate between *L. entomophila* and *Lepinotus reticulates* (Yang et al., 2012; Arif et al., 2012). A recent study indicated that both the 16S rDNA and COI genes were suitable for identification of seven *Liposcelis* species (Yang et al., 2013). Alternatively, a multiplex PCR method based on ITS2 sequences was used for rapid identification of six psocid species and this was an easier, faster, and more reliable method (Wei et al., 2011a). Results show DNA barcoding has great potential for use in fast and accurate liposcelid identification.

2. Genetic Diversity

To date, population genetic variation for psocids have been investigated using different molecular markers, such as allozymes (Ali and Turner, 2001), randomly amplified polymorphic DNA (RAPDs) (Mikac and Clarke, 2006), and microsatellites (Mikac, 2006; Mikac and FitzSimmons, 2010), as well as mitochondrial and nuclear ITS genes (Shreve et al., 2011; Wei et al., 2012a). Paradoxically, against the background of its obligatory parthenogenetic characteristics, *L. bostrychophila* showed a considerable degree of inter- and intra-population variation (Li et al., 2011; Wei et al., 2012a). In recent studies, the two Liposcelis species, *L. bostrychophila* and *L. entomophila* were sampled from 15 localities in China and analyzed for polymorphisms at the mitochondrial DNA (Cytb) and ITS (ITS1-5.8S-ITS2) regions. In total, 177 individual *L. bostrychophila* and 272 individual *L. entomophila* were analyzed, and both species displayed high genetic diversity within and between populations. Compared to sexually reproducing *L. entomophila*, asexually reproducing *L. bostrychophila* has a higher genetic diversity (Wei et al., 2012a). Additionally, population differentiations were detected and this widespread differentiation was mainly due to other factors, such as genetic drift, inbreeding and less by geographic distance since an Isolation By Distance (IBD) effect was not found on alarge geographic scale (Wei et al., 2012a).

Microsatellites or simple sequence repeats (SSRs) are tandemly repeated motifs of 1-6 genetic base pairs. Because of their ubiquity, neutrality, hyper-variability, co-dominance, sensitivity and high polymorphism, microsatellite markers have become a powerful tool for revealing genetic variation and subsequently the population structure (Zane et al., 2002). In our previous study, microsatellite-enriched libraries of *L. bostrychophila* and *L. entomophila* were constructed utilizing methodologies that exploit the strong affinity between biotin and the protein streptavidin. In total, 13 microsatellite enriched libraries were constructed: 6 for *L. bostrychophila*, and 7 for *L. entomophila*. The number of microsatellites detected for these two species was 260 (Wei et al., 2011b). Comparative analysis of microsatellite sequences for these two psocids revealed that the di-nucleotide repeats microsatellite sequences exist in multiple copies in the psocid genome, and they were found to have similar or almost identical flanking regions (Wei et al., 2011b).

3. Gene Cloning and Expressing Analysis

Psocids have developed high levels of resistance to various insecticides in grain storage systems worldwide and have proven difficult to control because they do not respond to management tactics that have been developed for other stored-product insect pests (Ren et al., 2008; Nayak and Collins, 2008; Nayak, 2010). In order to understand the mechanisms behind the tolerance of psocids to insecticides, studies were done on gene cloning of detoxification and target enzymes (such as AChE, CarE, P450, etc.) and also on RNA expression quantification and heterogeneous expression of functional genes. At present, two full length cDNAs encoding AChE have been cloned and sequenced from, *L. bostrychophila*, *L. entomophila*, *L. decolor* (Tang, 2009; Dou et al., 2010) and one from *L. paeta* (Wu et al.,

2010); Two cDNA encoding nAChR subunits were fully isolated from *L. bostrychophila* (Tang et al., 2009); The cDNAs of five novel P450 genes were sequenced and characterized from *L. bostrychophila* (Jiang et al., 2010a; Jiang et al., 2012); Furthermore, two full length cDNA encoding CarE sequences were cloned from *L. bostrychophila* using RT-PCR and RACE. For target genes, two homologs of sodium channels, *LbVGSC* and *LbSC1*, were identified from the genome of *L. bostrychophila*, and many alternative splicing phenomena were found in the *LbSC1*. The study represents the first steps towards molecular characterization of VGSCs and DSC1 orthologs in Psocoptera, and also provides the first sequences of both channels (Jiang et al., 2013).

The mRNA expression levels of the above genes from *Liposcelis* species in different strains, developmental stages, and under insecticide induction were tested using Quantitative Real-Time PCR (qPCR). For accurate gene quantification analysis, normalization of qPCR data is absolutely essential and normalization is most frequently achieved by the use of internal controls, often referred to as reference genes. For example, four housekeeping genes were cloned to evaluate endogenous references for insecticide-induced and developmental expression profiling in *L. bostrychophila* and the results indicated that *Lbβ-actin1* was an appropriate reference gene for profiling in this species (Jiang et al., 2010b; Jiang et al., 2011); In addition, mRNA relative expression level of *ace 1* gene indicated that there were population differences in organophosphate insecticide susceptibility of *L. paeta* (Wu et al., 2010); For P450 genes, the expression profiles advanced our understanding of the roles of P450s in psocids, suggested that some P450 genes are possibly associated with deltamethrin and paraoxon-methyl metabolism (Jiang et al., 2010a; Jiang et al., 2012); The expression levels of AChE and CarE genes in resistant strains of *L. bostrychophila* were significantly higher than those of a susceptible strain. After exposure to dichlorvos or phosphine, the expression levels of two AChE genes were significantly increased relative to untreated controls (Tang, 2009). Furthermore, the AChE genes expression profiles from other psocids have also been conducted using qPCR. The above results contributed to development of a molecular diagnostic technique for psocid resistance in the field and could help in developing new insecticides and new strategies for pest management, as well as facilitate the study of the role of each gene in the developmental process and in insecticide resistance.

4. Multipartite Mitochondrial Genome

The typical mitochondrial (mt) genome of bilateral animals was a single circular chromosome that is 13 to 20 kb in size and contains 37 genes (Boore, 1999). Presently, the fragmented insect mt genomes are only found in booklice and parasitic lice. To date, three barklice and four booklice (*Liposcelis* species) have been sequenced for complete mt genomes in previous studies. We found that psocids have the most dramatic variation of mt genomes oberved in insects, including genome architecture, gene order, gene content and gene strand asymmetry. Here, based on investigation of the evolutionary features of the multipartite mt genomes in Psocodean species, we introduced a new concept "mitochondrial chromosome karyotypes" to describe the complexity of the mt genome (Wei et al., 2014). According to the sequencing results of the mt genome of *Liposcelis* species, we proposed that the selection pressure for compact mt genomes favors small mt chromosomes when small mt chromosomes co-exist with the typical large mt chromosomes. We speculated that the mitochondrial chromosomes ls have the tendency of reducing their size and the fragmentation of mt genome is one of important strategies to streamline mt genome.

The mt genome of *L. bostrychophila* has fragmented into and been replaced by two medium-sized chromosomes with unequal copy numbers (Wei et al., 2012b). *L. decolor* has its mt

genes on a single chromosome, like most other insects (Chen et al., 2014a). Meanwhile, we found that *L. entomophila* and *L. paeta* also have multipartite mt genomes, like *L. bostrychophila*, with the mt genes we identified on two chromosomes (Chen et al., 2014b). In *L. bostrychophila*, the mt genes are distributed approximately equally between the two chromosomes. However, in *L. entomophila* and *L. paeta*, one mt chromosome has most of the genes whereas the other chromosome has numerous pseudogenes and non-coding regions. Furthermore, the arrangement of mt genes varies extensively among the four *Liposcelis* species; indeed, no gene boundary is shared by *L. paeta* and *L. bostrychophila*. Our results indicate unusually fast evolution in mt genome organization in the genus *Liposcelis*, and reveal different patterns of mt genome fragmentation among *Liposcelis* species. It also seems that the fragmentation of mt genome in *Liposcelis* is likely a recent evolutionary event and that it occurred after *L. decolor* and *L. bostrychophila* split. Thus, we also can conclude that most booklice have a multipartite mt genome which is different from barklice (Li et al., 2013; Shao et al., 2003). The fascinating structures of mt genomes of booklice will also give us invaluable information into the origin and evolution of mitochondria.

Many molecular systematic analyses were carried out to revise or evaluate the controversial phylogeny of different classification levels of psocids (Yoshizawa et al., 2011; Yoshizawa and Johnson, 2008; Yoshizawa et al., 2006; Yoshizawa, 2004; Johnson and Mockford, 2003). However, a more interesting issue is the phylogenic position of the family Liposcelididae. A close relationship between Psocoptera and Phthiraptera (the two "orders" have been recognized as the "superorder Psocodea") has been well established based on both morphological and molecular data sets (Lyal, 1985; Grimaldi and Engel, 2006; Yoshizawa and Lienhard, 2010; Murrell and Barker, 2005). The paraphyly of Psocoptera with regard to Phthiraptera is now widely accepted (Yoshizawa and Lienhard, 2010; Grimaldi and Engel, 2006). Recently, due to the unstable phylogeny status of the Liposcelididae in Psocodea, the evolutionary relationship between parasitic lice and booklice remain phylogenetic enigmatic. The monophyly of the order Phthiraptera was regarded as unresolved. To date, there are two contradictory hypotheses: 1) parasitic lice are monophyletic and booklice, as are the sister-group to the parasitic lice based on morphological data (Lyal, 1985; Grimaldi and Engel, 2006); and 2) parasitic lice are paraphyletic and the booklice are the sister-group to the lice suborder Amblycera based on DNA data sets (Johnson et al., 2004; Murrell and Barker, 2005; Yoshizawa and Johnson, 2010). However, using mitochondrial genome sequences, the phylogeny of booklouse, barklouse and parasitic lice indicated that the order Phthiraptera was monophyletic (Wei et al., 2012b; Li et al., 2013; Chen et al., 2014a; Chen et al., 2014b). Indeed, the exact phylogeny position of Liposcelididae in Psocodea is the key issue to determine the origins and evolution of parasitism in lice. We inferred that parasitism evolved once in the most recent common ancestor (MRCA) of the Anoplura, Rhyncophthirina, Ischnocera, and Amblycera and the order Phthiraptera is monophyletic (Wei et al., 2012b).

5. Transcriptome analysis

As a major stored-product pest insect, psocids have developed high levels of tolerance and possibly even resistance to various insecticides in grain storage systems. However, the molecular mechanisms underlying resistance and environmental stress have not been characterized. Presently, there is a lack of genomic information for psocids. Therefore, studies focuaed on profiling the psocids transcriptome would provide a better understanding of the biological functions at the molecular levels. In our previous studies, we applied Illumina sequencing technology to sequence the transcriptome of *L. entomophila* and *L. bostrychophila* (Wei et al., 2013; Dou et al., 2013). For *L. entomophila*, a total of 54,406,328 clean reads

were obtained and that de novo assembled into 54,220 unigenes, with an average length of 571 bp. For *L. bostrychophila*, we obtained more than 51 million sequencing reads that were assembled into 60,012 unigenes (mean size = 711 bp). All unigenes were further functionally annotated by non-redundant (Nr) protein database, gene ontology (GO), cluster of orthologous groups of proteins (COG), and KEGG orthology (KO).

A large number of genes potentially involved in insecticide resistance were manually curated. For *L. entomophila*, 68 putative cytochrome P450 genes, 37 putative glutathione S-transferase (GST) genes, 19 putative carboxyl/cholinesterase (CCE) genes, and other 126 transcripts containing target site sequences or encoding detoxification genes representing eight types of resistance enzymes were identified. For *L. bostrychophila*, total of 49 P450-, 31 GST- and 21 CES-specific genes representing the three enzyme families were identified. Meanwhile, 16 transcripts were identified to contain target site sequences of resistance genes. Furthermore, we profiled gene expression patterns based on exposure to malathion and deltamethrin using the tag-based digital gene expression (DGE) method for *L. bostrychophila* and 1,100 SSRs and 57,757 SNPs were detected and 231 pairs of SSR primes were designed for *L. entomophila*. The transcriptomes of two psocids and DGE data provide gene expression data that would further our understanding of molecular mechanisms in psocids. In particular, these sequences and putative molecular markers would further promote our understanding of the molecular mechanisms underlying insecticide resistance or environmental stress, and will facilitate studies on population genetics for psocids, as well as providing useful information for functional genomic research in the future.

6. Research gaps and future directions

For the molecular level, studies that explore the genes related to oxidative and heat stress, growth and development, resistance are all important for understanding the ecology and evolution of psocids. Research on proteomics of psocids will also play important role in discovering new resistance and oxidative stress relative proteins. A thorough understanding of the mechanism of genes mediating resistance, further verified by recombinant protein expression of these proteins as well as RNA interference of these genes needs to be conducted. It is unclear if endosymbiotic microorganisms affected the mitochondrial genetic diversity in relation to population genetics. Therefore, it will be necessary to investigate the effects of endosymbiotic bacteria infection on mtDNA variation in psocids. Also, more polymorphic microsatellite DNA markers will be isolated and these microsatellite loci will be used to further study psocid intra- and inter-specific differentiation and gene flow in China and worldwide. Furthermore, the fascinating mt genome architecture of *L. bostrychophila* made us to think two questions below. What drove the fragmentation of a typical mitochondrial chromosome into two or more smaller chromosomes? How could multiple small mitochondrial chromosomes replace a typical large mitochondrial chromosome? These answers could be seen in the light of sequencing more mt genomes in psocids and in functional studies of those genomes.

Acknowledgements

This study was funded in part by the National Natural Sciences Foundation (31301667), the Fundamental Research Funds for the Central Universities, China (XDJK2013C051; XDJK2013A005) to DDW and WD, the Doctor Foundation Project of Southwest University (SWU112046).

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