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The *doublesex* gene integrates multi-locus complementary sex determination signals in the Japanese ant, *Vollenhovia emeryi*

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ABSTRACT

A female diploid, male haploid sex determination system (haplodiploidy) is found in hymenopteran taxa, such as ants, wasps, bees and sawflies. In this system, a single, complementary sex-determination (*sl*-CSD) locus functions as the primary sex-determination signal. In the taxa that has evolved this system, females and males are heterozygous and hemi/homozygous at the CSD locus, respectively. While the *sl*-CSD system enables females to alter sex ratios in the nest, it carries a high cost in terms of inbreeding, as individuals that are homozygous at the CSD locus become sterile diploid males. To counter this risk, some of hymenopteran species have evolved a multi-locus CSD (*ml*-CSD) system, which effectively reduces the proportion of sterile males. However, the mechanism by which these multiple primary signals are integrated and how they affect the terminal sex-differentiation signal of the molecular cascade have not yet been clarified. To resolve these questions, we examined the molecular cascade in the Japanese ant *Vollenhovia emeryi*, which we previously confirmed has two CSD loci. Here, we showed that the sex-determination gene, *doublesex* (*dsx*), which is highly conserved among phylogenetically distant taxa, is responsible for integrating two CSD signals in *V. emeryi*. After identifying and characterizing *dsx*, genotypes containing two CSD loci and splicing patterns of *dsx* were found to correspond to the sexual phenotype, suggesting that two primary signals are integrated into *dsx*. These findings will facilitate future molecular and functional studies of the sex determination cascade in *V. emeryi*, and shed light on the evolution and diversification of sex determination systems in insects.

1. Introduction

Biologists have been challenged to clarify the fundamental basis of the various sex determination mechanisms that have evolved in organisms. While having two sexes is essential for sexually reproducing organisms, primary cues underlying sex determination are highly diversified across species (Bopp et al., 2014). Haplodiploidy is a somewhat peculiar sex determination system that has evolved independently in several animal groups, including nematodes, rotifers, mites, scale insects, thrips, whiteflies, micromalthid beetles, bark beetles and in all member of the insect order Hymenoptera (ants, bees, wasps, and sawflies (Mable and Otto, 1998). In haplodiploidy, males are haploid and develop from unfertilized eggs, while the sterile or reproductive females are diploid and develop from fertilized eggs. The evolutionary significance of haplodiploidy has received considerable attention, both empirically and theoretically, in the literature. One of the features of the system is that, through the haploid males, deleterious mutations can be purged from the population more rapidly than is possible in diplontic populations (Avery, 1984; Goldstein, 1994). In addition, female of haplodiploid species has acquired ability to control sex ratio of offspring by fertilizing or not fertilizing eggs depending on factors, such as resource availability and mating competition (Cremer and Heinze, 2002; Passera et al., 2001; Ratnieks and Keller, 1998). Especially in the Hymenoptera, the ability of taxa to adjust sex ratios is considered to be central to the development of eusociality (Gardner and Ross, 2013). Today, nearly 20% of animal species, including 200,000 hymenopteran species, use haplodiploidy (Crozier and Pamilo, 1996). Therefore, understanding the molecular mechanisms that underlie haplodiploidy is important for understanding why the system has become one of the most pervasive sex determination mechanisms in animals.

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The genetic and molecular components involved in the sex determination cascade of haplodiploid taxa have been well studied in the honeybee *Apis mellifera*, a hymenopteran model organism (Beye et al., 2003; Hasselmann et al., 2008; Nissen et al., 2012). It has been found that a single complementary sex determination locus (*sl*-CSD) is involved in the regulation of sex determination in honeybees, with the heterozygous or hemi/homozygous condition at the CSD locus leading to the production of females and males, respectively (Crozier, 1971; Mackensen, 1951; Woyke, 1963). Since individuals that are homozygous at the CSD locus are typically sterile or inviable, the copy number of CSD, rather than ploidy, is considered to be a key factor underlying sex determination (El Agoze et al., 1994; Stouthamer et al., 1992). Genetic components encoding Csd proteins have been identified in *A. mellifera* (Beye et al., 2003). Experimental crosses of hymenopterans, such as ants and parasitoid wasps, also suggests that the CSD locus is involved in sex determination, since inbreeding crosses lead to the production of diploid males that are homozygous at the CSD locus (Cook, 1993; Krieger et al., 1999) (Fig. S1 A).

Although the *sl*-CSD sex-determination system is considered to be the standard model for sex determination among members of the Hymenoptera (Butcher et al., 2000; Van Wilgenburg et al., 2006; Whiting, 1943, 1939), the cost of inbreeding is high because half of the offspring become sterile diploid males (Crozier, 1971). It has been shown that diploid male production increases the colony mortality (Ross and Fletcher, 1986) and decreases the colony growth rate (Plowright and Pallett, 1979). Consequently, species with *sl*-CSD generally have evolved strategies to avoid inbreeding, such as premating dispersal, single-sex production, or asynchronous hatching of male and female eggs (Cook and Crozier, 1995; Van Wilgenburg et al., 2006). However, inbreeding is not avoidable under situations of low genetic diversity, such as when there is a small mating group size or during the initial phase of invasion of a new area (Ross et al., 1993; Zayed and Packer, 2005).

As a counter strategy to the high cost of inbreeding, modifications in the primary signals of the sex determination cascade have occurred in some hymenopteran species. Previous QTL and bioinformatics analyses demonstrated that Japanese ant *Vollenhovia emeryi* has evolved multilocus CSD system (*ml*-CSD) with two loci (Miyakawa and Mikheyev, 2015). Laboratory inbreeding crosses showed that diploid male production is suppressed to 25% of all offspring, which means that the inbreeding load is half of *sl*-CSD system. In this case, diploid individuals that are heterozygous at least one of the two CSD loci develop into females, whereas individuals that are haploid hemizygous or diploid homozygous at all loci develop into males (Fig. S1 B). Field surveys confirmed that *V. emeryi* colonies have a patchy distribution, and that some of these patches are densely distributed because new colonies are produced by budding after intra-colony mating (Ohkawara et al., 2002). Population genetic analysis revealed that genetic diversity was low at each of the study sites in Japan, as well as in areas that have been invaded (USA) (Miyakawa and Mikheyev, 2015). Therefore, life history strategies, which have evolved in *V. emeryi*, facilitate inbreeding. Today, *ml*-CSD with two or more CSD loci have been reported in some ants and parasitoid wasps (De Boer et al., 2008; Miyakawa and Mikheyev, 2015; Paladino et al., 2015). Empirical and theoretical evidence has shown that the *ml*-CSD system may have a marked effect on reducing the cost of inbreeding. It is also helpful in the early stages of invasion of a new area when the likelihood of genetic bottlenecks arising is high (Crozier, 1971; Weis et al., 2017; Zayed and Packer, 2005). Although *ml*-CSD has spread in Hymenoptera, it is still unclear how multiple primary signals are integrated to produce a terminal sex differentiation signal in the molecular cascade. The maintenance of multiple primary sex determination signals has not typically evolved in other organisms. To address this question, *V. emeryi* provides an excellent model system to clarify the mechanism responsible for integrating the multiple primary signals with the terminal sex determination factor.

One possible genetic component involved in integrating the two independent primary sex determination signals in *V. emeryi* is the *doublesex-mab3* (DMRT) gene family, which is functionally well conserved among phylogenetically distant species (Kopp, 2012; Matson and Zarkower, 2012; Price et al., 2015; Yi and Zarkower, 1999). *Doublesex* (*dsx*) was first described in *Drosophila melanogaster* as terminal transcription factor in the fly sex determination cascade (Burtis and Baker, 1989; Robinett et al., 2010). The encoded protein is characterized by two functional domains, a DM domain and an oligomerization domain (Bayrer et al., 2005). *dsx* is transcribed into sex-specific mRNA of the encoded sex-specific proteins, which regulate downstream sexual differentiation. In *A. mellifera*, heteroallelic *csd* expression leads to the production of Csd proteins that direct female splicing of the downstream gene *feminizer* (*fem*) pre-mRNAs, and the Fem proteins then regulate female splicing of the *dsx* gene downstream (Hasselmann et al., 2008). On the other hand, hemi or homoallelic *csd* causes the absence of Csd protein activity. Consequently, the downstream *fem* and *dsx* pre-mRNAs are spliced into the male isoform. Alternative splicing of *dsx* has also been observed in other hymenopteran species, including ants (Klein et al., 2016; Mine et al., 2017; Shukla and Nagaraju, 2010).

Recent studies shown that *dsx* orthologs are important for controlling sexual dimorphism in organisms that undergo environmental sex determination, such as members of Cladocera (water fleas) (Kato et al., 2011; Taylor et al., 1999; Toyota et al., 2013). Furthermore, alternative splicing of the *dsx* ortholog is not only detected in cases of inter-sex dimorphism, but also in cases of intra-sex dimorphism, such as nutrition-dependent mandibular growth of male stag beetles (Kijimoto et al., 2012). The existence of different *dsx* traits that integrate a variety of upstream signals led us to hypothesize that in hymenoptera with *ml*-CSD including *V. emeryi*, multiple primary CSD signals are integrated by *dsx*, and these signals affect downstream sex differentiation processes.

Here, we investigated whether the *dsx* ortholog that is conserved in various insect species, is involved in sex differentiation in *V. emeryi* with two loci sex determination system. In *V. emeryi*, one of the two QTL regions contains two tandem *tra* homologs, *csd* and *fem* as with honeybee and other ants (Miyakawa and Mikheyev, 2015; Privman et al., 2013; Schmieder et al., 2012). The other QTL region does not contain these orthologs, but the genomic locus containing the QTL region is also conserved. In this study, to confirm whether a sex-specific variant of *dsx* is present in this species, first we obtained the full length of the *dsx* transcript by phylogenic analysis with DM-domain gene and Rapid Amplification of cDNA Ends PCR (RACE-PCR). Expression analyses of *dsx* were then conducted by Reverse Transcription PCR (RT-PCR) and real-time quantitative PCR (qPCR) using specimens from multiple sites. Finally, we conducted expression analysis for each individual produced by laboratory crosses to compare the expected allele patterns of two CSD loci, splicing variants of *dsx*, and sexual phenotypes. It is considered that identification and characterization of *dsx* in *V. emeryi* could help us to clarify the function of *dsx* within a sex determination cascade comprising multiple primary CSD signals.

2. Materials and methods

2.1. Sample preparation

Colonies of *V. emeryi* were collected from Tokyo, Tochigi and