Molecular characterization and evolutionary insights into potential sex-determination genes in the western orchard predatory mite *Metaseiulus occidentalis* (Chelicera: Arachnida: Acari: Phytoseiidae)

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Little is known about the process of sex determination at the molecular level in species belonging to the subclass Acari, a taxon of arachnids that contains mites and ticks. The recent sequencing of the transcriptome and genome of the western orchard predatory mite *Metaseiulus occidentalis* allows investigation of molecular mechanisms underlying the biological processes of sex determination in this predator of phytophagous pest mites. We identified four *doublesex-and-mab-3-related transcription factor* (*dmrt*) genes, one *transformer-2* gene, one *intersex* gene, and two *fruitless*-like genes in *M. occidentalis*. Phylogenetic analyses were conducted to infer the molecular relationships to sequences from species of arthropods, including insects, crustaceans, acarines, and a centipede, using available genomic data. Comparative analyses revealed high sequence identity within functional domains and confirmed that the architecture for certain sex-determination genes is conserved in arthropods. This study provides a framework for identifying potential target genes that could be implicated in the process of sex determination in *M. occidentalis* and provides insight into the conservation and change of the molecular components of sex determination in arthropods.

Keywords: Acari; Arthropoda; Phytoseiidae; phylogenetic analysis; sex determination

1. Introduction

Molecular mechanisms that govern sex determination have received increased attention in arthropods due to the recent advances of molecular approaches. From the production of all-male progeny of flies and beetles (Schetelig, Milano, Saccone, & Handler, 2012; Shukla & Palli, 2012), the feminization of water fleas (Kato, Kobayashi, Watanabe, & Iguchi, 2011), to the elucidation of a mimicry ‘supergene’ in sexually dimorphic butterflies (Kunte et al., 2014), genetic components of the sex-determination pathway have emerged as major factors for development, reproduction, behavior, and sexual dimorphism (Bellefroid et al., 2013; Kopp, 2012). A comprehensive understanding of arthropod sex-determination pathways can be valuable for gaining insight into developmental and evolutionary questions, as well as being directly applicable for novel pest-control programs (Alphey, 2002; Concha & Scott, 2009; Schetelig et al., 2012; Shukla & Palli, 2012).

The sex-determination pathway is best characterized in insects, especially the fruit fly *Drosophila melanogaster*. In *D. melanogaster*: alternative splicing of key regulatory genes into male- or female-specific transcripts results in the production of the male or female phenotype (MacDougall, Harbison, & Bownes, 1995; Nagoshi, McKeown, Burtis, Belote, & Baker, 1988). The primary cue for sex determination in *D. melanogaster* is due not only to the ratio of X chromosomes to autosomes, but to the dose of X-linked genes called X-signaling elements (XSE) (Erickson & Quintero, 2007). The master-switch gene *Sex-lethal* (*Sxl*) regulates the female-specific splicing of the pre-mRNA of *transformer* (*tra*) (Bell, Maine, Schedl, & Cline, 1998). The functional Tra protein interacts with the protein product of the *transformer-2* (*tra-2*) gene, which results in the female-specific splicing of *doublesex* (*d sx*) pre-mRNA.

The *doublesex* gene was first recognized as the major regulator of somatic sexual differentiation in *D. melanogaster* (Baker & Ridge, 1980). Subsequently, the *male abnormal-3* (*mab-3*) gene was identified in the nematode *Caenorhabditis elegans* (Shen & Hodgkin, 1988) and the *dsx* and *mab-3* genes were shown to share molecular and functional similarity. This led to the identification of related genes in vertebrates, which were named *doublesex-and-male abnormal-3-related transcription factor* (*dmrt*) genes (Raymond et al., 1998). The *dmrt* genes appear to function in all animals as tissue-specific transcription factors involved in sex determination as well as...
many other developmental processes (Bellefroid et al., 2013).

The intersex (ix) gene acts near the bottom of the sex-determination cascade in D. melanogaster and is required for somatic sexual development in females (Chase and Baker, 1995). The products of ix function together with the female-specific Dsx (Dsx3) protein and help to activate/repress target genes in a female-specific manner. For instance, the Ix protein interacts with the Dsx3 protein to regulate the expression of yolk protein (yp) genes in D. melanogaster (Garrett-Engele et al., 2002).

In D. melanogaster males, a single X chromosome, or lower dose of XSE, results in the default mode in which the mRNAs of Sxl and tra contain in-frame stop codons so that functional proteins are not produced (Bopp, Bell, Cline, & Schedl, 1991). The absence of functional Sxl and Tra proteins results in the default pathway in which dsx pre-mRNA is spliced into the male-specific form (Dsxm). In the absence of the functional Tra protein in males, the fruitless (fru) gene is expressed in the male neural system where it functions in building neural circuits required for proper courtship and sexual behavior (Anand et al., 2001). Molecular mechanisms involved in sex determination have been characterized to some degree in several other dipterans, including Anastrepha obliqua (Sarno et al., 2010), Anopheles gambiae (Scali, Catteruccia, Li, & Crisanti, 2005), Ceratitis capitata (Salvemini et al., 2009), Lucilia cuprina (Concha & Scott, 2009), and Musca domestica (Burghardt et al., 2005).

Sex-determination mechanisms have been studied in other insects including the red flour beetle Tribolium castaneum (Shukla & Palli, 2012, 2013), the honeybee Apis mellifera (Cho, Huang, & Zhang, 2007; Hasselman et al., 2008), the parasitoid wasp Nasonia vitripennis (Beukeboom & van de Zande, 2010), and the domesticated silkworm Bombyx mori (Suzuki, Suzuki, Aoki, & Ajimura, 2012). In all insects examined, the last gene in the sex-determination pathway is dsx, which is sex-specifically spliced and acts to regulate sexually dimorphic characters (Burtis & Baker, 1989; Shukla & Nagaraju, 2010). The gene tra is a splicing regulator for dsx and is in the key gene around which insect sex-determination mechanisms evolved (Verhulst, Zande, & Beukeboom, 2010).

There are relatively few functional studies on the sex-determination mechanisms of non-insect arthropods. The most comprehensive studies have been carried out in Daphnia magna (Arthropoda: Crustacea: Cladocera), a freshwater branchiopod crustacean with an environmental sex-determination system. D. magna contains two dsx genes, one of which is predominantly expressed in male embryos and male-specific structures (Kato et al., 2011). Knockdown of this dsx gene in D. magna male embryos results in the production of female traits, including ovarian maturation, while ectopic expression of the gene in female embryos results in the development of male-like phenotypes (Kato et al., 2011). Thus, the dsx gene appears to act as the key regulator of sex-specific traits in many arthropods. However, unlike dsx in insects, the Daphnia dsx genes do not encode sex-specific Dsx proteins, but instead exhibit sexually dimorphic differences in the abundance of transcripts. Despite a separation of over 400 million years between insects and crustaceans (Glenner, Thomson, Hebsgaard, Sorensen, & Willerslev, 2006), the dsx gene of D. magna has maintained the domain structure essential for establishing sexual dimorphism.

To date, possible molecular mechanisms for sex determination in chelicerates have only been briefly investigated in one species, the spider mite Tetanychus urticae (Grbić et al., 2011). A potential ortholog of doublesex was reported in the T. urticae genome but other insect sex-determination genes, including complementary sex determinant, transformer and transformer-2, were not found (Grbić et al., 2011). The subphylum Chelicerata split from the other arthropod lineages an estimated 540 million years ago (Rota-Stabelli, Daley, & Pisani, 2013). To our knowledge, no other chelicerate genomes have been evaluated in detail for sex-determination genes.

The western orchard predatory mite Metaseiulus (= Typhlodromus or Galendromus) occidentalis (Chelicerata: Arachnida: Acari: Phytoseiidae) is an economically important natural enemy of phytophagous pest mites in agricultural crops (McMurty & Croft, 1997; Roush & Hoy, 1981). Phytoseiid mites have an unusual genetic system known as parahaploidy in which the female must be fertilized to oviposit and both her male and female progeny begin embryonic development as diploids. During embryogenesis in males, however, the paternal set of chromosomes is eliminated by heterochromatinization and they develop to become functionally haploid adults (Nelson-Rees, Hoy, & Roush, 1980). The role of the paternal chromosomes before their elimination and the genetic basis for sex determination is unknown for phytoseiid mites. The transcriptome and genome of M. occidentalis have been sequenced and annotated (Hoy et al., 2013; Hoy et al., unpublished), allowing investigation of specific molecular components pertaining to this parahaploid mite’s sex-determination system. We expanded our sequence searches to include representative arthropod species from each major extant subphylum, and these data were used to evaluate the architecture and evolution of arthropod sex-determination genes.

2. Materials and methods

2.1. Screening for potential sex-determination genes

Sex-determination genes in D. melanogaster were used as queries in BLASTp and tBLASTn online searches
(Altschul et al., 1997). These searches were conducted against multiple species of insects, crustaceans, and arachnids using available data through the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Other online databases included the Human Genome Sequencing Center to evaluate the centipede Strigamia maritima genome (http://blast.hgsc.bcm.tmc.edu/blast.hgsc?organism=Smaritima) and the Online Research For Community Annotation of Eukaryotes to evaluate the spider mite T. urticae genome (http://bioinformatics.psb.ugent.be/orcae/overview/Tetur). The top BLAST hits for M. occidentalis with E-value scores of 1e-05 or lower were retrieved and used as queries to perform reciprocal BLAST searches against the NCBI non-redundant protein database. Results of the reciprocal BLAST match validated the sequence homology.

### 2.2. Sequence analyses and multiple sequence alignments

Sequences were downloaded from online databases and initially examined with the Geneious software package 6.1.6 (www.geneious.com). Exon-intron boundaries for genes of interest were confirmed by aligning the candidate gene sequences with their corresponding genomic DNA sequences with the Spidey program (www.ncbi.nlm.nih.gov/spidey/). Tertiary protein structure analyses were carried out using Phyre2 (Kelley & Sternberg, 2009) for known sex-determination genes in D. melanogaster and potential homologs in M. occidentalis. Multiple alignments of the amino-acid sequences were constructed using MAFFT 7.147 (Katoh & Standley, 2013) with the E-INS-i alignment algorithm and the BLUSUM 62 matrix. A complete list of sequences used in this study can be found in Supplementary Tables S1–S4. PartitionFinderProtein v1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012) was used to determine the best-fit model of molecular evolution for each protein alignment.

### 2.3. Phylogenetic analyses

Phylogenetic analyses were conducted on the amino-acid data-set of each sex-determination gene in a maximum likelihood (ML) framework using RAxML 7.3.2 (Stamatakis, 2006) implementing the optimal partitions estimated in PartitionFinderProtein. Bootstrap analyses that included 1000 bootstrap replications were applied to assess confidence in nodal support. We also conducted Bayesian phylogenetic analyses using MrBayes v3.2.2 (Ronquist et al., 2012) estimated with the model of amino-acid substitution produced from PartitionFinderProtein. Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed with one cold and three heated chains. Starting trees were random and were performed for one million generations, sampling every 100 generations, until the average standard deviation of split frequencies dropped below 0.01. Nodal confidence for the trees was assessed using node posterior probabilities.

### 3. Results and discussion

#### 3.1. Potential sex-determination genes identified in M. occidentalis

M. occidentalis shares homologs of somatic sex-determining genes lower in the model insect D. melanogaster pathway (transformer-2, doublesex, intersex, and fruitless) but does not contain upstream sex-determining genes utilized by D. melanogaster (Sex-lethal, transformer) (Table 1). The presence of genes lower in the sex-determination pathway and absence of genes higher in the pathway supports the postulation by Wilkins (1995) that sex-determination pathways evolve from the bottom up. While the putative M. occidentalis genes in this study have been identified and characterized, their biological function(s) in this species remains to be determined. The following discussion sections are organized by gene, starting with the dmrt genes, followed by tra-2, ix, and finally fru.

#### 3.2. doublesex-and-male abnormal 3-related transcription factor (dmrt)

We identified four distinct dmrt genes in M. occidentalis (Moccdmrt99B, Moccdmrt11E, Moccdsx1-like, and Moccdsx2-like) (Table 1). All Dmrt amino-acid sequences in M. occidentalis contain a single conserved DNA-binding motif known as the DM domain. One Dmrt sequence in M. occidentalis contains both the DM domain and a second motif that is known as the DMA domain (Table 1). The DM motif is a cysteine-rich DNA-binding domain that contains an intertwined CCHC and HCCZn2+-binding site and a putative nuclear localized signal consisting of KGHKR (Figure 1) (Zhu et al., 2000).

The Moccdmrt99B mRNA is 1,422 nucleotides (473 amino acids) in length and contains both a DM domain (in the amino-acid sequence positions 41–78) and a DMA domain (amino-acid sequence positions 287–321) (Supplementary material Figure S1(A)). A multiple sequence alignment of Moccdmrt99B to insect and crustacean Dmrt amino-acid sequences reveals that the DM domain in M. occidentalis shares 99% sequence identity to D. magna Dmrt99B and 98% identity to D. melanogaster Dmrt99B (Figure 1). The ML and Bayesian analyses reveal a distinct cluster of these Dmrt99B sequences from arthropods with bootstrap support of 78% and a posterior probability value of 0.95 (Figure 2). Predicted tertiary structure similarity to D. melanogaster Dmrt99B (Figure 3(A)
and (B)) and phylogenetic placement of this Dmrt sequence in *M. occidentalis* supports orthology to the arthropod Dmrt11E proteins.

The *Moccdmrt11E* mRNA is 1,269 nucleotides (422 amino acids) and contains a single DM domain (aa sequence positions 257–294) (Supplementary material Figure S1(B)). The DM domain shares 80% pairwise identity to the DM domain in *D. magna* Dmrt11E and 74% pairwise identity to the *D. melanogaster* Dmrt11E (Figure 1). The ML and Bayesian analyses reveal a distinct cluster of this *M. occidentalis* Dmrt sequence with other arthropod Dmrt11E sequences with 98% bootstrap support and a posterior probability of 1.0 (Figure 2). Predicted tertiary structural similarity to *D. melanogaster* Dmrt11E (Figure 3(C) and (D)) and phylogenetic placement of this *M. occidentalis* Dmrt sequence supports orthology to the arthropod Dmrt11E proteins.

### Table 1. Potential sex-determination genes identified in the *M. occidentalis* genome.

| Gene name          | mRNA accession no. and size (nucleotides) | Protein accession no. and size (amino acids) | Domain(s) and Pfam ID | Genomic scaffold accession and range |
|--------------------|------------------------------------------|---------------------------------------------|-----------------------|-------------------------------------|
| *Moccdmrt99B*      | XM_003742476.1 1422 nt                   | XP_003742524.1 473 aa                      | DM (PF00751), DMA     | NW_003805392.1 259,891–250,943 (inverse) |
| *Moccdmrt11E*      | XM_003748104.1 1269 nt                   | XP_003748152.1 422 aa                      | DM (PF00751)          | NW_003805515.1 1347,331–1340,247 (inverse) |
| *Moccdmrt11E-like* | XM_003740382.1 945 nt                    | XP_003740430.1 314 aa                      | DM (PF00751)          | NW_003804921.1 392,749–391,805 (inverse) |
| *Moccdmrt11E-like* | XM_003740381.1 888 nt                    | XP_003740429.1 295 aa                      | DM (PF00751)          | NW_003804921.1 375,405–376,292 |
| *Moccdsx1*         | XM_003746965.1 1329 nt                   | XP_003747013.1 242 aa                      | Med29 (PF11568)       | NW_003805180.1 1368,958–1373,140 |
| *Moccdsx2*         | XM_003741762.1 808 nt                    | XP_003741810.1 236 aa                      | BTB (PF00651)         | NW_003804532.1 394,039–395,218 |
| *Moccdsx2-like*    | XM_003739471.1 1785 nt                   | XP_003739519.1 380 aa                      | BTB (PF00651)         | NW_003805485.1 451,120–444,472 (inverse) |
| *Moccdsx2-like*    | XM_003746525.1 1315 nt                   | XP_003746573.1 401 aa                      | BTB (PF00651)         | NW_003805485.1 729,547–731,969 |

Note: Sequences in *M. occidentalis* were obtained from NCBI GenBank and were given abbreviated names for convenience in this study. Genes from *M. occidentalis* are designated with an *Mocc-* prefix.
The remaining two dmrt sequences in *M. occidentalis* (MoccDsx1-like and MoccDsx2-like) encode a single DM domain (aa sequence positions 199–245 and 177–219, respectively). They are found on the same genomic scaffold and consist of a single exon with no intron (Supplementary material Figure S1(C)). MoccDsx1-like and MoccDsx2-like share 69% aa sequence identity within the DM domain to one another, 60 and 46% sequence identity to *D. melanogaster* Dsx, respectively, and 50 and 33% sequence identity to *D. magna* Dsx, respectively (Figure 1). ML and Bayesian analyses reveal that these two Dmrt sequences in *M. occidentalis* cluster together with 98% bootstrap support and a posterior probability of 0.98 and group near the insect and crustacean Doublesex sequences (Figure 2). Due to the tertiary structural similarity to *D. melanogaster* Dsx.
(Figure 3(E)–(G)) and phylogenetic placement, these genes are here named Moccdsx1-like and Moccdsx2-like, and could represent a case of gene duplication.

Interestingly, D. magna contains two dsx genes that consist of single coding exons and are located on the same genomic scaffold (Kato et al., 2011). These two dsx genes are thought to have arisen by duplication specific to the Cladocera, an order of small crustaceans commonly known as water fleas (Toyota et al., 2013). In addition to the two Dsx-like sequences in M. occidentalis, two Dsx-like sequences were also recovered for the tick I. scapularis, the spider mite T. urticae, and the centipede S. maritima (Figure 2). Both ML and Bayesian analyses supported the Daphnia Dsx sequences as sister to the insect Dsx sequences, but the precise relationships of the acarine and centipede Dsx-like sequences to one another and to the insect/crustacean Dsx sequences were not clearly resolved, as support for these nodes were low (Figure 2).

Thus far, all metazoan species have been found to contain multiple DM-domain genes, and members of this gene family act as transcription factors that play roles in sex determination and sexual differentiation (Volff, Zarkower, Bardwell, & Schartl, 2003). D. melanogaster contains four DM-domain genes, mammals contain eight, and the nematode C. elegans holds the current record with 11. Interestingly, the spider mite T. urticae genome appears to contain at least eight DM-domain genes. The M. occidentalis genome appears to contain four distinct DM-domain genes that cluster into three dmrt groups that are present in insects and crustaceans (dmrt99B, dmrt11E, and dsx) (Figure 2). A fourth dmrt group named dmrt93B is present in D. melanogaster, but no apparent ortholog was found in M. occidentalis. High sequence conservation within the functional DNA-binding domain (DM domain) (Figure 1) and phylogenetic groupings (Figure 2) suggest that dmrt genes in M. occidentalis could act as transcriptional activators or repressors for functions related to sex determination and sexual differentiation, as they do in other arthropod species.

3.3. transformer-2 (tra-2)

We identified one transformer-2 gene in the M. occidentalis genome (Mocctra-2) (Table 1). Mocctra-2 harbors four exons and three introns, the mRNA consists of 1329 nucleotides, and there are 242 aa (Supplementary material Figure S2). Database searches also identified Tra-2 protein sequences in the ticks Amblyomma maculatum, A. variegatum, I. scapularis, and R. pulchellus, and the centipede S. maritima (Supplementary material Table S2). MoccTra-2 contains a single RRM domain (RNA Recognition Motif) (aa positions 89–160), two
arginine-/serine-rich regions on either sides of the RRM domain (aa 19–61 and aa 224–240), and has two glycine-rich regions (aa 185–190 and aa 217–223) (Figure 4). Within the RRM are two ribonucleoprotein (RNP) elements, RNP-1 and RNP-2, which are involved in RNA recognition (Amrein, Hedley, & Maniatis, 1994).

Multiple sequence alignment of MoccTra-2 with the Tra-2 proteins from other arthropods showed a high degree of aa sequence conservation within the RRM and Linker domain (Figure 4). The region encoding the RRM and Linker region of MoccTra-2 shares 72% pairwise aa sequence identity with the centipede S. maritima (SmarTra-2), 67% aa identity with the crustacean D. pulex (DpulTra-2), and 60% with D. melanogaster (DmelTra-2) (Figure 4). The aa sequences for the RRM domain and neighboring regions aligned well, but other parts of the Tra-2 aa sequences share less homology. The ML and Bayesian analyses reveal that the tick Tra-2 sequences form a distinct group with 99% bootstrap support and posterior probability of 1.0 (Figure 5). The Phyre2 program predicted similar tertiary structures for D. melanogaster Tra-2 and M. occidentalis Tra-2 (Figure 6(A) and (B)). MoccTra-2 groups with the tick and the centipede Tra-2 sequences, although bootstrap and posterior probability support were low, while the insect and Daphnia Tra-2 sequences form separate branches (Figure 5).

Transformer-2 belongs to a serine/arginine-rich protein family and is required for female sex determination in somatic cells and for spermatogenesis in male germ cells in D. melanogaster (Amrein, Maniatis, & Nothiger, 1990). The function of tra-2 has been documented in several insect species through RNA interference (RNAi) studies. RNAi of tra-2 in the housefly M. domestica and the fruit flies, Anastrepha suspensa, A. obliqua, and C. capitata showed that Tra-2 proteins are involved in female-specific splicing of transformer (tra) pre-mRNAs (Burghardt et al., 2005; Salvemini et al., 2009; Sarno et al., 2010). Knockdown of tra-2 in early embryos of B. mori led to morphological abnormalities in the testes (Suzuki et al., 2012). In T. castaneum, RNAi of tra-2 in female pupae resulted in the male-specific splicing of the dsx transcript and both male and female forms of tra, which suggests that Tra-2 is implicated in female-specific splicing of dsx and tra transcripts, and knockdown of tra-2 in embryos was lethal (Nissen, Muller, & Beye, 2012).

The tra-2 gene was isolated in the crustaceans Daphnia pulex, the giant tiger shrimp Penaeus monodon (Leelatanawit et al., 2009), and the oriental river prawn Macrobrachium nipponense (Zhang et al., 2013). In P. monodon, expression of tra-2 was not significantly different in ovaries and testes. In M. nipponense, tra-2 mRNA was found to be widely distributed in adult ovaries and testes. The function of tra-2 in female sex determination in M. nipponense and its implications for female-specific splicing of transformer (tra) pre-mRNAs, and its role in female sex determination in several insect species through RNA interference (RNAi) studies, and its role in female sex determination in several crustacean species through RNA interference (RNAi) studies.
tissues, and was more highly expressed in testes than in the ovary.

Taken together, the architecture of \( \text{tra}^{-2} \) appears to be conserved across arthropod evolution and is involved in sex determination in insects, but an apparent \( \text{tra}^{-2} \) homolog was not identified in the spider mite \( T. \ urticae \) genome (Grbić et al., 2011). The \( \text{tra}^{-2} \) gene appears to be present in \( M. \ occidentalis \) and the ticks \( I. \ scapularis, A. \ maculatum, R. \ pulchellus \) (identiﬁed in this study), although it is unknown what biological functions \( \text{tra}^{-2} \) plays in these acarine species.

### 3.4. \textbf{intersex (ix)}

We identiﬁed one \( \text{ix} \) gene in the \( M. \ occidentalis \) genome (\( Moccix \)) (Table 1). The \( Moccix \) mRNA is 808 nucleotides, there are 236 aa, and \( Moccix \) harbors three exons and two introns (Supplementary material Figure S3). Database searches also identiﬁed \( \text{ix} \) sequences for the ticks \( A. \ maculatum \) and \( R. \ pulchellus \), the spider mite \( T. \ urticae \), and the centipede \( S. \ maritima \) (Supplementary material Table S3). The deduced aa sequence of \( \text{ix} \) in \( M. \ occidentalis \) was aligned to \( \text{ix} \) sequences from selected arthropod species (Figure 7). The multiple alignments revealed that the \( Moccix \) full-length aa sequence shares 32% pairwise identity to the centipede \( S. \ maritima \), 24% identity to the crustacean \( D. \ pulex \), and 28% identity to the \( D. \ melanogaster \) \( \text{ix} \) proteins. Alignments of \( \text{ix} \) sequences show that \( Moccix \) contains regions high in proline, glycine, glutamine, and serine amino acids, which is characteristic of insect \( \text{ix} \) proteins (Figure 7).
Both ML and Bayesian analyses revealed that MoccIx grouped most closely with the Ix sequences from ticks, followed by the two Ix sequences identified in *T. urticae* with bootstrap support of 100% and a posterior probability of 1.0 (Figure 8). Both analyses reveal that Ix sequences in insects formed a distinct cluster, with the *Daphnia* Ix as the sister group. The Phyre2 program predicted similar tertiary structures for *D. melanogaster* Ix and *M. occidentalis* Ix with a high degree of alpha-helix turns (Figure 6(C) and (D)). Based on aa structure and phylogenetic relationships, it appears that we have identified an *ix* ortholog in *M. occidentalis*, which we named Moccix.
In *Drosophila*, the Ix protein interacts with the female-specific protein product of *dsx* (*Dsx*). The *ix* gene likely encodes a transcription factor with no known DNA-binding domain and the interaction of Ix and DsxF affect female differentiation such as regulation of yolk protein genes (Garrett-Engele et al., 2002). Additionally, an *ix* ortholog was examined in the silkworm *B. mori* and appears to have retained its functional conservation in sexual differentiation at the bottom of the sex-determination pathway (Arunkumar & Nagaraju, 2011). The architecture for *ix* seems to be conserved across arthropod evolution and is involved in sex determination in insects, but the biological roles of *ix* in *M. occidentalis* and other non-insect arthropods is not yet known.

### 3.5. *fruitless* (*fru*)

We identified two genes in *M. occidentalis* that contain conserved elements of the *fruitless* gene in *D. melanogaster* (Table 1). Database searches also identified structurally related sequences in the tick *I. scapularis*, the spider mite *T. urticae*, and the centipede *S. maritima* (Supplementary material Table S4). The aa sequences contain a conserved region known as the Broad complex, Tramtrack and Bric-a-brac (BTB) domain (Zollman, Godt, Prive, Couder, & Laski, 1994). This region of about 120 aa is present in a large family of transcriptional regulator proteins such as *fruitless*, *bric-a-brac* (*bab*), *broad complex* (*brc*), *longitudinals lacking* (*lola*), and *tramtrack* (*ttk*). One sequence in *M. occidentalis* has an mRNA sequence of 1785 nucleotides, coding for 380 aa; it contains a BTB domain (aa positions 23–122) and was named *MoccBTB1* (Supplementary material Figure S4(A)). The second *fru*-like gene has an mRNA sequence of 1315 nucleotides, coding for 401 aa, and contains a BTB domain (aa positions 22–118); it was named ‘*MoccBTB2’* (Supplementary material Figure S4(B)). The BTB domain of MoccBTB1 and MoccBTB2 is about 120 aa and is located near the N-terminal, which is characteristic of other BTB-domain zinc-finger DNA-binding proteins (Ito et al., 1996).

The MoccBTB1-deduced aa sequence within the BTB domain shares the highest sequence identity to the *I. scapularis* BTB1 (75%), followed by *M. occidentalis* BTB2.
S. maritima BTB1 (68%), I. scapularis BTB2 (68%), T. urticae BTB1 (68%), and T. urticae BTB2 (64%) (Figure 9). A comparison of the aa sequences within the BTB domains in M. occidentalis to those in D. melanogaster revealed that MoccBTB1 shares 59% sequence identity to D. melanogaster Tramtrack (DmelTtk), 57% sequence identity to Longitudinals lacking (DmelLola), 54% to Fruitless (DmelFru), and 53% sequence identity to Broad complex (DmelBrc). MoccBTB2 shares 61% aa sequence identity to DmelTtk, 58% sequence identity to DmelLola, 55% sequence identity to DmelBrc, and 51% sequence identity to DmelFru (Figure 9). The Phyre2 program predicted similar tertiary structures for D. melanogaster Fru and M. occidentalis BTB1 and BTB2 (Figure 6(E)-(G)).

The ML and Bayesian analyses revealed that MoccBTB1 and MoccBTB2 are grouped with BTB-domain sequences identified in other mite and tick species in this study (Figure 10). The Fruitless proteins in insects and Daphnia form a distinct cluster, while other BTB-domain proteins in insects form distinct clusters of Longitudinals lacking (Lola), Tramtrack (Ttk), and Broad complex (Brc). The BTB-domain sequences of M. occidentalis do not cluster with the Fruitless or other BTB-domain proteins in insects suggest that we have identified novel BTB-domain genes within the Acari (Figure 10). Based on the ML and Bayesian analyses, it appears that the diversification of the BTB-domain genes into distinct Fru, Lola, Brc, and Ttk families occurred after the split between mandibulate and chelicerae arthropods.

A genetic hierarchy governs many aspects of male behavior in D. melanogaster in which fruitless acts as the top regulatory gene. Male courtship behavior in D. melanogaster is an elaborate ritual that involves male vs. female orientation, males tapping the female with their forelegs, singing by vibrating the wings, licking the female genitalia and curling of the abdomen for copulation (Hall, 1994), and loss of fruit disrupts this process (Villella et al., 1997). Orthologs of fruit have been isolated in several insect species including grasshoppers (Ustinova & Mayer, 2006) and cockroaches (Clynen, Ciudad, Belles, & Piulachs, 2011). RNAi studies in grasshoppers and cockroaches showed that fruit is important in regulating successful copulation and sexual behavior in adult males and suggests that the function of the fruit gene as master regulator of male sexual behavior is conserved over insect evolution (Boerjan, Tobback, De Loof, Schoofs, & Huybrechts, 2011; Clynen et al., 2011).

M. occidentalis males display clear mating and sexual behaviors. Males exhibit a distinct ‘hovering’ behavior over female deutonymphs, likely in response to a sex pheromone (Hoy & Smilanick, 1979). Upon encountering a female, the male repeatedly touches the female with his forelegs and palps, climbs on top of the female’s dorsum and drums on her with his forelegs and palps, climbs underneath her and into a venter-to-venter position, and then appears to transfer spermatophores via the spermatodactyl on his chelicerae from his genital opening to one or both of the sperm induction pores of the female (Hoy & Cave, 1985). To date, there is no information regarding genetic factors that could be involved in male courtship behavior in M. occidentalis. Because BTB-domain genes are diverse (they are known to be present in over 40 protein families), it is unclear whether the BTB-domain genes identified in M. occidentalis play a role in male courtship behavior. Expression and functional studies are required to determine the biological roles of MoccBTB1 and MoccBTB2.
4. Conclusions

The Arthropoda is the most speciose phylum of animals with estimates of 5–10 million species (Odegaard, 2000). The phylum can be divided into four main extant subphyla: Hexapoda (includes the insects), Crustacea (crabs, lobsters, crayfish, shrimp, and krill), Myriapoda (centipedes and millipedes), and Chelicerata (spiders, scorpions, horseshoe crabs, mites, and ticks). The Chelicerata and the Mandibulata (consisting of Hexapoda, Crustacea, and Myriapoda) split over 540 mya (Rota-Stabelli et al., 2013). Our current understanding of the molecular processes underlying the sex-determination pathway in arthropods is confined to a handful of insects and even fewer crustaceans. We identified and characterized putative sex-determination genes in the western orchard predatory mite, *M. occidentalis*, by screening the genome and transcriptome with gene sequences known to be involved in sex determination in insects and other arthropods. We identified four *doublesex-and-mab-3-related transcription factor* (*dmrt*) genes, one *transformer-2* gene, one *intersex* gene, and two *fruitless*-like genes in *M. occidentalis* and conducted phylogenetic analyses to infer their molecular relationships to sequences in other arthropod species.

Figure 10. ML and Bayesian consensus tree of the BTB-domain proteins in selected arthropod species: *M. occidentalis* (MoccBTB1, MoccBTB2), *T. urticae* (TurtBTB1, TurtBTB2), *I. scapularis* (IscapBTB1, IscapBTB2), *Amblyomma variegatum* (AvarBTB), *S. maritima* (SmarBTB1, SmarBTB2), *D. melanogaster* (DmelFru, DmelBrc, DmelTtk, and DmelLola), *N. vitripennis* (NvitFru, NvitBrc, Nvit Ttk, and NvitLola), *B. mori* (BmorFru, BmorBrc, and BmorTtk), *T. castaneum* (TcasFru, TcasBrc, TcasTtk, and TcasLola), and *D. pulex* (DpulFru). Sequences from mites and ticks are highlighted in red, the centipede is in green, the insects are in blue, and *Daphnia* is in orange. The amino-acid substitution model used was the JTT model. The numbers above or below branches indicate branch support: bootstrap values are on the left and posterior probabilities are on the right. Hyphens indicate branches that had a posterior probability <0.5. The scale bar represents substitutions per site. The BTB-domain proteins separate into distinct groups: Broad complex (*Brc*), Fruitless (*Fru*), Longitudinals lacking (*Lola*), and BTB-domain proteins from acarine species and *S. maritima*. The BTB sequences identified in *M. occidentalis* are boxed. Accession numbers for BTB sequences are in Table S4.
To our knowledge, this is the first study in which the conservation and evolution of genes putatively involved in the process of sex determination (dmrt, tra-2, ix, and fru) have been expanded to include members of the four extant subphyla of the Arthropoda. Future work will be conducted on the expression and function of the candidate genes in an attempt to elucidate components of the sex-determination pathway in M. occidentalis and could have implications for genetic improvement of a predatory mite that is used in biological control programs in agriculture.

Supplementary material
The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2014.941402.

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