Evidence for involvement of a transformer paralogue in sex determination of the wasp Leptopilina clavipes

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Abstract

Transformer (tra) is the central gear in many insect sex determination pathways and transduces a wide range of primary signals. Mediated by transformer-2 (tra2) it directs sexual development into the female or male mode. Duplications of tra have been detected in numerous Hymenoptera, but a function in sex determination has been confirmed only in Apis mellifera. We identified a tra2 orthologue (Lc-tra2), a tra orthologue (Lc-tra) and a tra paralogue (Lc-traB) in the genome of Leptopilina clavipes (Hymenoptera: Cynipidae). We compared the sequence and structural conservation of these genes between sexual (arrhenotokous) and asexual all-female producing (thelytokous) individuals. Lc-tra is sex-specifically spliced in adults consistent with its orthologous function. The male-specific regions of Lc-tra are conserved in both reproductive modes. The paralogue Lc-traB lacks the genomic region coding for male-specific exons and can only be translated into a full-length TRA-like peptide sequence. Furthermore, unlike LC-TRA, the LC-TRAB interstrain sequence variation is not differentiated into a sexual and an asexual haplotype. The LC-TRAB protein interacts with LC-TRA as well as LC-TRA2. This suggests that Lc-traB functions as a conserved element in sex determination of sexual and asexual individuals.

Keywords: transformer orthologue, transformer-2, Hymenoptera, protein interactions, reproductive modes

Introduction

Sex determination is a ubiquitous developmental process in eukaryotes. It entails the differentiation of two sexual functions and leads to the development of female and male morphologies and behaviours. Being a basic developmental process, sex determination may be expected not to tolerate modifications in the underlying developmental pathway as these would disrupt the correct specification of the two sexes. Sex determination is nevertheless characterized by a wide variety of fast-evolving mechanisms, including duplication and subsequent recruiting of sex-determining genes (Beukeboom and Perrin, 2014; Herpin and Schartl, 2015).

Insects constitute a particularly suitable group for studying the regulation of sex determination as they have shown rapid turnover in sex determination mechanisms. The signal-transducing elements of their sex determination cascade are well conserved, but they exhibit a wide variety of upstream signals (Bopp et al., 2014). A hallmark of insect sex determination is sex-specific splicing of the transducing genes transformer (tra) and doublesex (dsx). The male splice variants of tra include exons with in-frame early STOP-codons, resulting in a truncated TRA protein. The female splice variants code for a TRA protein that belongs to a class of SR-type proteins characterized by regions rich in arginines (R) and serines (S). Despite its conserved function, tra displays high sequence divergence amongst insects, possibly as a result of accommodating many different upstream primary signals in the cascade (Verhulst et al., 2010b). It contains a number of distinctive domains of which the most conserved is the Ceratitis-Apis-Musca (CAM) domain, which is believed to implement the...
autoregulatory splicing loop of tra (Hediger et al., 2010). It has been found in all investigated tra orthologues, including those of Tribolium castaneum, Apis mellifera, Nasonia vitripennis, Asobara tabida and various dipterans, with the exception of drosophilids (Pane et al., 2005; Lagos et al., 2007; Ruiz et al., 2007; Hasselmann et al., 2008a; Hediger et al., 2010; Verhulst et al., 2010b; Saccone et al., 2011; Shukla and Palli, 2012; Geuverink et al., 2018). TRA also possesses order-specific domains, one of which is only shared amongst the Diptera (the DIP domain) and another that is only present in the Hymenoptera (the HYM domain) (Verhulst et al., 2010b). Despite the rapid evolution of sex determination cascades, most insect species have a functionally conserved tra orthologue as the transducer of the primary signal.

TRA and Transformer-2 (TRA2) form a complex that controls the sex-specific splicing of dsx pre-messenger RNA (pre-mRNA; Nagoshi et al. 1988; Nagoshi and Baker, 1990; Hedley and Maniatis, 1991; Hoshijima et al. 1991; Inoue et al. 1992; Tian and Maniatis, 1992, 1993; Amrein et al., 1994). TRA and TRA2 are essential components of the autoregulatory tra loop and required for splicing of dsx transcripts in T. castaneum, various dipterans, Ap. mellifera and N. vitripennis (Pane et al., 2002; Lagos et al., 2007; Ruiz et al., 2007; Hasselmann et al., 2008a; Salvemini et al., 2009; Sarno et al., 2010; Nissen et al., 2012; Shukla and Palli, 2012; Shukla and Palli, 2013; Liu et al., 2015; Geuverink et al., 2017). Tra2 can be transcribed either into a single isoform or into multiple isoforms that each code for an RNA binding domain (RBD) flanked on both sides by arginine-serine rich regions (Burghardt et al., 2005; Niu et al., 2005; Concha and Scott, 2009; Salvemini et al., 2009; Sarno et al., 2010; Martin et al., 2011; Nissen et al., 2012; Schetelig et al., 2012; Shukla and Palli, 2013; Liu et al., 2015).

Tra is considered to be ancestral to the holometabolous insects based on its conserved domains (Verhulst et al., 2010b; Geuverink and Beukeboom, 2014). However, tra orthologues appear absent in some insect groups, notably the Lepidoptera (Salvemini et al., 2013; Geuverink and Beukeboom, 2014). In the order of the Hymenoptera, tra has been found in nearly all families [except Athalia rosae, which represents the basal lineage of Tenthredinoidea (Mine et al., 2017)]. In addition, paralogues of tra have been identified in multiple branches of the Aculeata (Schmieder et al., 2012; Privman et al., 2013; Koch et al., 2014). A sex-determining function of a tra parologue has thus far only been documented in Ap. mellifera, where it constitutes the complementary sex-determining locus (csd) (Beye et al., 2003; Hasselmann et al., 2008a). Interestingly, tra paralogues have been found predominantly in families that have tested positive for a complementary sex determination system (CSD), although a recent study has demonstrated tra paralogues in the suggested non-CSD clade of Chalcidoidea (Jia et al., 2016).

All Hymenoptera reproduce by haplodiploidy. Their sexual mode of reproduction is arrhenotoky, in which haploid males develop from unfertilized eggs and diploid females from fertilized eggs. A large number of hymenopteran species are known to reproduce by thelytoky. Thelytokous females produce diploid females from unfertilized eggs parthenogenetically, without a paternal genome contribution. Thelytoky can be the result of infection with endosymbionts, but can also be caused by nuclear factors (Stouthamer, 1997; Lattorff et al., 2005; Sandrock and Vorburger, 2011). The manipulation of host reproduction by endosymbiotic bacteria, such as Wolbachia and Cardinium, is widespread amongst arthropods, in particular amongst Hymenoptera (O'Neill et al., 1997).

In the wasp Leptopilina clavipes (Hymenoptera; Cynipidae) both arrhenotokous and Wolbachia-infected thelytokous populations exist (Pannebakker et al., 2004b; Kraaijeveld et al., 2011). The cytological mechanism of thelytokous reproduction is gamete duplication, i.e. diploidy is restored by skipping the first mitotic anaphase division (Pannebakker et al., 2004a). This results in identical chromosome pairs and thus complete homozygosity. In northern Europe, L. clavipes populations are fixed for Wolbachia infection, meaning that they consist of infected thelytokous females only. Conversely, several southern European populations lack this Wolbachia infection, and reproduce sexually. Theory predicts that genes with sexual function will degenerate through accumulation of deleterious mutations under asexual reproduction (Kraaijeveld et al., 2016). Potential divergence or decay of the sex determination cascade in thelytokous systems has, however, not yet been studied. Furthermore, how endosymbionts achieve their host manipulation is poorly known and requires more knowledge of hymenopteran sex determination mechanisms. The L. clavipes system provides this opportunity because the sequences and regulation of sex determination genes can be directly compared between arrhenotokous and thelytokous individuals.

Here we investigate whether and how tra and tra2 func-
ction in the sex determination cascade of arrhenotokous and thelytokous lineages of L. clavipes. We also screen for paralogues of both genes in both lineages. Splicing patterns of the tra orthologue, parologue and tra2 are compared in both reproductive modes. Genes are expected to degenerate if they have no function in a particular reproductive mode. Therefore, if genes are only degenerated in thelytokous wasps this would suggest a loss of a sex determining related function at the onset of asexuality induction. However, if these genes are conserved in both reproductive modes and their proteins interact, it would indicate an active function in sex determination. An interaction between TRA and TRA2 is hypothesized.
to occur as a requirement for female development. Based on our results, a model for the sex determination system of *L. clavipes* will be presented and compared to known mechanisms within the Hymenoptera.

**Results**

*Identification of tra homologues and their structure in L. clavipes*

Two homologous sequences of *tra* were found in the *L. clavipes* reference genome assembly [from the thelytokous GBW strain (Kraaijeveld et al., 2016)], in two genomic scaffolds (scf7180005166757 and scf71800051664248). The two homologues shared 90.5% identity in their coding region sequence. The two homologues could also be detected in the arrhenotokous wasps by rapid amplification of cDNA ends-PCRs (RACE-PCRs) and reverse-transcription (RT-)PCRs. Although the two loci have a distinctly different genomic structure (Fig. 1), they code for similar mRNA sequences. The gene in scf7180005166757 has sex-specific splice variants that match those of known *tra* genes with a function in sex determination. The female splice variant codes for a peptide of 417 amino acids with all known functional domains of hymenopteran *tras*: the HYM domain, the CAM domain, an arginine-serine (RS)-region and a proline-rich region (Fig. 2). The predominant male-specific splice variant contains a premature STOP-codon shortly after the HYM domain, resulting in a 242-amino-acid protein. Another, less abundant male-specific splice variant...
variant contains a STOP-codon at the same position, but merges the sixth and seventh exons (Fig. 2). Based on these observations we concluded that the gene in scf7180005166757 is the \textit{tra} orthologue and named it \textit{Lc-tra}.

\begin{verbatim}
Amel_fem  PEDVMLKRRT GEKKPSIFER EEIKN 25
Amel_csd  I V I                    L               25
Bter_traA . LV . GE                      G 25
Bter_traB F  LV . E                    S               25
\end{verbatim}

\textbf{Figure 3.} Alignment of the \textit{Ceratitis-Apis-Musca} (CAM) domain (putative autoregulatory region) amongst Hymenoptera. The first 10 amino acids of the transformer (TRA) orthologues are present in both males and females, the latter 15 amino acids are only translated from the female-specific splice variant. CSD and the TRA paralogue (TRAB) contain a full CAM domain in both sexes. Amel, \textit{Apis mellifera}; Bter, \textit{Bombus terrestris}; Aech, \textit{Acromyrmex echinatior}; Acep, \textit{Atta cephalotus}; Pbar, \textit{Pogonomyrmex barbatus}; Cfio, \textit{Camponotus floridanus}; Nvit, \textit{Nasonia vitripennis}; Lcla, \textit{Leptopilina clavipes}; fem, feminizer.

In contrast, the gene in scf7180005164248 is not sex-specifically, indeed not even alternatively, spliced and lacks the region corresponding to male-specific exon sequences and the intron corresponding to that between exons 10 and 11 in \textit{Lc-tra} (Fig. 1). The single splice variant contains an open reading frame (ORF) that closely matches the female-specific splice variant of \textit{Lc-tra}. The conserved domain coding sequences of \textit{tra} are present (HYM domain and CAM domain plotted in Fig. 2), whereas the coding part for the putative autoregulatory region, referred to as the CAM-domain, displays stronger divergence from other hymenopteran sequences (Fig. 3 and Supporting Information Figure S1). Based on these data, we concluded that the gene in scf7180005164248 is a paralogue of \textit{Lc-tra} and named it \textit{Lc-traB}. The peptide sequence measures 429 amino acids and the amino acid sequence similarity is 74% compared to LC-TRA.F.

\textbf{Differential splicing of Lc-tra and Lc-traB in arrhenotokous and thelytokous \textit{L. clavipes}}

Arrhenotokous \textit{L. clavipes} produce both female and male offspring, whereas thelytokous wasps only produce females. Thelytokous male production can however be induced by antibiotic treatment of the infected females. RNA was extracted from individual adults of each sex and both reproductive types to assess splice variation of sex determination genes. In males of either reproductive
mode \(Lc-tra\) pre-mRNA was spliced solely into the male variant, whereas arrhenotokous females contained a mix of the female and the male splice variants (Fig. 4). This is in contrast to the non-treated thelytokous females, which displayed only the female-specific form of \(Lc-tra\). The single transcript of \(Lc-traB\) was abundantly present in females and males of both reproductive modes (Fig. 4). This full-length \(Lc-traB\) transcript results in the presence of a CAM-like domain coding sequence in males. No splice variation of \(Lc-traB\) was detected in either sex.

Faint traces of other amplicons were detected in the \(Lc-tra\) RT-PCR (Fig. 4). The faint band above the \(Lc-tra\) arrow was the less abundant \(M2\) splice variant. The \(Lc-tra\) isotig of \(Lc-tra\) the transcriptome of strain EPG, which only contains an initial alternative splice variants could not be detected in individual and the faint lower band in some thelytokous individuals were not successfully cloned. These potential alternative splice variants could not be detected in the transcriptome of strain EPG, which only contains an isotig of \(Lc-tra\) \(M2\) (accession: GAXY02017594) as well as \(Lc-traB\) (accession: GAXY02017595) (Peters et al., 2017), nor could they be predicted from the genomic sequence.

Sequence divergence of \(Lc-tra\) and \(Lc-traB\) in thelytokous and arrhenotokous individuals

Fragments of the two \(tra\) homologues were sequenced from stored samples of a range of arrhenotokous and thelytokous \(L. clavipes\) strains, used previously for genetic diversity assays (Pannebakker et al., 2004b; Kraaijeveld et al., 2011). An overview of these 12 thelytokous strains (AR1, AR2a, AR3a, Aust, BB1, CDB1a, GBW, KBH, MGS4, STP, WB1a, WB3) and nine arrhenotokous strains (CBY, DC, EJ, EPG, Mol, MS, PdA, PIB, TL) is presented in Supporting Information Table S1.

A coding region upstream of the sex-specifically spliced exons in \(Lc-tra\), containing two non-sex-specific exons separated by an intron, was amplified from both arrhenotokous and thelytokous individuals. The two \(tra\) homologues differ in this region by 33 single nucleotide polymorphisms (SNPs) and a 3-bp deletion (Fig. 5). No intrastrian variation was present in \(Lc-tra\) and \(Lc-traB\). The nucleotide polymorphisms in the two \(tra\) copies were used to assess the genetic divergence between the lineages. \(Lc-tra\) polymorphisms between lineages resolves into one cluster of arrhenotokous and one cluster of thelytokous variants, with the exception of lineage KBH (which was also an outlier in Kraaijeveld et al., 2011). The arrhenotokous and thelytokous \(Lc-tra\) haplotypes can be separated by a single nonsynonymous SNP. By contrast, such separation by reproductive mode is not evident for \(Lc-traB\), for which three haplotypes were detected (Fig. 5). The arrhenotokous lineages, except EPG, share the same haplotype of \(Lc-traB\). The thelytokous lineages are divided into two clusters with two nonsynonymous and one synonymous SNPs separating their \(Lc-traB\) haplotypes. Notably, the \(Lc-traB\) haplotype that is found in both arrhenotokous and thelytokous lineages contains a longer intron that is similar in length (1-bp difference) to the \(Lc-tra\) intron. The other \(Lc-traB\) haplotypes contain an intron that is 76 bp shorter. The distinction of thelytokous lineages into two clusters was also observed with neutral markers (microsatellites) and mtDNA (Kraaijeveld et al., 2011).

The region between exons 3 and 9 of \(Lc-tra\) contains the male-specific exons that are spliced out in the female form. Thelytokous lineages do not produce males and this region could potentially have degenerated in these lineages without affecting the functionality of \(tra\). This large region in \(Lc-tra\) could not be amplified by PCRs with primers located on exons 3 and 9. As an alternative approach genomic HiSeq data of thelytokous strain MGS4 and arrhenotokous strains EJ and PdA were mapped against the thelytokous GBW reference genome. Comparison of the thelytokous (MGS4/GBW) and arrhenotokous (EJ/PdA) consensus sequences yielded only one intronic
SNP in this male region of \textit{Lc-tra} (Table 1). This indicates that the entire male-specific region is intact in the thelytokous \textit{Lc-tra} lineage (Figs 1, 2). By contrast, the prospective promoter region, the 5' untranslated region (5'UTR), and introns 9 and 10 contain a large number of SNPs and deletions (Table 1). Furthermore, two nonsynonymous SNPs are present between the exonic consensus sequences of the thelytokous and arrhenotokous lineages, but no synonymous SNPs. These patterns confirm the separation of \textit{Lc-tra} into an arrhenotokous and a thelytokous haplotype.

Whereas the thelytokous and arrhenotokous consensus sequences of \textit{Lc-tra} contain a large number of intronic SNPs, \textit{Lc-tra}\textsubscript{B} is almost identical between the two reproductive modes (Table 1). There is some sequence variation amongst strains, but only a single nonsynonymous mutation on exon 2 separates the sequences by reproductive mode. The \textit{Lc-tra}\textsubscript{B} genomic region in the thelytokous \textit{L. clavipes} genome lacks the region that in \textit{Lc-tra} contains male-specific exons (Fig. 1). To examine potential divergence of these regions between the \textit{tra} copies of different lineages, we amplified this intronic region of \textit{Lc-tra}\textsubscript{B} in DNA samples of the arrhenotokous and thelytokous strains. The length of the intronic regions appear conserved in all lineages, regardless of reproductive mode (Fig. 6).

\textbf{Conservation of \textit{Lc-tra} and \textit{Lc-traB}}

The two \textit{tra} copies of \textit{L. clavipes} are more similar to each other than to any other hymenopteran \textit{tra} homologue (Fig. 7). This matches a pattern observed in bumblebees and ants (Schmieder et al., 2012; Privman

### Table 1. Comparison of promotor-region, 5' untranslated region (5'UTR), coding DNA sequences (CDS), male-specific region and 3' region between \textit{Leptopilina clavipes} transformer (\textit{Lc-tra}) of arrhenotokous strains (EJ/PdA) and \textit{Lc-tra} of thelytokous strains (GBW/MGS4), the \textit{Lc-tra} paralogue (\textit{Lc-traB}) of arrhenotokous strains and \textit{Lc-traB} of thelytokous strains, and \textit{Lc-tra2} of arrhenotokous and \textit{Lc-tra2} of thelytokous strains. The number of segregating sites includes both single nucleotide polymorphisms (SNPs) and deletions. SNPs located on exons are marked as nonsynonymous or synonymous in the right-most two columns

<table>
<thead>
<tr>
<th></th>
<th>Exon+intron #segregating sites</th>
<th>Exon #nonsynonymous mutations</th>
<th>Exon #synonymous mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Lc-tra} thelytokous vs. arrhenotokous</td>
<td>11</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>5'UTR</td>
<td>14</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CDS exons 1–3</td>
<td>1</td>
<td>1 (P in thelytokous vs. S in arrhenotokous)</td>
<td>0</td>
</tr>
<tr>
<td>Male-specific exon region (intron after exon 3 until start exon 9)</td>
<td>1 (intron)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>3' (exon 9 until transcript stop)</td>
<td>15 (14 of which in introns)</td>
<td>1 (P in thelytokous vs. A in arrhenotokous)</td>
<td>0</td>
</tr>
<tr>
<td>\textit{Lc-traB} thelytokous vs. arrhenotokous</td>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>5'UTR</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CDS exons 1–3*</td>
<td>1</td>
<td>1 (K in thelytokous vs. E in arrhenotokous)</td>
<td>0</td>
</tr>
<tr>
<td>Intron between exons 3 and 4</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>3' (exon 4 until transcript stop)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{Lc-tra2} thelytokous vs. arrhenotokous</td>
<td>4</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>5'UTR</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CDS</td>
<td>6</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>3'UTR</td>
<td>2</td>
<td>1 on exon, 1 in intron)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

interstrain variation in this region, see Figure 4

\textbf{Figure 6.} Amplification of intronic regions of the \textit{Leptopilina clavipes} transformer paralogue (\textit{Lc-traB}) in nine arrhenotokous strains (lanes 1–9: CBY, DC, EJ, EPG, Mol, MS, PdA, PlB, TL) and 12 thelytokous strains (lanes 10–21: AR1, AR2a, AR3a, Aust, BB1, CDB1a, GBW, KBH, MGS, STP, WB1a, WB3). The negative control in lane 22 contains no cDNA (ntc). The amplified fragment is 1129 bp and includes the truncated intronic region in \textit{Lc-traB} that is homologous to the region containing male-specific exons in \textit{Lc-tra}.
et al., 2013; Koch et al., 2014). The tra homologue in honeybees (Ap. mellifera, Apis cerana, Apis dorsata) is called feminizer (fem) and is duplicated. This paralogue contains a hypervariable region and was identified as the complementary sex determiner (csd) locus (Hassellmann et al., 2008a, 2008b). The hypervariable region is not present in tra paralogues of bumblebees and ants, and also does not appear in Lc-traB. The similarity of Lc-tra and Lc-traB sequences between reproductive types points to a duplication event after the divergence from other hymenopterans, but before the split between thelytokous and arrhenotokous lineages of L. clavipes (Figs 5, 7).

Identification and sequence variation of Lc-tra2

The nucleotide sequence of the Lc-tra2 coding DNA sequences (CDS) was predicted from isotig C57958, nucleotide position 134 to 958 (GenBank accession: GAXY02014083) (Peters et al., 2017). This translates into a 275-amino-acid sequence containing two RS domains flanking the RBD, consistent with previously identified tra2 orthologues.

Alternative splicing was detected in Lc-tra2, but the alternative splice variants are not sex-specific (Fig. 8). Splicing variation in exon 2 results in a different length of the N-terminal RS domain. The splicing variation at the last three exons results in highly conserved peptides compared to N. vitripennis and Ap. mellifera (Nissen et al., 2012; Geuverink et al., 2017). Lc-tra2A and Lc-tra2B translate at the 3’ end of the coding region to a FESRGIG motif, whereas Lc-tra2C translates into RY immediately followed by a STOP codon through the inclusion of exon 4. Owing to an A-rich region in the 3’ region it was impossible to obtain 3’RACE PCR fragments of Lc-tra2. Thus, a splice variant with a poly-A tail at the end of exon 4 could exist, but would not yield a different protein sequence or UTR compared to the variants reported here. Lc-tra2C is visible in the female samples of Fig. 4.

Figure 7. Gene tree of Transformer/Feminizer (TRA/FEM) and the duplications TransformerB/Complementary sex determiner (TRAB/CSD) in Hymenoptera. The analysis is based on predicted protein sequences of the female splice variants (including putative autoregulatory Ceratitis-Apis-Musca domain, arginine/serine-rich region and protein-rich region). All 578 positions of the 22 amino acid sequences were used to construct the tree with the maximum likelihood method based on the Jones et al. w/freq model with gamma distribution (Jones et al., 1992). Bootstrap values (1000 replicates) are shown on the branches. The scale bar shows the number of substitutions per site. Amel, Apis mellifera; Ador, Apis dorsata; Acer, Apis cerana; Bter, Bombus terrestris; Bimp, Bombus impatiens; Cflo, Camponotus floridanus; Pbar, Pogonomyrmex barbatus; Aech, Acromyrmex echinatior; Acep, Atta cephalotus; Nvit, Nasonia vitripennis; Atab, Asobara tabida; Ajap, Asobara japonica; Lcla, Leptopilina clavipes. CSD absence or presence is noted on the right.
N. vitripennis with LC-TRA2 and with TRA2 of length ORF constitutes the only trA (LC-TRA) in both female and male wasps and Lc-tra (LC-TRA2) was selected based on transcript abundance. Lc-traB and Lc-tra2 were cloned in the yeast 2-hybrid vectors. 1994), but in vitro tests of this molecular interaction have not been performed for the TRA and TRA2 homologues of other insects. We examined the N. vitripennis model to assess conservation of this protein interaction in Hymenoptera, using the Yeast 2-Hybrid protein interaction assay (Fields and Song, 1989). Yeast 2-Hybrid vectors containing either a DNA-binding or a transcriptional activation domain were fused to full-length CDS of N. vitripennis tra (NV-TRA) and N. vitripennis tra2 (NV-TRA2). Yeast 2-Hybrid assays demonstrated a weak interaction between NV-TRA and NV-TRA2 (Table 2). NV-TRA, as well as NV-TRA2, interacts with itself, a feature also observed in Drosophila (Amrein et al., 1994; Table 2).

Subsequently, full-length CDS of Lc-tra2A, Lc-traF and Lc-traB were cloned in the Yeast 2-hybrid vectors. Lc-tra2A (LC-TRA2) was selected based on transcript abundance in both female and male wasps and Lc-traF (LC-TRA) constitutes the only tra splice variant that codes for a full-length ORF.

As the results in Table 2 demonstrate, LC-TRA interacts with LC-TRA2 and with TRA2 of N. vitripennis (NV-TRA2).

Whereas an alternative faint amplicon is visible in the males, but based on various RT-PCRs in this 3' region these transcripts do not seem sex-specific, and are low in abundance. No splicing variation was found between these transcripts do not seem sex-specific, and are low in abundance. No splicing variation was found between reproductive modes.

Comparison of the genomic region of Lc-tra2 between the two thelytokous and two arthetokous strains yielded a limited number of SNPs (Table 1). None of these SNPs is located on exons in the coding region, confirming the high level of tra2 conservation between the reproductive types. No sequence variation is present within the reproductive types.

Protein interactions between Lc-tra, Lc-traB and Lc-tra2
Protein–protein interaction between TRA and TRA2 is required to regulate female-specific splicing of dsx pre-mRNA in Drosophila melanogaster (Amrein et al., 1994), but in vitro tests of this molecular interaction have not been performed for the TRA and TRA2 homologues of other insects. We examined the N. vitripennis model to assess conservation of this protein interaction in Hymenoptera, using the Yeast 2-Hybrid protein interaction assay (Fields and Song, 1989). Yeast 2-Hybrid vectors containing either a DNA-binding or a transcriptional activation domain were fused to full-length CDS of N. vitripennis tra (NV-TRA) and N. vitripennis tra2 (NV-TRA2). Yeast 2-Hybrid assays demonstrated a weak interaction between NV-TRA and NV-TRA2 (Table 2). NV-TRA, as well as NV-TRA2, interacts with itself, a feature also observed in Drosophila (Amrein et al., 1994; Table 2).

Subsequently, full-length CDS of Lc-tra2A, Lc-traF and Lc-traB were cloned in the Yeast 2-hybrid vectors. Lc-tra2A (LC-TRA2) was selected based on transcript abundance in both female and male wasps and Lc-traF (LC-TRA) constitutes the only tra splice variant that codes for a full-length ORF.

As the results in Table 2 demonstrate, LC-TRA interacts with LC-TRA2 and with TRA2 of N. vitripennis (NV-TRA2).

Table 2. Protein–protein interactions in the Yeast 2-Hybrid system.

<table>
<thead>
<tr>
<th>Insert 1 (binding domain)</th>
<th>Insert 2 (activation domain)</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptopilina TRA</td>
<td>Leptopilina TRA2</td>
<td>++</td>
</tr>
<tr>
<td>Leptopilina TRAB</td>
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</tr>
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<td>Leptopilina TRA</td>
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<td>Leptopilina TRAB</td>
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<tr>
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<td>Leptopilina TRAB2</td>
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<td>Leptopilina TRA2</td>
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<tr>
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<td>Leptopilina TRA2</td>
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<td>Nasonia TRA2</td>
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<tr>
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</tr>
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<td>Lam (negative control)</td>
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<td>-</td>
</tr>
<tr>
<td>p53 (negative control)</td>
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<td>-</td>
</tr>
<tr>
<td>Lam (negative control)</td>
<td>Nasonia TRA2</td>
<td>-</td>
</tr>
</tbody>
</table>

Conversely NV-TRA interacts with LC-TRA2. This shows the conserved ability of TRA2 to bind diverged TRA homologues. In D. melanogaster TRA interacts with itself (see above), but we only observed this for NV-TRA and not for LC-TRA (Table 2). LC-TRAB showed interactions with LC-TRA2 and NV-TRA2, confirming TRA2 binding recognition of TRA-like sequences (Table 2). LC-TRAB also interacts with LC-TRA (Table 2) allowing the possibility of a trimeric protein complex of LC-TRA, LC-TRAB and LC-TRA2. LC-TRAB additionally interacts with itself.

Figure 8. Exon–intron structure of the splice variants of Leptopilina clavipes transformer 2 (Lc-tra2). White boxes represent the 5' and 3' untranslated regions, the black boxes contain the coding sequence. Grey boxes depict the RNA binding domain.
As most test combinations yielded an interaction we verified the likelihood of false positive interactions through inclusion of control plasmids that contained nonrelated proteins (murine p53 or human Lamin C). The L. clavipes constructs did not interact with these constructs, or with empty constructs (which only express either the binding or the activation domain), indicating that the observed interactions between the sex determination genes are specific (Table 2 and Supporting Information Table S2).

### Ploidy of arrhenotokous and thelytokous L. clavipes

Males and females were compared for ploidy between arrhenotokous and thelytokous strains. No differences were detected between sexes of the different reproductive modes. All females were diploid and all males were haploid (Table 3).

### Discussion

**The transducing level of sex determination is conserved in L. clavipes**

Two homologues of *tra* were detected in *L. clavipes* and both displayed strong amino acid sequence conservation compared to hymenopteran TRA orthologues. *Lc-tra* is probably the *tra* orthologue based on the sex-specific splicing of its transcripts, sequence conservation within reproductive type and the high conservation of domains and structure of the LC-TRA protein. It retains all elements required for a conserved sex determination function. Comparison of the arrenhotokous and thelytokous *Lc-tra* mRNAs and peptides did not reveal much divergence. Genes that have become redundant in thelytokous wasps have been observed to decay (Kraaijeveld et al., 2016). In the thelytokous (all-female) lineage this decay was not observed in the genomic region of *Lc-tra* specifying the male-specific exon containing the premature stop codon. Haploid males that are produced by thelytokous females after antibiotic treatment contain the same male-specific splice variants as arrenhotokous haploid males. However, *Lc-tra* transcripts are not present in thelytokous females infected with Wolbachia endosymbionts. This is a notable difference compared to arrenhotokous females, which, beyond their expression of *Lc-tra*, also express abundant male-specific *Lc-tra* transcripts. Apparently, Wolbachia infection prevents the generation of male specific splice forms from *Lc-tra* in females. Yet this entire genomic region of *Lc-tra* is highly conserved between arrenhotokous and thelytokous wasps, and production of male splice forms is possible upon removal of Wolbachia endosymbionts. This suggests that the functionality of *tra* is retained in either reproductive mode.

*Lc-tra2* contains all conserved regions associated with *tra2*. Its splicing variation at the 3′end corresponds with known variants in *Ap. mellifera*, *N. vitripennis* and *As. tabida* (Nissen et al., 2012; Geuverink et al., 2017, 2018). We performed protein–protein interaction assays in *N. vitripennis* to test the hypothetical TRA/TRA2 binding complex in Hymenoptera. Both *tra* and *tra2* in *N. vitripennis* are required for the splicing of *tra* pre-mRNA as well as *dsx* pre-mRNA (Verhulst et al., 2010a; Geuverink et al., 2017). In this study we demonstrated an interaction between TRA and TRA2 in *N. vitripennis*. The conservation of this interaction in *L. clavipes*, combined with the cross interaction of TRA and TRA2 between *L. clavipes* and *N. vitripennis*, enables the possibility that *tra* and *tra2* are also involved in *tra* and *dsx* pre-mRNA splicing in this species.

**Involvement of Lc-traB in sex determination of L. clavipes**

*Lc-traB* is neither sex-specifically nor alternatively spliced. The absence of the corresponding male-specific exon region of *Lc-tra* results in a default splicing of *Lc-traB* transcripts, similar to the female-specific *tra*. This suggests that *traB* does not need autoregulation of its splicing once switched on. The *Lc-traB* sequence coding for the CAM region associated with autoregulation (Hediger et al., 2010) is distinctly different compared to *Lc-tra* (Fig. 3). If *traB* had become obsolete in thelytokous sex determination this would be visible in sequence degeneration. However, this is not observed in any of the four (re)sequenced lineages. *Lc-traB* does group into three haplotypes independent of reproductive mode. This could reflect the evolutionary history of the lineages, rather than a functional implication, as the clustering matches divergence patterns observed with neutral and mitochondrial markers (Kraaijeveld et al., 2011). The strong sequence conservation and lack of degeneration in thelytokous lineages suggest a
conserved function. Yet, the lack of male-specific region and default splicing indicate a function different from Lc-tra. As LC-TRAB interacts with LC-TRA and LC-TRA2, this function may still be a part of the process of sex determination. Thus, we propose that a combination of Lc-tra, Lc-traB and Lc-tra2 may be required for female development in L. clavipes.

Implications for the sex determination mechanism of L. clavipes

One of the widespread mechanisms of sex determination in Hymenoptera is CSD. Under CSD female development ensues when one (single-locus CSD) or multiple (multi-locus CSD) loci are heterozygous. The only csd locus identified thus far is in Ap. mellifera and it is a parologue of fem, the Ap. mellifera orthologue of tra (Hasselmann et al., 2008a). Other paralogues of tra have been found in species with CSD, but little is known about their functionality (Schmieder et al., 2012; Privman et al., 2013). This association potentially reflects a bias in study effort, rather than a true link to CSD. Recently, three homologues of tra were reported from the fig wasp Ceratosolen solmsi, a species belonging to the Chalcidoidea in which CSD appears absent (van Wilgenburg et al., 2006; Heimpel and de Boer, 2008; Jia et al., 2016). Transcripts of the two duplicates in C. solmsi are only detected in females, but their possible role in sex determination remains unknown. Hence, the presence of a tra parologue is not informative about the presence or absence of CSD.

The lack of evidence for CSD in the Leptopilina genus (Biémont and Bouletreau, 1980; Hey and Gargiulo, 1985) requires consideration of the only other reported sex determination mechanism in Hymenoptera: maternal effect genomic imprinting. This has been described for the wasp N. vitripennis (Beukeboom and van de Zande, 2010; Verhulst et al., 2010a, 2013) and consists of a maternally imprinted (inactivated) sex determination gene [the putative womanizer (wom) gene] that can perform a feminizing function in the zygote. The non-inactivated wom of paternal origin in fertilized eggs acts in combination with maternal provisioning of tra and tra2 mRNA to effectuate female development. It is not known if this mechanism, which requires sex determination gene transcripts to be maternally provided to the eggs and involves a paternally provided factor in the fertilized egg, is present in other groups. The presence of two tra homologues in L. clavipes provides multiple options for maternal effect genes. Additional studies are required to elucidate the thelytokous (uniparental, all-female) mode of sex determination, as under thelytoky a paternally provided element is impossible. How can female development be activated in a zygote containing only maternally provided chromosome sets and gene products? One intriguing possibility is that Wolbachia provides this signal. Wolbachia may directly interfere with the splicing regulation of Lc-tra itself, resulting in the fixed splicing pattern observed in thelytokous adult females. Transcriptomes of early developmental stages need to be procured to identify these signals. This may shed more light on the diversity of sex determination mechanisms in hymenopteran insects and open the possibility of testing endosymbiont interference in insect sex determination.

Experimental procedures

Source material

Stored samples of 12 thelytokous strains (AR1, AR2a, AR3a, Aust, BB1, CDB1a, GBW, KBH, MGS4, STP, WB1a, WB3) and nine arrhenotokous strains (CBY, DC, EJ, EPG, Mol, MS, PdA, PIB, TL), as described in Pannebakker et al. (2004b, Kraaijeveld et al. (2011) and Table 1, were used to screen divergence of the tra genes. The additional SCA strain was collected in Santa Cristina d’Aro (Spain) in October 2015. The wasps were cultured on second-instar Drosophila phalerata host larvae at 25 °C under constant light. Individuals from the KBH strain were kindly provided by Todd Schlenke. Females of the KBH strain were cured from their Wolbachia infection by feeding honey with 0.5% rifampicin (Schidlo et al., 2002); this results in haploid bacteria that develop into males (referred to as ‘thelytokous males’).

Identification of tra homologues and structure of tra in L. clavipes

Scaffolds containing putative tra homologues were identified from the L. clavipes genome assembly (Kraaijeveld et al., 2016) using the protein sequence of N. vitripennis tra (NP_001128299) as a query in translated BLAST (tblastn) (Altschul et al., 1997). Adult males and females of the arrhenotokous strain EPG and thelytokous strain GBW were collected from laboratory cultures that were terminated immediately afterwards. RNA extractions were performed with TRIzol according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). All isolated total RNA was primed with oligo(dT) and random hexamers (in a mixture of 1:6) and reverse transcribed with a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). Reverse transcription for 3’RACE adapter synthesis was also performed with a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) using all isolated total RNA primed with a 3’RACE adapter (5'-GGC AGC ACA GAA TTA ATA CGA CTC ACT ATA GGT 12VN-3'). A 5’RACE adapter containing cDNA was produced according to the manufacturer’s instructions (FirstChoice RLM-RACE kit, Ambion, Austin, TX, USA). Sequences of all primers used in this study are shown in Table 4. To assess the Lc-tra splice variants present in adult males and females 5’RACE-PCR was performed with outer primer Lcla_tra_5RACE1

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and inner primer Lcla_tra_5RACE2 in a reaction at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 60 s, with a final extension of 10 min at 72 °C. Outer primer Lcla_tra_3RACE1 and inner primer Lcla_tra_3RACE2 were used in 3’RACE-PCR in a reaction with DreamTaq (Fermentas). Cycling conditions were 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 2 min, with a final extension of 7 min at 72 °C. Resulting PCR fragments were visualized on ethidium bromide-containing 1.5% agarose gel with 1x TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA).

All RACE-PCR products were ligated into pGEM-T vector (Promega, Madison, WI, USA) after purification using a GeneJET Gel Purification Kit (Fermentas). Ligation products were used to transform competent JM-109 Escherichia coli (Promega). Colony PCR was conducted by use of pGEM-T primers at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, with a final extension of 7 min at 72 °C.

As the lowered specificity of RACE-PCRs (one gene-specific primer per PCR) rarely permitted the detection of Lc-traB, RT-PCRs were used to detect splice variation in this gene. These PCRs were performed with primers Lcla_5’_F Lc-traB_frontF and Lcla_3’_F Lc-traB_R in a reaction at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, with a final extension of 7 min at 72 °C.

PCR-fragments were sequenced on an ABI 3730XL (Applied Biosystems, Foster City, CA, USA) and reads were inspected in Chromas (Technelysium, South Brisbane, Australia) and aligned in MEGA7 (Kumar et al., 2016). Exon–intron structure of the genes was constructed by comparing the mRNA sequences to the genomic assembly scaffolds (Lc-tra: scf7180005166757, Lc-traB: scf7180005164248) and visualized with EXON–INTRON GRAPHIC MAKER (https://wormweb.org/exonintron). Transcript sequences were deposited in GenBank (accession numbers: MG963997–MG964000).

**Table 4.** Overview of primers used in this study

<table>
<thead>
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<th>Primer name</th>
<th>Gene/construct</th>
<th>Application</th>
<th>Primer sequence 5’–3’</th>
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<td>Lc-traB</td>
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</table>

**Overview of primers used in this study**

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arrhenotokous strain SCA and thelytokous strain KBH were collected from laboratory cultures. RNA extractions were performed as described above.

The presence of sex-specific splice variants of Lc-tra in adults was tested with primers Lcla_tra_spliceA_F and Lcla_tra_spliceA_R. Transcripts of Lc-traB were detected with primers Lcla_spliceB_F and Lcla_spliceB_R. The cycling-conditions were 94 °C for 3 min, 45 cycles of 94 °C for 30 s, 57 °C (tra)/55 °C (traB) for 30 s and 72 °C for 2 min, with a final extension of 7 min at 72 °C. The resulting fragments of each category were sequenced to verify their identity as Lc-tra male- and female-specific splice variants and Lc-traB.

Sequence divergence of Lc-tra and Lc-traB in thelytokous and arrhenotokous individuals

To assess variation in the tra genes between different populations of L. clavipes DNA was individually extracted from five females per strain with a standard high salt protocol (Aljanabi and Martinez, 1997). Population variation was tested with the primer Lcla_traAB_F (5'-GTCCATCATTCAGAGACAGAC-3') in combination variation was tested with the primer Lcla_traAB_F (5'-AGGTCATTATTTACAATGATGG-3').

Whole genome sequencing was performed on DNA of three L. clavipes lineages (EJ, PdA and MGS4) that had been stored in 96% ethanol at 4 °C. DNA was extracted from groups of 20 females following the animal tissue protocol (Qiagen, Valencia, CA, USA). In short, wasps were air-dried and crushed in 180 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA) in a 1.5-ml microcentrifuge tube using a pestle. The DNA was individually extracted from each sample and purified using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). In short, wasps were air-dried and crushed in 180 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA) in a 1.5-ml microcentrifuge tube using a pestle. The DNA was eluted in 100 µl DNAse-free MilliQ (EMD Millipore, Burlington, Massachusetts, United States) water. The DNA was eluted in 100 µl DNAse-free MilliQ (EMD Millipore, Burlington, Massachusetts, United States) water. Length and integrity of the DNA molecules were checked on a 2100 Bioanalyzer lab-on-chip with a High Sensitivity DNA kit (Agilent, Santa Clara, CA, USA) and purity was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific Waltham, Massachusetts, United States).

For Illumina library preparation, each DNA sample was fragmented and size-selected to 350 bp using a KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, Boston, MA, USA) according to the supplied protocol (KR1145v215-1). Size range and concentration were assessed using a 2100 Bioanalyzer lab-on-chip with a High Sensitivity DNA kit (Agilent). Library concentration and correct adaptor ligation were assessed using quantitative (q) PCR according to basic protocol 5 documented in Bronner et al. (2014). The libraries were stored at −20 °C in 100 µl DNAse-free MilliQ water and sequenced within 4 weeks on an Illumina HiSeq4000 (Illumina, San Diego, California, United States) (150 bp paired-end) at the Leiden Genome Technology Center (Leiden, the Netherlands).

Initial quality checks of the raw reads were conducted using fastqc (Andrews, 2010). Reads were mapped to the L. clavipes reference genome using BOWTIE2 (Langmead & Salzberg, 2012). Duplicate reads were removed using PICAERTOOLS (https://broadinstitute.github.io/picard/) and indel realignment was conducted using GATK (McKenna et al., 2010). Consensus sequences of each strain were constructed by comparing the aligned reads of EJ, PdA and MGS4 to the GBW reference sequence. Full-length amino acid sequences of arrhenotokous and thelytokous LC-TRA and LC-TRAB were aligned with MUSCLE (Edgar, 2004) in GENEIOUS 8 (Biomatters Ltd, Auckland, New Zealand). The intronic regions between traB exons 3 and 4 were amplified in DNA samples of the different strains with the same primers as used for RT-PCR: Lcla_spliceB_F

<table>
<thead>
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<td>Y2H_Res_Nvit-tra2_R</td>
<td>BamHI (3')</td>
<td>56</td>
</tr>
</tbody>
</table>

*Lc, Leptopilina clavipes; Nv, Nasonia vitripennis; tra, transformer; traB, tra parologue.*

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and Lcla_spliceB_R. The cycling-conditions were 94 °C for 3 min, 45 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, with a final extension of 7 min at 72 °C.

Conservation of Lc-tra and Lc-traB

The following sequences were used in alignments and the gene tree: Ap. mellifera fem (AAS86667) and csd (AAS86653), Ap. dorsata fem (ABV56232) and csd (ABW36165), Ap. cerana fem (ABV56230) and csd (ABV58877), Bombus terrestris traA (NP_001267853) and traB (XP_003394693), Bombus impatiens traA (XP_003493796) and traB (XP_003491525), N. vitripennis tra (NP_001128299). Ant protein sequences were obtained from Privman et al.'s (2013) supplementary materials. Alignments were produced in CLC WORKBENCH (CLCbio, Aarhus, Denmark).

Identification and sequence variation of Lc-tra2

5'RACE-PCR was performed with outer primer Lcla_tra2_5RACE1 and inner primer Lcla_tra2_5RACE2. The PCR cycles were as follows: 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, with a final extension of 10 min at 72 °C. Outer primer Lcla_tra2_3RACE1 and inner primer Lcla_tra2_3RACE2 were used in 3'RACE-PCR with the same cycling conditions as the 5'RACE-PCR. RT-PCRs to verify and detect further splice variation were performed with primers Lcla_tra2_F1/Lcla_tra2_R1, Lcla_tra2_F2/Lcla_tra2_R2 and Lcla_tra2_F3/Lcla_tra2_R3 under the following conditions: 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 2 min, with a final extension of 10 min at 72 °C. Purification of PCR products, ligation, transformation, colony PCR and sequencing were performed according to the procedures described for Lc-tra identification. Expression plasmids were deposited in GenBank (accession numbers: MG963994–MG963996). Lc-tra2 consensus sequences of each resequenced strain were obtained through the methodology described above. The selected transcripts were the female-specific variant of Lc-tra and Nv-tra (Werren et al., 2010) and the single splice variant of Lc-traB. The chosen splice variants of tra2 (Lc-tra2α and Nv-tra2β) were most abundant in N. vitripennis and conserved in other Hymenoptera (translated into FESRGIG motif) (Geuverink et al., 2017, 2018). Primers containing adapters that add restriction sites for EcoRI/Ndel (5’end) and BamHI (3’end) were used to amplify full-length transcripts in RT-PCRs. These primers and corresponding restriction sites are displayed in Table 5 and the primer sequences are given in Table 4. The cycling conditions were 94 °C for 3 min, 45 cycles of 94 °C for 30 s, 53–64 °C for 30 s and 72 °C for 90 s, with a final extension of 7 min at 72 °C. Annealing temperatures per primer pair are shown in Table 4. PCR products were visualized on ethidium-bromide-containing 1.5% agarose gel with 1x TAE buffer and were purified from gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol.

All PCR products were digested with restriction enzymes (Table 5) using a double digestion. Plasmids pGBK7 and pGADT7 (Clontech, Mountain View, CA, USA) were also double digested with both EcoRI/BamHI and Ndel/BamHI. Digestion reactions for transcripts Lc-tra, Lc-traB, Nv-tra and Nv-tra2 consisted of: 1 µg cleaned-up PCR product and 1 µg of both plasmids pGBK7 and pGADT7, 1 µl EcoRI, 0.5 µl BamHI and 2 µl BamHI buffer; the volume was increased to 20 µl with MilliQ. These reactions were incubated at 37 °C for 16 h, followed by a 20-min incubation at 80 °C. Digestion reactions for transcript Lc-tra2 consisted of: 1 µg cleaned-up PCR product and 1 µg of both plasmids pGBK7 and pGADT7, 4 µl Ndel, 1 µl BamHI and 2 µl BamHI buffer; the volume was made up to 23.5 µl with MilliQ. These reactions were incubated at 37 °C for 16 h, followed by a 20-min incubation at 80 °C. Digested PCR products were ligated into pGBK7 and pGADT7 using T4 DNA Ligase (New England Biolabs, Beverly, MA, USA) to yield both bait and prey vectors containing the genes listed in Table 5. Control plasmids pGBK7-53 (murine) and pGBK7-Lam (human) (Clontech) were included to account for the possibility of false positive detections.

Plasmids containing bait and prey constructs were transformed into competent JM-109 Escherichia coli (Promega). Colony PCR was performed using primers: Y2H_T7promotor_F and Y2H_3’DNA-BD_R for pGBK7 constructs and Y2H_T7promotor_F and Y2H_3AD_R for pGADT7 constructs. The cycling-conditions were 94 °C for 3 min, 45 cycles of 94 °C for 30 s, 50 °C (AD)/55 °C (BD) for 30 s and 72 °C for 90 s, with a final extension of 7 min at 72 °C. PCR products were sequenced to confirm that no PCR errors were generated and that the protein is fused in-frame with the vector promoter. Plasmids containing the correct genes were isolated from the colonies using a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific).

pGBK7 vectors containing binding domains were introduced into yeast strain AH109, and pGADT7 vectors containing activation domains were introduced into yeast strain Y187 to test for protein interactions.

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All experimental procedures were conducted according to the Matchmaker GAL4 Two-Hybrid System 3 manual (Clontech). Protein interactions were identified by observing the growth of transformants on SD-Ade/-His/-Leu/-Trp plates as a result of the transcription of reporter genes (Fig. S2).

**Ploidy of arrhenotokous and thelytokous L. clavipes**

Ploidy of arrhenotokous males, arrhenotokous females, thelytokous males and thelytokous females was confirmed by flow cytometry analysis. Newly collected arrhenotokous strain CA1 and thelytokous strain LS1 were used in this assay (Table S1). A thin layer of yeast mixture containing 2.5 mg tetracycline per gram of dry yeast was added to agar bottles. Second-instar *D. phalerata* larvae were added to the bottle and parasitized by thelytokous LS1 females. All emerging F1 offspring were still female, but cured of their *Wolbachia* infection. They were hosted on regular bottles containing second-instar *D. phalerata* host larvae for parasitization. The emerging F2 offspring solely consisted of males. These thelytokous males, their cured thelytokous mothers and nontreated thelytokous females were used to assess ploidy. Adult wasp heads were homogenized in Galbraith buffer (21 mM MgCl₂, 30 mM tri-sodium citrate hydrate, 20 mM MOPS, 0.1% Triton X-100, 1 mg/l RNAse A) using a motorized pestle, filtered by 35-µm cell strainer caps (BD Falcon Cell strainer #352235, BD Biosciences, San Jose, CA, USA) and stained with propidium iodide (Sigma, St Louis, MO, USA). Samples were loaded on a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analysed with FLOWLOGIC software (Miltenyi Biotec).

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**References**


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Alignment of female-specific TRA and non-specific TRAB amino acid sequences of strains EJ, PdA, GBW and MGS4 (populations previously described in Kraaijeveld et al., 2011). The HYM and CAM domain are depicted on top of the sequences.

Figure S2. Displayed on the left are pictures of Yeast-2 hybrid matings showing growth on SD-Adel-His/Leu-Trp plates (QDO). Tables summarizing each set of matings are displayed on the right.

Table S1. Strains of *L. clavipes* used in this study.

Table S2. Protein-protein interactions in the Yeast 2-Hybrid system. Protein-protein interaction was assessed using the Matchmaker Gal4 Two-Hybrid System 3 as provided by Clontech. Sex determination genes constructed in combination with either the binding domain or the activation domain were tested against empty constructs.