university of groningen

MASTER THESIS

Analysis of the *transformer* gene in gynandromorph producing and normal strains of *Nasonia vitripennis*

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Abstract:

Sex determination is an important and widespread developmental programme that involves a cascade of genes. Insect exhibits a high diversity of sex determination mechanism and diversification is found on the top of the cascade with various cues, including genetic, environmental or maternal signals. However, the bottom of the sex determination cascade is conserved with *doublesex (dsx)* and its splicing regulator *transformer (tra)*. The regulator of *tra* has been proposed as the key aspect of diversification in insect sex determination as different species adopt various ways to turn on or off the production of TRA protein.

Nasonia vitripennis, a parasitoid wasp, is an emerging model species in developmental and evolutionary biology. *Nasonia,* like all hymenoptera, has haplodiploid sex determination: females are diploid and develop from fertilized eggs, whereas males are haploid and develop from unfertilized eggs. Recently, the maternal effect genomic imprinting model (MEGISD) was proposed as the sex determination mechanism of *N.vitripennis*, although many mysteries still remain. A naturally occurring strain of *N.vitripennis* produces gynandromorph offspring, which are individuals with both female and male phenotypes. Previous study has revealed that a temperature sensitive maternal effect gene *gyn1* is responsible for the production of gynandromorph offspring but cytoplasmic component are also essential. The proposed gene *gyn1* is upstream of *tra* and is proposed to cause defects in silencing zygotic *tra.* However, the involvement of *tra* in producing gynandromorphs has not been elucidated. In this project, we first identified polymorphism on the sequence of t*ra* between the gynandromorph producing strain (HiCD12) and a normal lab strain. Then association between *tra* polymorphism and the trait of producing gynandromorph progeny was tested with an introgression line created by crossing HiCD12 females to males from *N.giraulti* because that *N.giraulti* shows no gynandromorph production and there are species specific SNPs between these two sister species. Following that, with the same introgression line, we investigated whether gyn1 has an effect on the expression of *tra* in adult females by comparing the tra expression among gynandromorph-producing females and those who did not produce gynandromorph progeny. Lastly, species-specific SNPs analysis at the candidate region identified in previous study around *dsx* was carried out on the introgression line to search for location of *gyn1*. Our results suggest that suggest that mutations on the sequence of tra is not likely to be contributing to the production of

gynandromorph progeny and gyn1 does not have a strong effect on the *tra* expression level in adult females.

CONTENTS

Introduction

Transformer in insect sex determination

Sex determination, an important and widespread developmental programme that commits the embryos to either female or male pathways, is a long-standing research interest. Decades of research on sex determination have revealed a wealth of varieties for deciding sex in the animal world, in particular among the insect order, that contains all known types of sex determination mechanisms (Sánchez, 2008). Studies on insect sex determination also unveiled that this pathway is composed of a cascade of genes (Baker, 1989; Schütt and Nöthiger, 2000; Shearman, 2002; Sánchez, 2008) and the bottom of the cascade is conserved while new components are recruited to a preexisting pathway on the top (Wilkins, 1995). Thus high diversity is observed on the top of the sex determination cascade, allowing it to be initiated by various types of cues, including genetic, environmental or maternal signals (Schütt and Nöthiger, 2000; Saccone et al., 2002; Sánchez, 2008; Bopp, 2010; Nagaraju and Saccone, 2010; Verhulst et al., 2010b; Gempe and Beye, 2011). In contrast with this diversification on the top of the cascade, the bottom components of the cascade is conserved. One key gene shared by these sex determination pathways in several groups of insects, such as Dipteran and Hymenoptera, as the master switch is the transformer gene (*tra*). (O'Neil and Belote, 1992; Shearman and Frommer, 1998; Pane et al., 2002; Lagos et al., 2007; Ruiz et al., 2007b; Concha and Scott, 2009; Gempe et al., 2009; Hediger et al., 2010; Shukla and Nagaraju, 2010; Verhulst et al., 2010b; Shukla and Palli, 2012b). *Tra* achieves its female promoting role by splicing its downstream target, the doublesex (*dsx*) gene to its female specific form, which as a global effector in sex determination, will subsequently turns on female development. A female signal is required to start the female promoting role of *Tra*. Once it is initiated, *tra* will subsequently engage a positive feedback loop to sustain its production. The splicing state of *tra* determines whether there will be functional TRA protein to achieve its female promoting role. Only the female specific splicing variant produces functional TRA protein, while a male signal will lead to the incorporation of in-frame stop codon and generate functionally inactive splicing variant of *tra*. The gene product of *tra* is a splicing regulator that is necessary to maintain its own female specific splicing. Thus, the decision of to be male or female is depend on whether there is an early

supply of functional TRA protein in the zygote to start and sustain the positive feedback look of TRA production.

The regulation of *tra* has been proposed as the key aspect of diversification in insect sex determination as different types of female or male signals are present in the upstream of the sex determination pathway to regulate the zygotic form of *tra* (Pomiankowski 2003, Verhulst et al., 2010). Several different female signals that activate zygotic *tra* have been identified among insect. In dipteran *C. capitata* and *M. domestica* (Pane et al., 2002; Hediger et al., 2010) and hymenopteran *Nasonia vitripennis* (Verhulst et al., 2010a) maternally provided *tra* product serve as a loop starter for zygotic *tra*; in hymenopteran *Apis*, being heteroallelic for the *complementary sex determination (csd)* gene constitutes the female signal to activate *tra*; in *drosophila* the presence of 2 X chromosomes and active *sex-lethal (sxl)* gene imposes *tra* to its functional, female-specific splicing form. In the absence of female signals or the presence of male determiners, the M factors that interfere with female signals, *tra* will be left in its inactive form and leads to male development (see Bopp et al. 2013 for a recent review).

Sex determination in haplodiploid *Nasonia vitripennis*

Nasonia vitripennis, a parasitoid wasp, is an emerging model species in the developmental and evolutionary biology, owing to its recently sequenced genome, low number of chromosomes, short generation time and naturally occurring mutant strains (Shuker et al., 2003; Pultz &Leaf, 2003; Beukeboom, 2003, Lynch at al., 2006, Ferree et al., 2006). There are three sister species: *N.longicornis*, *N.giraulti* and *N.oneida*. These *Nasonia* species readily inbreed, facilitating introgression of genes between species (Werren &Leohlin, 2009). The sex determination of *Nasonia* is governed by haplodiploidy: females are diploid and develop from fertilized eggs, whereas males are haploid and develop parthenogenetically from unfertilized eggs (Beukeboom et al., 2006; Beukeboom et al., 2010). In recent years, much progress in understanding the sex determination of *Nasonia* has been achieved by the use of mutant strains (Beukeboom et al., 2007b; Kamping et al., 2007). Experimental evidence rejected models based on complementary sex determination, fertilization only, ploidy and balance of cytoplasmic and nuclear components (for a review of this research process see van de Zande &Verhulst, 2013), but pointed to epigenetic modification is likely to be involved in the

sex determination process. Incorporating evidence that maternal components are also involved in sex (Trent et al., 2006; Kamping et al., 2007), the maternal effect genomic imprinting model (MEGISD) was proposed (Beukeboom et al., 2007a). Under this model, upon gametogenesis a maternal effect gene (*msd*) actively imprints a zygotic sex determiner (*zsd*). This prevents the timely zygotic expression of *tra* and thus leaves *tra* in an off state. As a result, without functional TRA protein, a haploid individual will develop into a male. In a paternally inherited genome without the maternal effect gene (*msd),* the *zsd* is active and thus able to initiate *tra*. So in a diploid individual a paternally inherited genome with the active *zsd* enables the timely expression of zygotic *tra*, coupled with the maternally provided female specific *tra* mRNA, which starts the auto regulation loop of embryonic splicing of *tra*, ensures the female development. In sum, both maternal effect (provision of female-specific *Nvtra* mRNA) and imprinting (regulation of zygotic transcription of *Nvtra*) are critical to regulate timely *transformer* production (Verhulst et al., 2010). Recent experimental studies found an unknown upstream component to be involved in zygotic activation of *Nvtra*. This new factor, termed *womanizer,* is maternally silenced to ensure male development in haploid zygote (Verhulst et al., 2013). Although the basic pattern of *N.vitripennis* sex determination has been unveiled, several key factors remain mysterious. The identity and relationship among *msd, zsd, wom* genes and the mechanism through which *wom* is silenced maternally need to be further investigated.

Gynandromorph producing strain of N.vitripennis

Gynandromorphs are individuals that display both male and female phenotypes. The use of gynandromorphs has provided a unique opportunity for the study of sex determination, as the defects in their components of the sex determination cascade are indicative for the unknown components in the pathway (Kamping et al., 2007; van de Zande & Verhulst, 2013). A natural *N. vitripennis* strain collected in Canada was found to produce gynandromorphs at 5% among unfertilized haploid eggs (Beukeboom et al., 2007a). A clear anterior/posterior gradient of feminization is evident in these gynandromorphic individuals. This gynandromorphic strain has been selected for high (HiCD12) and low (LoCD12) gynandromorphic offspring production, indicating the genetic basis of gynandromphmism in *N. vitripennis* (Kamping et al., 2007). Exposing

the mothers or early embryos to high temperatures during specific stages of their development increases the proportion of gynandromorphs in the offspring (Kamping et al., 2007). A temperature sensitive mutation (*gyn1*) has been suggested to be responsible for the uniparental gynandromorph and daughter production and this locus has been mapped to chromosome IV close to the gene *doublesex*, but both genomic and cytoplasmic components are necessary for producing gynandromorph (Kamping et al. 2007). The gynandromorph producing stain has already been used to study the function of *dsx* gene and *tra* gene in Nasonia sex determination by investigating possible splice aberrations; both female and male variant of *dsx* and *tra* gene were found in gynandromorphic individuals, which indicates that *gyn1* is likely to be upstream of *tra*. However, it is possible that *wom,* msd *or zsd* is playing a role in producing the gynandromorph phenotype, but whether *tra* is involved in the producing the gynandromorph is not clear. It is necessary first to elucidate the role of *tra* in producing gynandromorph and how *tra* is affected by *gyn1* in *N.vitripennis*.

Project overview

This project started with sequencing *tra* from the gynandromorph producing strain HiCD12 and compared the sequences from a normal lab strain AcymC to identify polymorphism on the sequence of *tra*. After a 19 bp deletion on tra from HiCD12 was identified, the project took advantage of an introgression line to explore the relationship between tra polymorphism and the production of gynandromorph son. As both nucleic and cytoplasmic components are essential for the development of gynandromorph, this introgression line is created by crossing HiCD12 females to males from *N.giraulti*, because that *N.giraulti* shows no gynandromorph production and there are species specific SNPs between these two sister species. The following phase of the project continued the introgression experiment and collected females that produced gynandromorphic offspring in positive category, and no gynandromorphic offspring in negative category. *Tra* expression in these two categories of adult females was compared to test whether *gyn1* could affect *tra* expression. In the last part of the project, species – specific SNPs analysis around *dsx* were performed on the hybrid females form the introgression line to search for the location of *gyn1*. Our results suggest that mutations on the sequence of tra is not likely to be contributing to the production of gynandromorph

progeny and gyn1 does not have a strong effect on the *tra* expression level in adult females.

Materials and Methods

Preliminary experimental results

Previous experiments mapped the *gyn1* locus to chromosome IV (Kamping et al., 2007) and also showed that this locus is likely to be in the vicinity of *Nvdsx*. To further investigate and pin down the location of *gyn1*, an introgression line was created. This introgression line was created by crossing HiCD12 females, as both cytoplasmic and genomic components are necessary for producing gynandromorph progeny (Kamping et al., 2007), to males from *Nasonia giraulti*, a sister species that does no produce gynandromorph (**Figure 1**). During the introgression experiment, it was noted that the hybrid females lost the trait of producing gynandromorphic offspring until crossed back to the males of *N.vitripennis* HiCD12 strain, which indicates the *gyn1* is likely to be recessive or having two copies of it greatly increase the chance of producing gynandromorphic offspring. The aim of the introgression line is to isolate the components responsible for producing gynandromorph in *N.vitripennis* in the genetic background of *N.giraulti*. To differentiate genetic material between the two sister species, species-specific SNPs analysis was developed. The estimated the size of Nasonia chromosome IV is about 87.6 CM (Niehuis et al.2010), 410958bp were used as 1CM on chromosome IV. Within 10CM upstream and downstream of dsx, species specific SNPs were identified for every 1CM and primers were designed to amplify the regions containing these SNPs (**Figure 2**).

The sequencing of *tra* from the gynandromorph producing strain HiCD12 started before this project. Primers were designed based on the available genomic sequence of *Nvtra* from the lab strain AcymC (NCBI). The sequences of all nine exons and most introns of HiCD12 individuals were obtained and little differences were found between them and those from the lab strain AcymC. However, it was noted that regions on intron 2 were particularly difficult to amplify with primers designed based on AcymC sequences and leaves two regions of size 503bp and 741bp unsequenced (**Figure 3**). This is an indication that these regions within intron2 are likely to be more variable between the two strains.

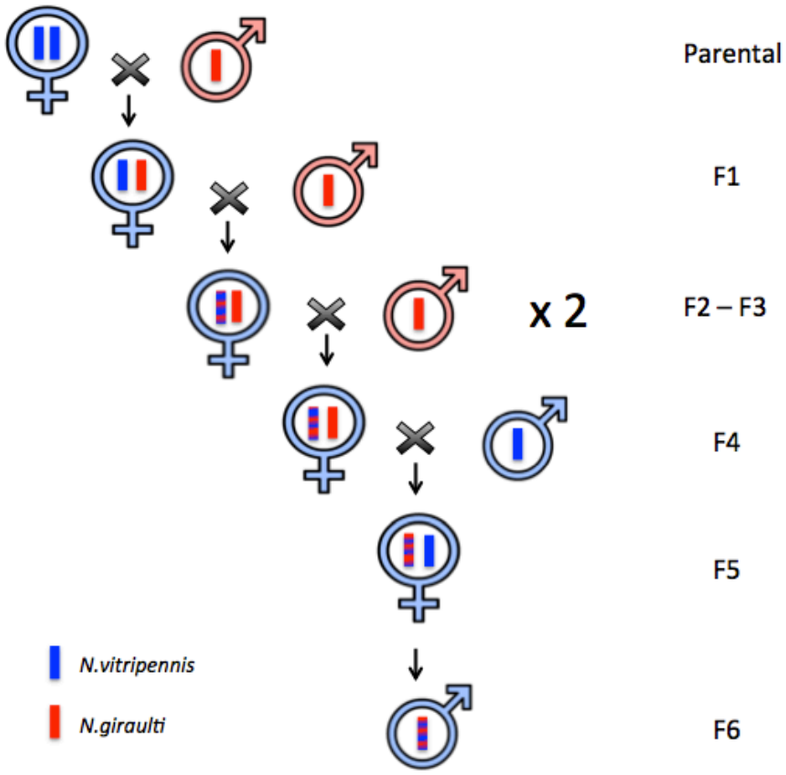


Figure1. Design of crossing
Females from *Nasonia vitripennis* ‘ gynandromorph strain (HiCD12) were crossed to *Nasonia giraulti* males as the parental line. The resulted female offspring were crossed again to *Nasonia giraulti* males, and this crossing was carried on for 3 generations: F2, F3, and F4 (performed by Mariia Chapliska). F4 females were crossed back to *Nasonia vitripennis* ‘ gynandromorph strain males and the females produced by this crossing (F5) were kept as virgins and they produced all male offspring (F6). F5 females are put to two categories (+ or -) depending on whether they produce gynandromorphic sons in F6 or not.

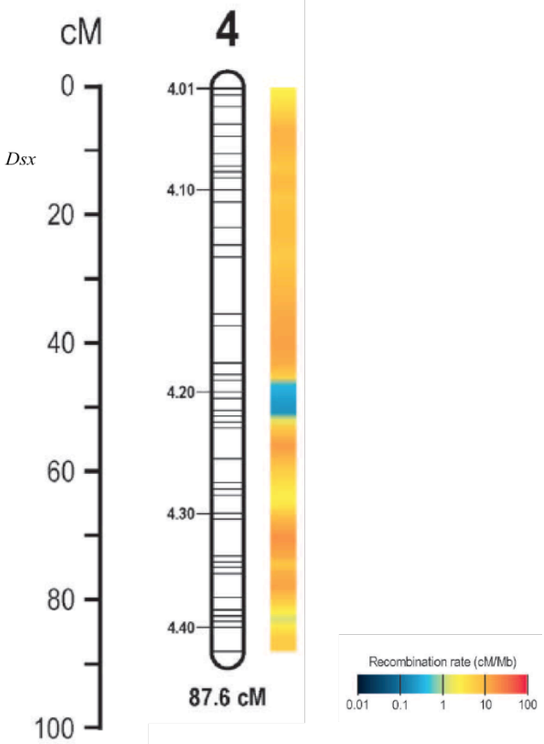


Figure 2: size and estimated recombination rate of *Nasonia vitripennis* Chromosome 4. The heat map next to the linkage map depicts the estimated local recombination rate (cM/Mb) in the corresponding region of the genome (Adopted form Niehuis et al., 2010).
On average, 1cM=410958 bp
Red arrow indicates the approximate location of Doublesex.

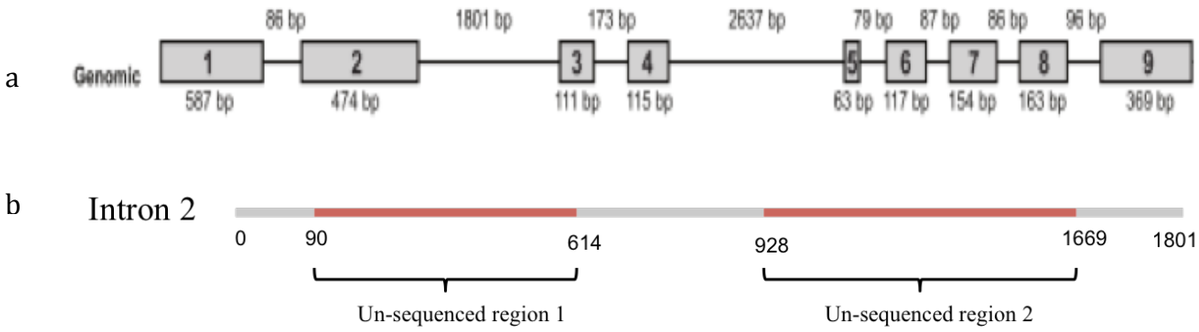


Figure 3, Structure of the Nvtra gene. (a) Genomic region of Nvtra with exon size indicated in base pairs (bp) below and intron size in bp above. (b) Intron2 of Nvtra with size indicated in bp below. Red segments indicate the previously sequenced regions, which are the focus of experiment 1.

Nasonia strains

The *Nasonia vitripennis* lab strains AsymC and HiCD12, and *Nasonia giraulti* lab strain RV tet were used throughout the experiments. All stains have been cured from *Wolbachia* infection, and were cultured under constant light at 25°C and reared on *Calliphora sp*. Fly pupae as hosts.

Experiment 1: Searching for tra sequence polymorphism

Sample collection:

10 male individuals from the Nasonia vitripennis lab strains AsymC and HiCD12 were obtained and stored in -20°C overnight before DNA extraction.

DNA isolation, Primer design and PCR:

Genomic DNA of the 10 male samples from these two lab strain were isolated following a standard high-salt DNA isolation protocol (SUPPLEMENTARY FILES). DNA concentration was quantified using Nano Drop ND2000.

In preparation of sequencing Nvtra intron2, primers were designed in Perl-Primer (Marshall 2004). To amplify the two missing regions within intron 2, primers were designed to anneal to the sequenced segment of intron 2 and have amplicon size of around 600bp and 900bp for the 503bp and 741bp unsequenced regions respectively (**Figure 3**). All primers were aligned to the *N.vitripennis* AcymC 'reference' genome by running BLASTn on NCBI. Settings were adjusted to short sequence searches. Primers were discarded if either the total primer length annealed more than 3x to the reference

genome without mismatches, or more than 5x if number mismatches ≤3. An over view of the primers used is shown in TABLE 1. The primers were diluted to 100 µM in nuclease-free water to create a stock solution and further diluted to 10 µM to create working solution.

PCR was performed according to a standard protocol (SUPPLEMENTARY FILES). PCR programme was adjusted for the amplicon size of the primers. The PCR programme started with 3 min denaturation (94°C), followed by 35 cycles of 30 sec at 94 °C, 60 sec at 55 °C, and 45 sec at 72 °C, and finished with 7 min at 72 °C. All PCR products were tested on a 1% Agarose gel, containing 1x TAE buffer and 10µg/µl EtBr. The Gene ruler 100 bp DNA ladder (Fermentas Life Sciences) was used for detection of DNA fragment sizes.

Sequencing and sequence analysis

After verifying PCR products of size and concentration with Agarose gel, the PCR products of expected size were cleaned using ExoSAP-IT, then Sanger-sequenced on a ABI 3130XL gene analyzer (Applied Biosystems). Obtained sequences were checked for quality (Phred quality > 20), reading frame, and were aligned in Mega. Sequence files were converted into fasta format and were aligned to the reference genome of *N.vitripennis* AcymC strain and *N.giraulti* in the database whole genome shotgun contigs (wgs) to search for strain specific polymorphism and species-specific polymorphism by running BLASTn on NCBI.

Experiment 2: association between tra polymorphism and the production of gynandromorphic offspring

After identifying the polymorphism on *Nvtra* between strains of *N.vitripennis* on intron 2, it is also noted that the same regions is variable between the Nasonia sister species. For this reason, the previously mentioned introgression line created between *N.vitripennis* HiCD12 strain and *N.giraulti* was used to explore the association between *tra* polymorphism and the production of gynandromorphic offspring. Restriction enzyme that cuts different times in the polymorphic site for the sister species of *Nasonia* was used to differentiate species-specific *tra*.

Sample collection:

The hybrid virgin females from the 4th generation of the introgression line were separated into positive and negative categories, based on whether they produced haploid gynandromorph male offspring. The genomic DNA samples from 6 positive category females and 9 negative category females were collected.

Primer design and PCR

To facilitate the use of restriction enzyme, primers were designed to amplify the region of the polymorphic site with minimum amplicon size. Primers were designed in Perl-Primer (Marshall 2004), with an amplicon size of 442bp from N.vitripennis HiCD12 strain (**TABLE 1**, primer set In2_del F and In2_del R2).

PCR was performed according to a standard protocol (SUPPLEMENTARY FILES). PCR programme was adjusted for the amplicon size of the primers. The PCR programme started with 3 min denaturation (94°C), followed by 40 cycles of 30 sec at 94 °C, 30 sec at 55 °C, and 40 sec at 72 °C, and finished with 7 min at 72 °C. To obtain higher concentration of PCR products, a secondary PCR was performed. The first PCR products were diluted 50times, and used as the template for the secondary PCR. The same PCR programme was used. All PCR products were tested on a 1% Agarose gel, containing 1x TAE buffer and 10µg/µl EtBr. The Gene ruler 100 bp DNA ladder (Fermentas Life Sciences) was used for detection of DNA fragment sizes.

Restriction enzyme digestion and Gel Electrophoresis

Restriction enzymes were searched with software NEBcutter (V2.0) with both *N.vitripennis* HiCD12 and *N.giraulti* sequence of the polymorphic region on intron2. Unshared restriction enzyme between the two species Hin1II (Thermo scientific), which cuts at the site 'CATG', was selected. With Hin1II, it will cut the amplified *tra* intron 2 regions containing the polymorphic site into 3 pieces with size of 207bp, 161bp and 72bp on *N.vitripennis* HiCD12 strain, and 2 pieces with size of 283bp and 161bp on *N.giraulti*.

After verifying the size and concentration of the PCR products with Agarose gel, 10µL of PCR products were transferred into a 0.5 tube and incubated with 2µL of Buffer G, 4µL of MilliQ water and 1µL of Hin1II at 37C for 1hour. Afterwards, the digestion products were loaded to a 3% Agarose gel, containing 1x TAE buffer and 10µg/µl EtBr. Electrophoresis was performed at a constant voltage of 90V for 3h.

Experiment 3: *Tra* expression and the production of gynandromorphic offspring

Introgression line and Sample collection:

Virgin females form the 4th generation of introgression line were collected and mated with males from *N.vitripennis* HiCD12 strain (**Figure 1**). Mated females were given hosts for oviposition at 31◦C, and parasitized hosts were put at 25◦C for completion of Nasonia offspring development. After 12 days, 5th generation female pupae were collected as virgins separately and kept at 31◦C. In total, 120 females were collected. After females emerged, they were given new hosts for oviposition. Females were placed into tubed with new hosts every 2 days, and in total three hosting was carried out. After the third hosting, all alive females were snap-froze with liquid nitrogen and then stored at -80 ◦C until RNA extraction. In total, 97 females were alive after the third hosting and were collected. All parasitized hosts were put at 25◦C for completion of *Nasonia* offspring development for about 2 weeks. Emerging haploid gynandromorphs were selected based on their morphological resemblance to either males or females (**Figure 4**, Kamping et al., 2007). Females were put into 'positive' category if gynandromorphs were observed among its offspring. In total, 8 positive category females were collected. 8 negative category females, 8 AcymC females and 8 HiCD12 females were also collected for the following experiment.

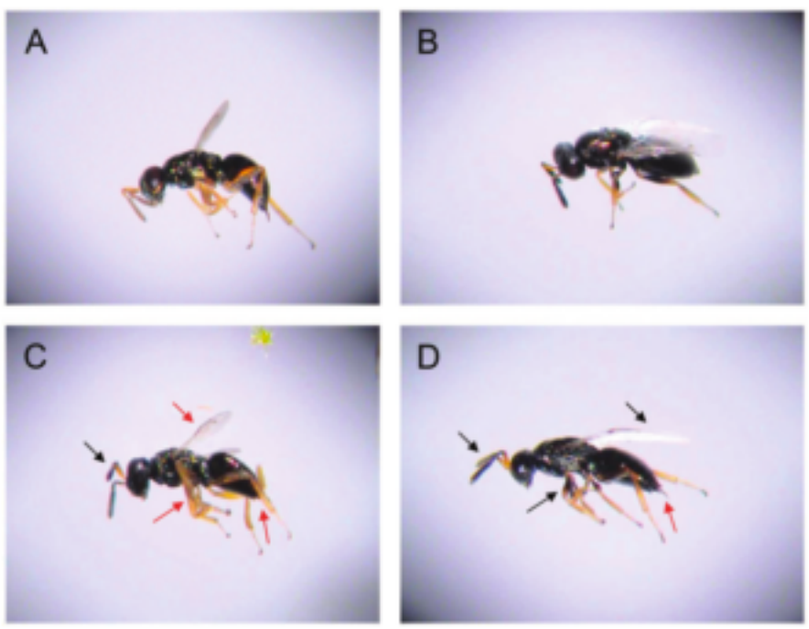


Figure 4.Morphology of *N. vitripennis*: Male (A), female (B), gynandromorph with female antennae (C), gynandromorph with female antennae, wings, and legs (D). Black

arrows indicate femaleness; red arrows indicate maleness (Picture taken from Kamping et al., 2007).

RNA isolation, cDNA conversion and Quantitative real-time PCR (qPCR)

Head and the rest of the body was separated for all insect sample, while head was stored at -20 ◦C until DNA extraction, RNA of the rest of body was isolated with TriZol (Invitrogen) and the entire isolated RNA sample was subsequently reverse transcribed with a mix of one part oligo-dT and six parts random hexamer both provided with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas).

The *Nvtra* primer set was designed in Perl-Primer (Marshall 2004), and was developed on non-sex specific parts of the mRNA. Two technical replicates were performed for all samples. One µl cDNA template was mixed with ABsolute™ QPCR SYBR Green ROX (500nM) Mix (ABgene) on an Applied Biosystems 7300 Real Time PCR System with 300 nM Nvtra qPCR primers NvTra qPCR F1 and NvTra qPCR R1 (**TABLE 1**). The elongation factor 1 alpha (*EF1α*) was used as reference gene, with 300 nM of primers NvEF1α F1 and NvEF1α R1 (**TABLE 1**). The following qPCR profile was used: enzyme activation of 95°C for 15 min, followed by45 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 30 s; and a final step extension (data collection point) of 72°C for 30 s. 1 µl of milliQ water was used instead of cDNA template as negative control. Samples were checked for non-specific amplification and primer-dimers using a standard ABI7300 dissociation curve and were also tested on a 1% Agarose gel, containing 1x TAE buffer and 10µg/µl EtBr.

LinReg- PCR (Ramakers et al., 2003) was used for calculating starting concentrations of both *NvTra* and the reference gene *EF1α.* Relative expression levels of each of the two technical replicates of each sample for *Nvtra* were calculated by comparing Nvtra starting concentrations to starting concentrations of the reference gene *EF1α*. The average of relative expression value of Nvtra of the 8 biological replicated from each category plus their standard errors were calculated to construct the graph.

Statistic analysis was performed in R (version 3.1.1). A linear model with mixed effect was used with relative expression of *tra* as response variable, category of female as fixed effect and sample ID as random effect (Package Plotrix, lme4 and nlme). Models

were constructed for two categories of females at one time to compare whether there is significant difference in *tra* expression between the categories.

Experiment 4: SNPs analysis around *doublesex* on gynandromorph-producing females and their offspring

Sample collection:

The head of the 8 positive category females used in the previous experiment and 8 negative categories females were collected, together with 4 gynandromorph males and 4 normal males from the offspring produced by the 5th generation introgression line.

DNA extraction and PCR:

Genomic DNA of all samples from was isolated following a standard high-salt DNA isolation protocol (SUPPLEMENTARY FILES). DNA concentration was quantified using Nano Drop ND2000. DNA isolated from the 8 negative categories females were diluted 10 times to a final concentration around 30ng/ µl for better PCR performance.

Primers set were already designed for SNPs analysis on *dsx* and nearby regions. After preliminary testing, primer set for SNPs analysis on *dsx*, 1cM and 8cM downstream of *dsx* and 7cM upstream of *dsx* were used in this experiment (**TABLE 1**). In total 15 species-specific SNPs between *N.vitripenni*s and *N. giraulti* were present.

PCR was performed as previously described. The PCR programme started with 3 min denaturation (94°C), followed by 40 cycles of 30 sec at 94 °C, 40 sec at 55 °C, and 30 sec at 72 °C, and finished with 7 min at 72 °C.

Sequencing and sequence analysis

Sequencing reaction and the following sequence analysis were performed as previous described.

Table 1: Overview of primers. Primers indicated by † were designed by Maria Chaplinska

Gene	Location	Application	Primer name	Sequence 5'-3'
Transformer	Intron 2	Sequencing	M1_2F	AAATGGAAGATTTGCTGTG
Transformer	Intron 2	Sequencing	M1_2R	ATCTTCAGTTGCTGATGGT
Transformer	Intron 2	Sequencing	M2_3F	AATTCTAGACAATCATACCAG
Transformer	Intron 2	Sequencing	M2_3R	TATTTCTTATCAAATCCTACCT
Transformer	Intron 2	Restriction enzyme digestion	In2_Del F	AGTGACTTCTGCAACTCTG
Transformer	Intron 2	Restriction enzyme digestion	In2_Del R2	TCCACTTCAATCGACTTAG
Transformer	Exon 3	qPCR	NvTra qPCR F1	CGCCGTTCTAAGTCATTGAG
Transformer	Exon 4/5	qPCR	NvTra qPCR R1	ATCGGAATAATGCCTATCGT

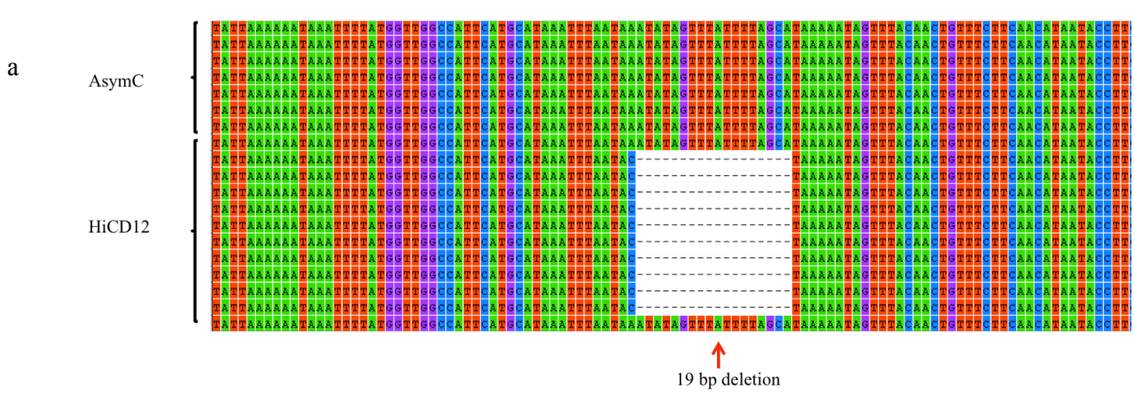
EF1α		qPCR	Nv EF1α F1	CACTTGATCTACAAATGCGG
EF1α		qPCR	Nv EF1α R1	GAAGTCTCGAATTTCCACAG
Doublesex		SNP analysis	Dsx F	GAAGCCTCGAGCCTGCTATA †
Doublesex		SNP analysis	Dsx R	TCTACTCGCGCCTTTAATGAC †
Minus_1cM		SNP analysis	M_1 F	CGCTCTTCATATCCGACAAG †
Minus_1cM		SNP analysis	M_1 R	TACTTTCATCTCCCAGCAAAC †
Minus_8cM		SNP analysis	M_8 F	CGCTTGTTATGGGCAATGGG †
Minus_8cM		SNP analysis	M_8R	ACATTACGGAGAGTCGGTACAC †
Plus_7cM		SNP analysis	P_7 F	GTCAGCGAAACTATTTCCGCA †
Plus_7cM		SNP analysis	P_7 R	GCTGTTATCAGAGACAACGGA †

Results

Experiment 1: Searching for *tra* sequence polymorphism

In total, 10 *N.vitripennis* males from the gynandromorph producing HiCD12 strain and 8 males from AcymC strain were used for sequencing. Two previously un-sequenced regions within *Nvtra* intron 2 were sequenced. After aligning all sequences with Mega, a 19 base pair deletion in the previously un-sequenced fragment 2 (**Figure 5a**) of the HiCD12 strain in comparison with AcymC strain was discovered. There is no difference in sequence among the males from the same strain. This deletion located at 142 base pair upstream of Exon3 of *Nvtra* (**Figure 5b**).
(All aligned sequencing results with can be found in SUPPLEMENTARY FILE 3.)

To check whether this deletion is located in evolutionary conserved region of *Nvtra,* consensus sequencing results of this fragment from each strain were blasted in the available genome of a sister species *Nasonia giraulti* on NCBI. The 19 bp deletion is shown to fall in a 36 bp variable region between *N.vitripennis* and *N.giraulti* (**Figure 6**).



b

Figure 5, a) Aligned sequences of 8 AsymC individuals, 10 HiCD12 individuals and available Nasonia vitripennis (AsymC strain) genome, showing a consistent 19bp deletion on the HiCD12 strain. b) Location of the 19bp deletion on *tra*.

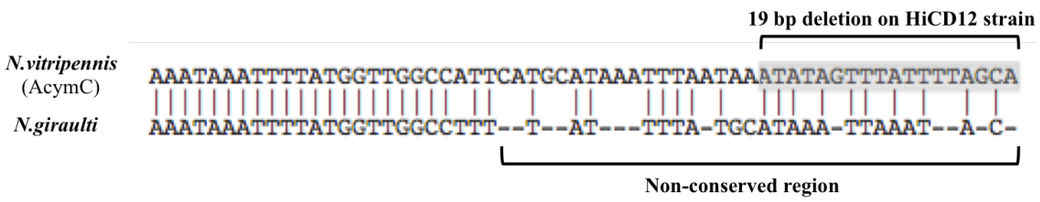


Figure 6, aligned sequences of *N.vitripennis* and *N.girault* of the area around the 19bp deletion showing that the deletion falls in a non-conserved region between the two sister Nasonia species

Experiment 2: association between *tra* polymorphism and the production of gynandromorph progeny

Restriction enzyme digestion was performed on the PCR products of a 440bp segment on *Nvtra* intron 2. This experiment includes 5 positive females and 9 negative females. These females were created by crossing 3rd generation introgression line female to HiCD12 males, as a result, they are either homozygous for HiCD12 *tra*, or heterozygous with one copy of *tra* from HiCD12 and one from *N.girault*.

For homozygous HiCD12 *tra,* the expected digestion result is 3 pieces of 207bp, 161bp and 72bp respectively.

For heterozygous *tra,* the expected digestion result is 4 pieces of 283bp, 207bp, 161bp and 72bp respectively.

3 positive females are shown to be heterozygous on tra, while 3 are homozygous.

6 negative females are shown to be heterozygous on tra, while 3 are homozygous (**Figure 7**).

This result indicates that being homozygous with HiCD12 specific *tra* is neither sufficient nor necessary for producing gynandromorphic offspring.

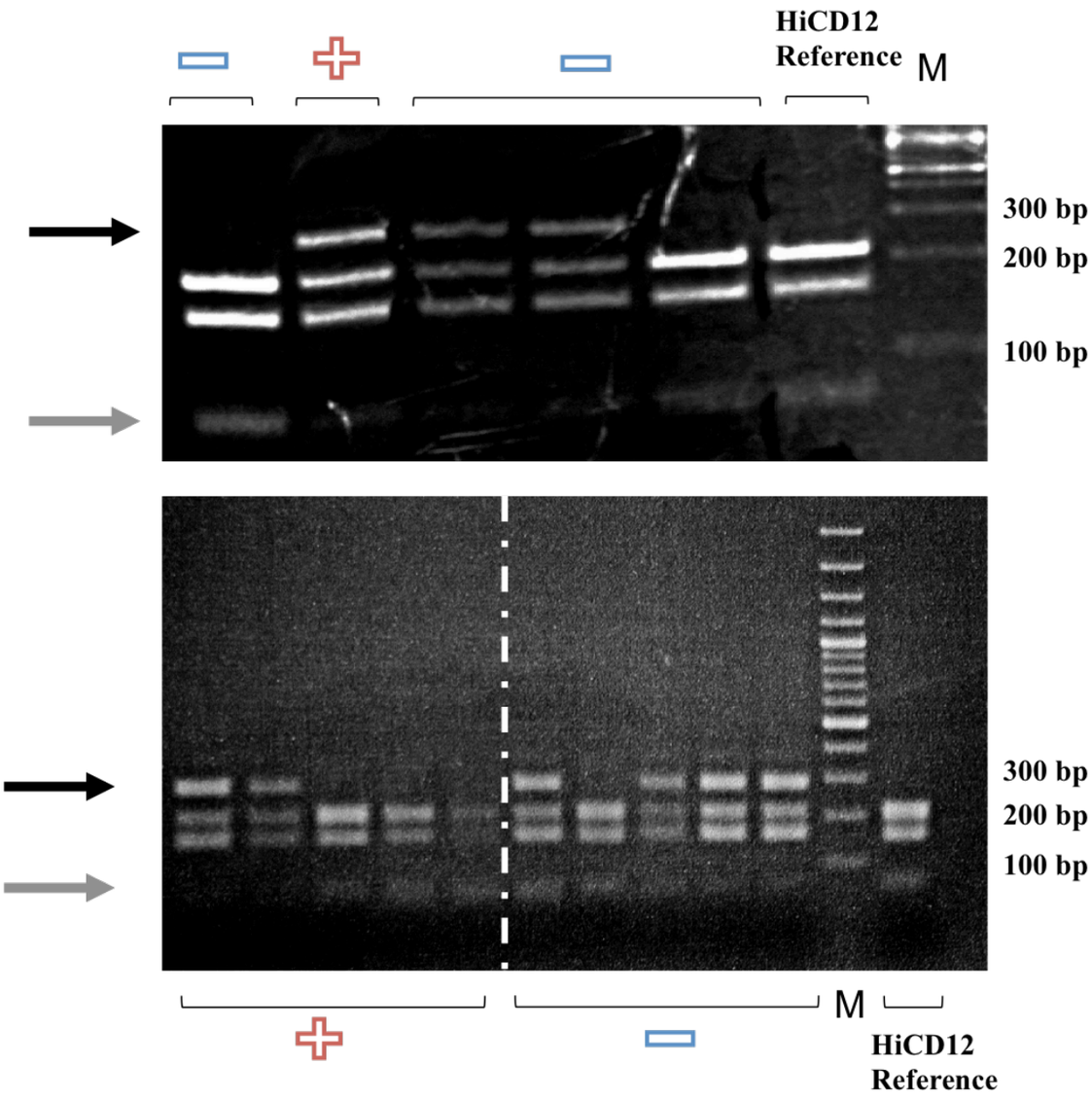


Figure 7, Digestion results with digestion enzyme HinII. Plus(+)sign indicates female from the positive category, while minus(-) sign indicates female from the negative category. A HiCD12 sample was used as reference. M is 100bp molecular size marker. Black arrow indicates *N.giraulti* specific band, and grey arrow indicate *N.vitripennis* specific band.

Experiment 3: *Tra* expression and the production of gynandromorph progeny

In this experiment, the relative expression of *tra* mRNA between the categories of positive (n=8) and negative females (n=8) from the 5th generation of introgression line were compared with qPCR. Females from HiCD12 (n=7) and AcymC strain (n=9) were also included in this comparison.

Among the four groups, females from the positive category show the highest level of *tra* expression, followed by females from AcymC strain and negative category. In contrast with previous preliminary results that the gynandromorph producing HiCD12 strain show higher tra expression than normal lab strain AcymC, in this experiment females from HiCD12 strain showed the lowest level of *tra* expression (**Figure 8**).

However, variance is big among females from the same category, especially among females from AcymC strain. The difference between *Nvtra* expression levels of positive category females and HiCD12 strain is significant (p=0.01), but none of the other paired group comparison in *Nvtra* expression level differences is significant.

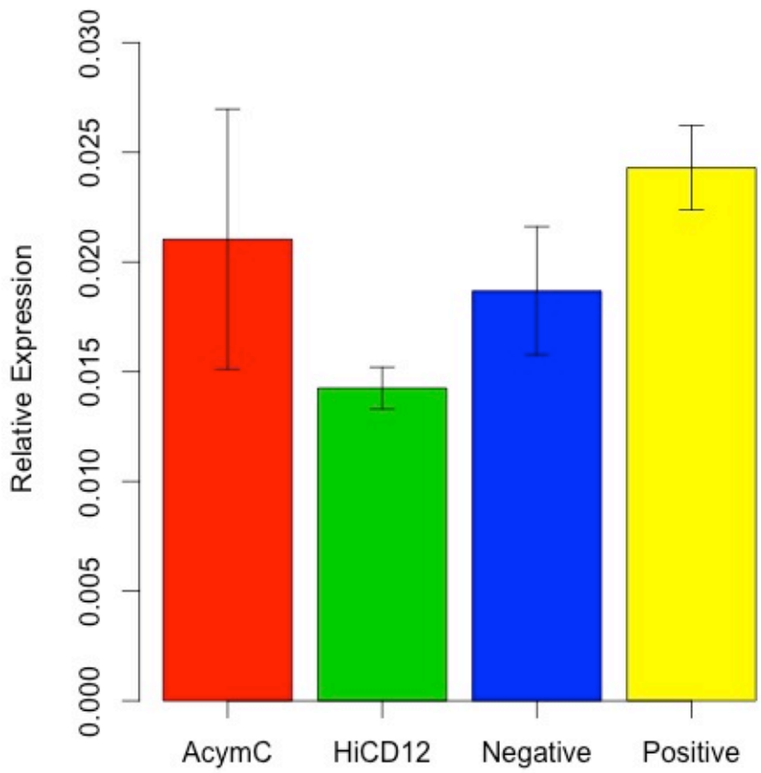


Figure 8, Relative levels of *Nvtra* mRNA in adult females from N.vitripennis AcymC strain (red bar), HiCD12 strain (green bar), and 5th generation of negative (blue bar) and positive (yellow bar) category of female from the introgression line.

Experiment 4: SNPs analysis around *doublesex* to search for *gyn1* location

The same 8 positive females from experiment 3, 4 phenotypically gynandromorphic and 4 phenotypically male offspring, together with 8 negative category females were used for this experiment. Primer set for SNPs analysis on *dsx*, 1cM and 8cM downstream of *dsx* and 7cM upstream of *dsx* were used, with in total 15 species-specific SNPs between *N.vitripenni*s and *N. giraulti*.

All 16 females are heterozygous for the sequenced segment on *dsx*, 8cM downstream of *dsx* and 7cM upstream of *dsx.* For the segment 1cM downstream of *dsx* 2 positive females and 1 negative female are homozygous for *N.vitripennis*, while the rest is heterozygous. Females' SNPs on one segment is consistent with each other (**Table 2**).

While 3 phenotypically male offspring from the positive females show a combination of *N.vitripenni*s and *N. giraulti* SNPs, 1 phenotypically male offspring and all phenotypically gynandromorphic offspring have all 15 *N.vitripenni*s SNPs (**Table 3**)

(Tables containing entailed results of SNPs analysis of the offspring are in SUPPLEMENTARY FILE. Aligned sequencing results are in SUPPLEMENTARY FILE).

Table 2, SNPs analysis result for positive and negative category female on *dsx*, 1cM(M_1) and 8cM (M_8) downstream of *dsx* and 7cM upstream (P_7) of *dsx*

Sample / SNPs position		Dsx	M_1	M_8	P_7
5th generation introgression line females	Positive (n=8)	All heterozygous	2 homozygous *N.vitripennis* 6 heterozygous	All heterozygous	All heterozygous
	Negative (n=8)	All Heterozygous	1 homozygous *N.vitripennis* 7 heterozygous	All heterozygous	All heterozygous

Table 3, SNPs analysis results for Haploid offspring of the positive category females on *dsx*, 1cM(M_1) and 8cM (M_8) downstream of *dsx* and 7cM upstream (P_7) of *dsx.*

Sample / SNPs position		Dsx	M_1	M_8	P_7
Haploid offspring of 5th generation introgression line positive females	Phenotypic Gynandromorphs (n=4)	All *N.vitripennis* SNPs	All *N.vitripennis* SNPs	All *N.vitripennis* SNPs	All *N.vitripennis* SNPs
	Phenotypic Males (n=4)	12 *N.vitripennis* *4 N.giraulti* SNPs	7 *N.vitripennis* *13 N.giraulti* SNPs	10 *N.vitripennis* *6 N.giraulti* SNPs	4 *N.vitripennis* *4 N.giraulti* SNPs

Discussion

Is there polymorphism on tra that contributes to the production of gynandromorph in HiCD12 strain?

The first part of the project leads to the identification of a 19 bp deletion polymorphism on *transformer* intron 2 between the lab strain AsymC and the gynandromorph producing strain HiCD12 of *Nasonia vitripennis*. This deletion also falls in a variable region between *Nasonia vitripennis* and it sister species *Nasonia giraulti*, which facilitates the designing of using restriction enzyme to differentiate the *tra* not only from the two strains of *N.vitripennis*, but also between the two *Nasonia* species. This result can be used in any future project that requires the discrimination of strain or species origin of *Nvtra*.

Afterwards, the project takes advantage of the introgression line between females from HiCD12 strain of *Nasonia vitripennis* and males from *Nasonia giraulti*. The association between *tra* type and the trait of producing gynandromorph offspring was tested among hybrid females from the 4th generation of the introgression line. Observation during the introgression experiment suggests that the locus contributing to producing gynandromorph offspring is likely to be recessive, however, being homozygous for HiCD12 *tra* is neither sufficient nor necessary for producing gynandromorph offspring. So, the results of this experiment do not indicate any strong link between *tra* type and the production of gynandromorph offspring.

Previous studies suggested that in gynandromorph producing strain a mutation occurred, which caused incomplete imprinting in the maternal germ line, generating a (partial) active *Nvtra* gene. A partial imprinting may give a reduced transcription rate of Nvtra in the haploid offspring, which would cause a partial start up of the auto regulatory loop. This would then lead to gynandromorph development (Kamping et al., 2007; Versulst et al., 2010). However, these studies did not exclude the possibility that mutations on *tra* in the HiCD12 strain could render the zygote more susceptible to the defect in the imprinting mechanism and thus increase the probability of gynandromorph production. As *tra* is the key gene in *Nasonia* sex determination, it is attractive to think

that mutations on this gene could affect the sex determination pathway and contribute to defects in sexual development. In this project, this hypothesis is explored and the result does not support the association of *tra* type with producing gynandromorph offspring. As *gyn1* is shown to be upstream of tra in the sex determination pathway (Verhulst et al., 2010), our result suggests mutations on *tra* do not have strong inference on *gyn1*'s effect.

Could gyn1 affect the expression of tra?

The second part of the project continued the introgression line for one more generation and 97 virgin females were collected for analyzing the expression of *tra* mRNA after producing offspring. Based on whether gynandromorph was found or not among their offspring, females were put into positive (gynandromorph found among her offspring) and negative (no gynandromorph found among offspring) categories.

Following that, qPCR was used to compare *tra* expression among positive females, and negative females, together with females from HiCD12 and AsymC strain. The females in the positive category show the highest level of tra expression, followed by AsymC females, then the negative category females and HiCD12 females. However, the difference between the positive females and the negative female is not significant, so is the difference between positive females and AcymC females, which are served as a reference for *N.vitripennis* adult female tra expression level. Contrary to previous observations, AcymC females show higher tra expression than HiCD12 females, however the difference is also not significant. Taken together, these results suggest that producing gynandromorph offspring is not correlated with higher or lower *tra* expression in adult females, and the locus responsible for gynandromorphism, *gyn1*, is not likely to affect the expression of *tra* in adult females.

However, some limitations of this experiment need to be noted. First of all, the sample size in this experiment is small, due to the limited amount of positive females from the introgression line. The experiment started with 120 5th generation hybrid females and 97 were collected after hosting for three times and fewer had three successful hosting. Among them, gynandromorphs were only found among the offspring of 8 females. Secondly, the negative category has a risk of being false positive. The number of offspring produced by these females ranges from around 30 to around 130 and proportion

of gynandromorph offspring also fluctuated drastically. As a consequence, the low number of offspring in certain cases could cause failing to find gynandromorph offspring. The negative category females used to be compared to positive females could be positive ones and subsequently increase the mean of *tra* expression in this category. Third, the age of the females was not controlled, while whether the expression level of tra in adult females is constant with age is not established and age was shown in previous experiment to affect produce gynandromorph (Kamping et al., 2007). The females from the introgression line were collected one week after emergency, while the age of females from AsymC and HiCD12 strain was not discriminated, which might contribute to the relative high variance observed among AsymC females.

The result of this experiment does not exclude that *gyn1*affects the expression of *tra,* but this effect might not be strong in adult females. It would be more revealing for future experiment to investigate the *tra* expression at the larvae stage of haploid offspring of positive females. Though it would still be challenging to collect some of the offspring at larvae stage and the rest for detecting gynandromorph phenotype from the same female. This experiment focused on non-sex-specific tra mRNA expression, it would also be interesting to explore sex specific variant of tra in different categories of females as the maternally provided *tra* mRNA is the female specific splicing variant.

SNPs analysis around dsx on chromosome IV to narrow down gyn1 location

The third part of the project uses the same introgression line females as in the previous part, and both the gynandromorph and male offspring of the positive females were also collected for species-specific SNPs analysis. Four segments on chromosome IV were used, which locates on *dsx*, 1cM, and 8cM downstream of *dsx* (M_1 and M_8) and 7cM upstream of *dsx* (P_7). Most females from both categories are heterozygous for the segments analyzed, expect 2 positive females and 1 negative female are homozygous on segment M_1.

Observation during introgression experiment suggests that gyn1 is likely to be recessive, as hybrid females created by mating HiCD12 females to N.giraulti males lose the trait of producing gynandromorph offspring and the trait is regained after these crossed the hybrid females back to HiCD12 males. In this experiment, the positive

category females produced gynandromorph offspring without being homozygous for the four segment analyzed indicates that *gyn1* does not locate in these regions. Alternatively, it could be that being homozygous for *gyn1* is not necessary to produce gynandromorph offspring, but having two doses of *gyn1* could greatly increase the chance of it and there are other factors or stochastic processes affect the production of gynandromorph.

Another interesting result is that all four haploid gynandromorph offspring showed all the 15 HiCD12 SNPs, while one male showed the same pattern and other males show a mix of HiCD12 SNPs and N.giraulti SNPs. This suggests that having all HiCD12 material on these regions could increase the chance of gynandromorph development, however, the sample size for the gynandromorphs is too small to be conclusive.

The SNPs analysis was only carried out on 4 segments on and around *dsx*, future experiment should continue on the other surrounding regions of *dsx* to narrow down the location of *gyn1*.

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