**Speciation**

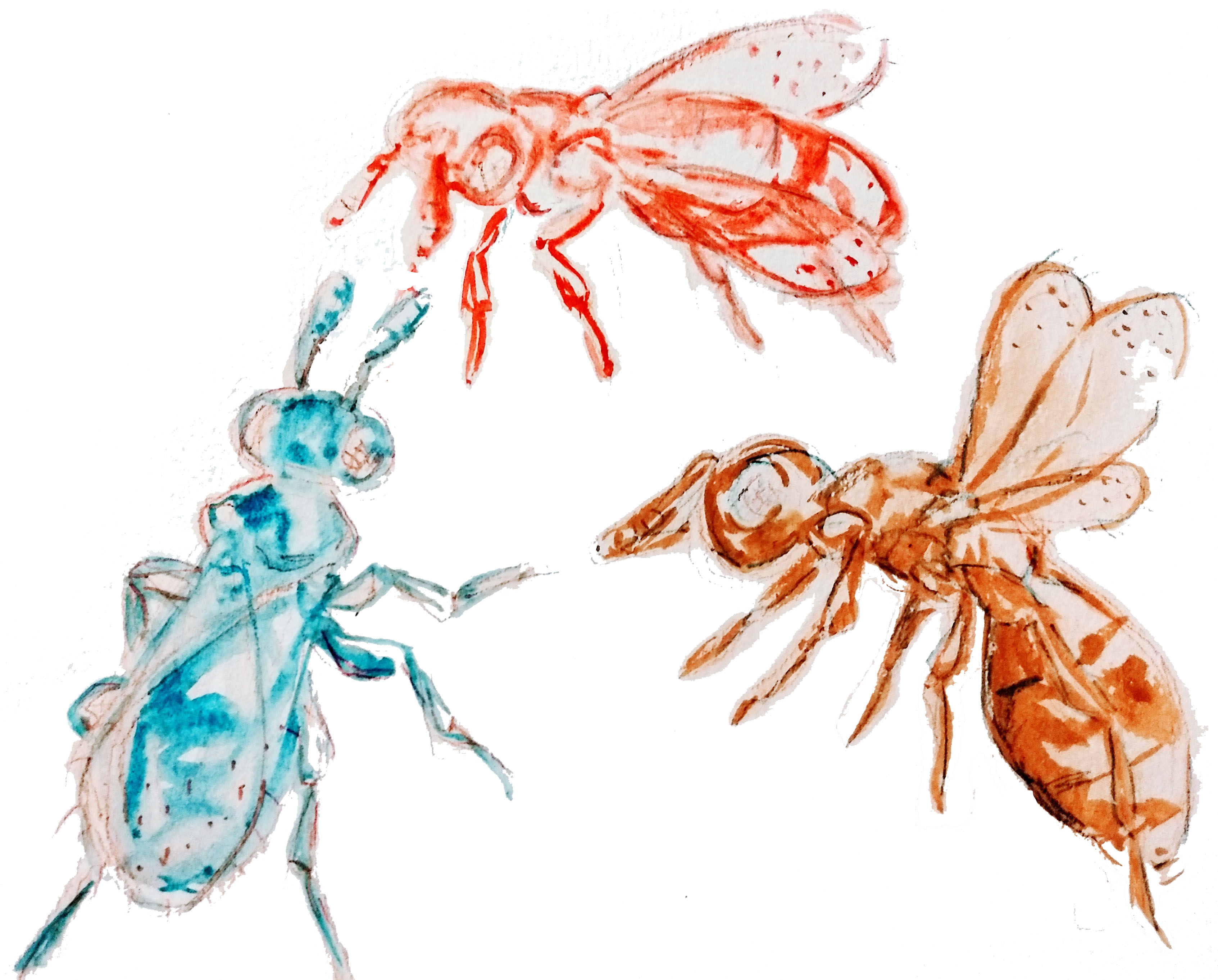
*Species identification and reproductive isolation*

Elzemiek Geuverink, Kelley Leung, Leo W. Beukeboom

**Part A) Morphological Differences and Prezygotic Isolation**

**Part B) Postzygotic Isolation**

**Part C) Phylogenetics**

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**Outline Note to instructor:** If the practical is being conducted with pre-existing data only and a physical experiment will not take place, in the student version delete sections that are in red and add the sections that are in blue. If a physical experiment is included, the practical will require set up on Week 1 and data collection 2 weeks later (Week 3). If only using pre-existing data, all work can be done in one day.

**Activities (Week 1, Week 3)**

Part A) (Week 1) Read background about speciation, reproductive isolation, and *Nasonia* biology. Experiment 1: Describe morphological differences Examine pictures of *Nasonia* species and answer questions about species differences. Experiment 2: Conduct prezygotic isolation experiment. (Week 3) Collect data, analyse data, and Using pre-existing data, answer questions about prezygotic isolation based on data analyses.

Part B) (Week 3) Experiment 3: Analyse provided dataset about postzygotic isolation (hybrid breakdown). Answer questions on post-zygotic isolation based on data analyses.

Part C) (Week 3) Experiment 4: Construct and interpret phylogenetic trees with provided dataset

**Materials**

For each team of 2-3 students:

1 work laptop with Microsoft Word, Microsoft Excel, and MEGA installed

1 dissection microscope

2-3 small paintbrushes (for transferring wasps)

100 small vials

Fresh cotton (for plugging small vials)

4 small vials each ofvirgin males (N=~20 each vial), one for each following strain: AsymCx (*N. vitripennis*), labelled only as “A”; RL MN 8150 (*N. longicornis)*, labelled only as “C”; STDR (*N.vitripennis*, red eye mutant), labelled only as “E”; and RVU2 (*N. giraulti*), labelled only as “G”. It is important to use these strains, as they have high laboratory fitness and are cured of antibiotic-cured of their Wolbachia endosymbiotic bacteria, which would prevent cross-strain mating.

4 small vials of virgin females (N=~20 each vial) , one for each following strain, with AsymCx (*N. vitripennis*), labelled only as “B”; RVU2 (*N. giraulti*), labelled only as “D”; RL MN 8150 (*N. longicornis)*, labelled only as “F”; andSTDR (*N.vitripennis*, red eye mutant), labelled only as “H”.

1 large vial of fresh blowfly pupae (~200 hosts). Commercially purchased *Sarcophaga bullata* or *Calliphora* spp. are recommended

2 racks for small tubes

1 marker for labelling tubes and racks

1 roll masking tape for labelling racks and euthanizing wasps

1 squirt bottle 70% ethanol for cleaning and euthanizing wasps

Paper towels for cleaning

**Objectives**

-Learn concepts of speciation and reproductive isolation

-Learn concept of haplodiploid sex determination

-Learn background of the *Nasonia* system

-Learn experimental handling of *Nasonia* as a laboratory model

-Learn analyses of experimental behavioural, reproductive, and fitness data

-Learn how to construct and interpret a phylogenetic tree

-Answer questions on biological knowledge based on experimental data

**Goal**

To enhance the knowledge of students on speciation and reproductive isolation, experimental design, and data analyses.

**Feedback**

Instructor will provide feedback on interpreting given background information to answer questions, following the scientific method to design and conduct an experiment, and drawing conclusions from experimental data.

**Part A — Morphological Differences and Prezygotic Isolation**

**Introduction**

Species form the building blocks of the tree of life. However, there are multiple species concepts and the definition of a species is constrained by the concept used. The **biological species concept** defines a species as a group of individuals that can interbreed and produce viable and fertile offspring. However, the barriers that reproductively isolate two species may not entirely prevent hybridization. Speciation processes can be studied in young species complexes, such as the *Nasonia* wasps used in this practical. This wasp complex consists of **allopatric** and **sympatric** species, enabling us to test different reproductive barriers under different conditions. This practical will additionally demonstrate phenomena that may not be easily studied in wild populations. For example, you will be asked to distinguish between **sexual dimorphism** and species-specific morphological variation. The distinction between **intraspecific** and **interspecific** **variation**, and how this variation is used in partner recognition and species recognition, is one of the major study areas of contemporary **speciation** research.

**Reproductive isolation**

Species are isolated from one another by **reproductive isolation barriers**. The barriers that exist between different species do not necessarily have to be the same as those that caused the separation of the species during the speciation process (they could have developed after the initial speciation). Reproductive isolation is divided into **prezygotic isolation mechanisms,** isolation barriers that take place before the formation of the zygote, and **postzygotic isolation barriers** barriers that act after the formation of the zygote – (Table 1).

Table 1. Overview of prezygotic and postzygotic isolation mechanisms (Ridley M, Evolution, Blackwell Science, Boston, 3rd ed, 2004).

1. Premating or prezygotic mechanisms prevent the formation of hybrid zygotes
   1. Ecological or habitat isolation.  
      The populations concerned occur in different habitats in the same general region.
   2. Seasonal or temporal isolation.  
      Mating or flowering times occur at different seasons.
   3. Sexual or ethological isolation.  
      Mutual attraction between sexes of different species is weak or absent.
   4. Mechanical isolation.  
      Physical non-correspondence of the genitalia of the flower parts prevents copulation of the transfer of pollen.
   5. Isolation by different pollinators.  
      In flowering plants, related species may be specialized to attract different insects as pollinators.
   6. Gametic isolation.  
      In organisms with external fertilization, female and male gametes may not be attracted to each other. In organisms with internal fertilization, the gametes or gametophytes of one species may inviable in sexual ducts or in the styles of other species.
2. Postmating or postzygotic mechanisms reduce the viability or fertility of the hybrid zygotes
3. Hybrid inviability  
   Hybrid zygotes have reduced viability or are inviable
4. Hybrid sterility.  
   The F1 hybrids of one sex of both sexes fail to provide functional gametes.
5. Hybrid breakdown.  
   The F2 or backcross hybrids have reduced viability or fertility.

|  |
| --- |
| **Box 1 - *Nasonia* biology**    *Nasonia* are small parasitoid wasps (Hymenoptera: Pteromalidae) that parasitize blowflies and fleshflies (see Figure 1 for the life cycle). These wasps have become a popular model organism for evolutionary biologists and geneticists. They are easy and quick to culture (generation time of 14-16 days at 25ºC), their gender is easy to determine (see Box 2) and they can be handled without anesthesia. Like all wasps, *Nasonia* has haplodiploid reproduction (see Figure 1 on page 5). Males develop from unfertilized eggs and are haploid, while females develop from fertilized eggs and are diploid. Sons only have a mother and no father. Furthermore, females decide whether or not to fertilise eggs, so mothers control production of daughters versus sons.  *Nasonia* uses different fly species as its host (mainly *Protocalliphora)*. The fly larvae feed on blood of bird chicks and pupate in the bottom of nests. After young birds have fledged, *Nasonia* appear in the bird nests to parasitise the fly pupae. The flies are also found on animal carcasses where the fly larvae feed and pupate on meat. Bird nests and carcasses are only suitable as for fly larval feeding and pupation for a short time, and only on a local scale. This means that *Nasonia* populations and their hosts are actually made up of many small, isolated subpopulations (demes). *Nasonia* males have a slightly shorter development time than females. In contrast to the females, *Nasonia* males cannot fly and mate locally with females from the same patch. Depending on the number of females (foundresses) that laid eggs in that deme, the males often mate with their own sisters. Females can fly, and so disperse and migrate to colonize new hosts.    There are four closely related species in the *Nasonia* genus, *N. vitripennis*, *N. longicornis*, *N. giraulti and N. oneida*. *N. vitripennis* is found throughout the world, but the other species are endemic to North America.    Figure 2. Life cycle of the parasitoid wasp *Nasonia* *vitripennis* and their *Protocalliphora* fly hosts. The fly larvae live inside bird nests and carcasses. After pupation they are vulnerable to parasitisation by *Nasonia*. A *Nasonia* female lays about 10-40 eggs per fly pupa, the wasp larvae will feed on the fly pupa. The wasps will emerge after approximately two weeks at 25ºC (unless they are in developmental diapause, a winter survival strategy that occurs when mothers are exposed to shorter photoperiod, in which case they can be kept in 5ºC storage for two years), The males emerge first. The males have short wings and cannot fly. They will mate with the emerging females, which will then disperse and colonize new hosts. The flies are ~10 mm, the wasps are 2-3 mm. |

**Biogeography of *Nasonia* wasps**

The *Nasonia* genus consists of four species that differ in distribution. *Nasonia vitripennis* is a cosmopolitan species (Figure 1a), whereas the other species have a more restricted geographical distribution to North America (Figure 1b). *Nasonia longicornis* occurs sympatrically with *N. vitripennis* in western North America. In eastern North America *N. vitripennis* occurs sympatrically with *Nasonia giraulti* and *Nasonia oneida*.

The species also differ in niche use. Whereas *N. vitripennis* is a generalist parasitoid, capable of parasitizing a wide range of blowflies and fleshflies, the other species are specialists and strongly prefer to parasitize true blowflies (*Protocalliphora*) that occur in bird nests.

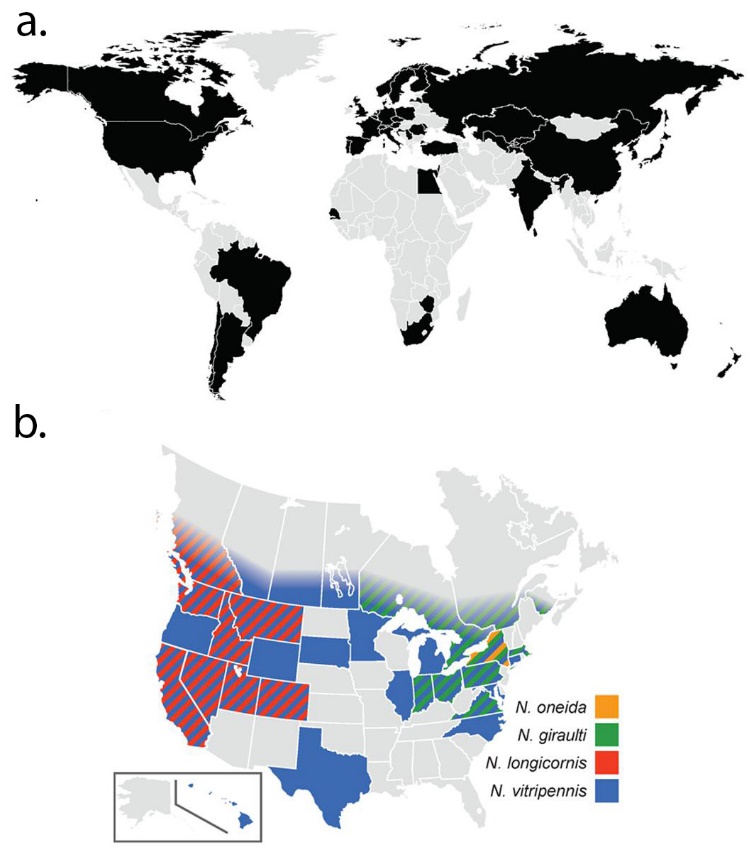


Figure 2. (a.) Geographical distribution of *N. vitripennis*. *N. vitripennis* has been reported from the countries marked in black. (b.) Distribution of the four *Nasonia* species in North America. Figure from Dittmer, J. *et al.* 2016. Disentangling a holobiont – Recent advances and perspectives in *Nasonia* Wasps. Front. Microbiol. 7:1–18.

***Nasonia* mating behavior and haplodiploid reproduction**

The mating behavior and reproductive mode of *Nasonia* make it possible to assess a range of different **behavioral isolation barriers**. The male will chase after the female to try to mount her. If successful, he will display a range of courtship behaviors while positioned on top of the female. After a series of head nods, the female may accept the male and allow him to copulate. She will signal receptivity by lowering her antennae and the male will move backwards to copulate with the female. Afterwards he will reposition himself on top of the female again and display post-copulatory behaviors, which decrease the likelihood of the female re-mating. In various stages of this mating process, the female can attempt to move away, or the male can fail in performing the species-specific features of the mating behavior and dismount if the female does not assume a copulatory position. These courtship steps allow for a detailed study of behavioral and mechanical reproductive barriers between the species.

**Gametic isolation barriers** can be detected using the haplodiploid mode of reproduction. *Nasonia* females are diploid and lay viable haploid eggs without fertilization. These haploid eggs will develop into males (Figure 2). If a *Nasonia* female mates with a male, she will fertilize a large proportion of her haploid oocytes with the male’s haploid spermatozoa (Figure 2). These diploid embryos will develop into females. Thus, if a female has mated with a male, but does not produce female offspring, gametic isolation barriers may have prevented fertilization (Table 1). Alternatively, this lack of female offspring may result from **hybrid inviability**, in which fertilization occurred but the embryo did not survive(Table 1). As the female will still produce male haploid offspring, it is easy within the the haplodiploid system to distinguish between speciation barriers (whether gametic or postzygotic) and any fecundity issues.

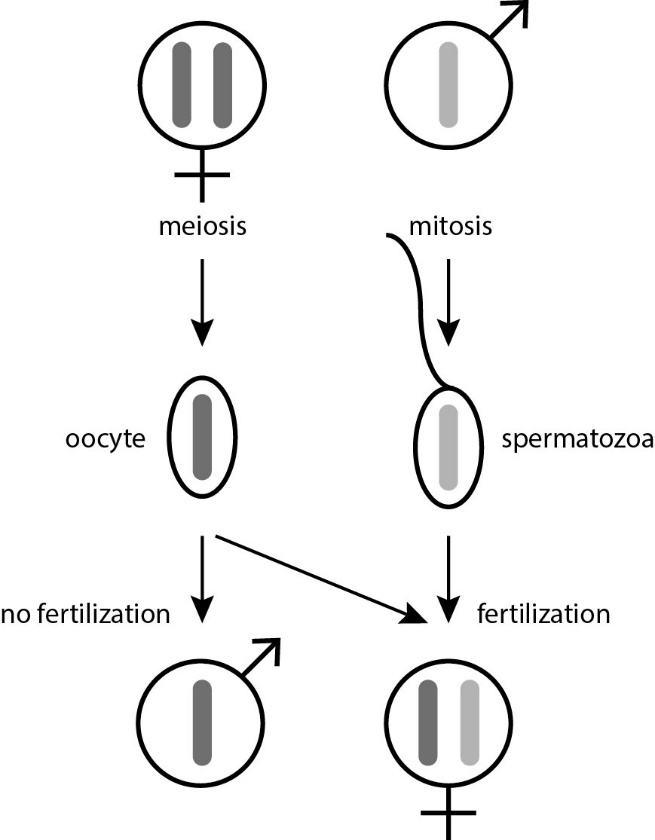
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Figure 3. Haplodiploid systems consist of diploid females and haploid males. Both sexes produce haploid gametes, but the eggs (oocytes) of the female can develop without fertilization. This results in haploid male offspring. If the oocyte is fertilized by a sperm (spermatozoa),, the resulting diploid embryo will develop into a female. In a cross between two species these females (bottom-right) will be F1 hybrids, whereas her brothers are not hybrids and are the same species as their mother (bottom-left).

**Using haplodiploidy to test postzygotic isolation**

The F1 females emerging from a hybrid cross between two *Nasonia* species can be used to assess both **hybrid sterility** as well as **F2 hybrid breakdown**. They will be separated as pupae and kept in individual tubes. This ensures their virginity and allows separation of postzygotic isolation effects from any potential prezygotic isolation effects in an F1 cross. Sterility can be detected if the virgin hybrid F1 female fails to lay eggs in the provided fly hosts. F2 hybrid breakdown is categorized as a reduced fitness of later generation hybrids. Screening the resulting F2 (all male) offspring will detect any developmental effects in this hybrid generation that indicate potential hybrid breakdown.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Box 2 – How to handle Nasonia Even though *Nasonia* are wasps, they cannot sting people. Males and females of *N. vitripennis* are easily distinguished: males have short rudimentary wings, while females have larger wings that extend over the tip of the abdomen (see Figure 4). The males of the other *Nasonia* species have longer wings than the *N. vitripennis* males, but not as long as the females, making it harder to tell the sexes apart in the other species. However, males have yellow antennae and legs and females have black antennae and darker legs. In addition to that, females have an ovipositor at the ventral side of the abdomen.  Adult wasps can be shaken in small numbers from the main vial onto the working surface. The wasps are more visible on a light-colored background. When the wasps are walking around on the working surface, you can isolate them by placing a small vial over them. Most of the times the wasps will walk up the vial. If necessary, the wasps can be pushed by using a small paintbrush. The vials are closed off with cotton wool and placed in styrofoam trays. The wasps are cultured in a climate-controlled room at 25ºC, but to speed up development to ensure wasps emerge in time for the second practical, after the first day transfer to 30ºC (Figure 5). You may euthanize loose wasps with masking tape or spraying and wiping with 70% ethanol, and wasps still in tubes by freezing overnight.  Differences between *Nasonia* ♂ and ♀   |  |  | | --- | --- | | ♂ ( | ♀ | | Yellow antennae and legs (all species) | Black antennae and partially black legs | | Tip of abdomen rounded (all species) | Tip of abdomen pointed with ovipositor |   Description: femaleDescription: male  Figure 4. Morphological differences between the *Nasonia vitripennis* sexes. Photo by P. Koomen    Figure 5. *Nasonia* cultures incubated in plastic vials in an incubator |

**Experiment 1: Morphological differences in the *Nasonia* genus**

The *Nasonia* wasps The pictures of *Nasonia* wasps that you will receive are of laboratory strains. The species have been described and studied for decades, enabling a fast identification, if you know which features to look for! In the field, or with a new study system, you would not have this luxury. This is the unfortunate reality of most ecological and evolutionary research projects. We will mimic this situation here. When encountering a new study species, the first step towards determining the species boundaries and potential speciation mechanisms is describing distinguishable morphological features.

Question 1.1

You will receive vials pictures with *Nasonia* wasps. Describe their morphological features.

|  |  |  |
| --- | --- | --- |
| Vial | Sex | Description |
| A | Male | *Short wings* |
| B | Female | *“Standard female”* |
| C | Male | *Mid-length wings* |
| D | Female | *“Standard female”* |
| E | Male | *Short wings and red eyes* |
| F | Female | *“Standard female”* |
| G | Male | *Long wings* |
| H | Female | *Female with red eyes* |

Question 1.2

How many species do you think you have received?

*Answers will vary, but check for logic (e.g. it should be at least 3 species, and based on male morphology rather than female morphology as male wings vary in size whereas females of the different species look similar).*

**Experiment 2 Prezygotic isolation in *Nasonia***

The males and females that you have identified in experiment 1 can be used in mating trials. This will screen any potential prezygotic reproductive isolation barriers. The most informative experimental set up is **reciprocal crosses** (think this over—why?). This means that the female of species 1 will be tested with the male of species 2, as well as the other way around in a test of the female of species 2 with the male of species 1. Each mating trial consists of a single male and a single female.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Prezygotic isolation  4 trials per combination | | Female | | | |
| B | D | F | H |
| Male | A | AB | AD | AF | AH |
| C | CB | CD | CF | CH |
| E | EB | ED | EF | EH |
| G | GB | GD | GF | GH |

Use 4 mating trials per combination to ensure enough replicates (particularly for the combined group data set), so test 4 vials with each one A male and one B female. At this point, you presumably do not know yet which exact cross is the reciprocal one. However, as you will test all possible combinations (16 types of crosses), the reciprocal cross will be in the scheme. Thus, in total you will have 64 mating trials. Start with just one trial at once, but you will notice that you will quickly be able to manage multiple trials at the same time.

To set up a mating trial:

* Mark the vial with the letters of the combination and the number of the replicate (e.g. AB1)
* Place a single male in a small plastic vial
* Add a single female to the vial
* Observe their behavior within a 5-minute time frame: score whether the male mounts the female and how much time transpired until the mounting, and whether copulation occurred. Fill this out in datasheet 2.1. You will copy this data into a shared datasheet for the whole group.

At the end of the experiment:

* Provide 2 fly hosts and place the vial in a styrofoam rack.
* Incubate the wasps at 25°C.
* Complete the excel sheet for day 1 (Week 1) and e-mail this file to your partner(s) (this is essentially a back-up on a server).

**This is the end of speciation practical day 1 (Part A, Week 1).**

You will receive a dataset for which each group conducted four mating trials per combination (16 types of crosses, accounting for all crosses), totaling 64 mating trials each group. The data provided will be:

**Week 1 (Datasheet pre-zygotic isolation)**

Within a 5 minute observation period,

-Whether the male mounts the female

-How much time transpired until the mounting

-Whether copulation occurred

**Week 3 (Datasheet post-zygotic isolation )**

-Whether trials produced offspring

-Whether trials produced female offspring

These data were pooled across groups for you to conduct analyses with greater statistical power.

Sheet 2.1

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| *Crosses* | | | *Score on practical day 1 (Week 1)* | | | *Score on practical day 2 (Week 3)* | |
| Male | Female | Replicate | Time to mount (in seconds) | Mating behavior (yes/no) | Copulation (yes/no) | Offspring (yes/no) | Female offspring (yes/no) |
| A | B | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| A | D | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| A | F | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| A | H | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| C | B | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| C | D | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| C | F | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| C | H | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Male | Female | Replicate | Time to mount (in seconds | Mating behavior (yes/no) | Copulation (yes/no) | Offspring (yes/no) | Female offspring (yes/no) |
| E | B | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| E | D | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| E | F | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| E | H | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| G | B | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| G | D | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| G | F | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |
| G | H | 1 |  |  |  |  |  |
| 2 |  |  |  |  |  |
| 3 |  |  |  |  |  |
| 4 |  |  |  |  |  |

**Experiment 2 Prezygotic isolation in *Nasonia***

Continuation after 2 weeks (Week 3): the next generation of wasps has emerged and the vials will be returned to each team for scoring.

Complete the last two columns of datasheet 2.1:

* Did the trials yield offspring?
* Was female offspring (daughters) produced? (No need to determine the exact sex ratio.)

Email your datasheet to your instructor

Answer the questions on the next sheet with the overall group data. While you are waiting for this to be uploaded to the group datasheet, continue with experiments 3 and 4. An overview of the data will be put together in a table, while you are working on the other experiments. A clear way to represent the data is to make a graph of the table, so we will ask you to do this for both experiment 2 as well as experiment 3. Ask for assistance if needed.

Questions 2.1 *(Use the combined group results!)*

Which type of graph would you use to display the data? Include it here.

*There will be two types of data-points. The timing will be an average in seconds. The other categories will be calculated (by the instructor or in the summary that the students get back after handing in their raw data) as proportions. Barplots are the easiest way to visualize the proportions.*

Which crosses display behavioral mating barriers? Can you recognize different behavioral patterns?

*Male A and E will approach the female rapidly, C is slower and G is likely the slowest. Males A and E are also most likely to exhibit mating behavior and approach all types of female they encounter with similar interest. Females D and F are, however, a lot less willing to mate with them.*

*Female F will be most discriminatory, particularly towards male A and E, but also against G. Female D will also discriminate against A and E, but less strongly against male C.*

*All interspecific crosses should display some level of isolation. These are the AD, AF, CB, CD, CH, ED, EF, GB, GF and GH crosses. The intraspecific crosses AB, AH, CF, EB, EH and GD are expected to have high rates of acceptance, but this may be beyond the 5 minute mark that will likely be observed for CF and GD.*

Do the mating barriers result in lack of daughter production? Or do you see daughters produced where no matings were observed?

*There may be no trials with successful matings in CF and GD, which will not produce daughters. Some trials with A and E males may have been scored as successful by the students, when in fact the male did not manage to copulate, also resulting in no daughters. It is also acceptable for students to speculate that copulation did occur, but male did not succeed at transferring sperm or the female chose not to use transferred sperm.*

Based on the behavioral observations and fertilization successes, which females and males do you think belong to the same species?

*Here it should be noticeable that A, B, E and H are the same species.*

How many species were present in the dataset, based on the observations of the prezygotic isolation experiment?

*Here the likely answer is 3 (or lower if female D is receptive to multiple male types).*

**Experiment 3 Postzygotic isolation in *Nasonia***

The female offspring obtained in the previous experiment, designated generation F1, can be used to test postzygotic isolation barriers. Specifically, their offspring, generation F2, can potentially suffer from hybrid breakdown. Testing this in this practical would be tricky, as the F1 females have to be collected as virgins (to test hybrid males in the F2) and the subsequent culturing would take another 2-3 weeks. So, instead, you will be provided with a dataset from a previous experiment.

Use these data to answer the questions on the next page. An overview of the data is provided, but it works better to visualize the data. Make a graph of the table.

Material and methods postzygotic isolation in *Nasonia*

F1 females were collected in the pupal stage and kept separate from the males. After emergence as adults, individual virgin females were placed in a small plastic vial with two *Calliphora* sp. hosts. The parasitizing females were incubated at 25 degrees Celsius and their offspring was scored after 18 days of incubation. The male F2 offspring was categorized in four types: emerged adults, adults remaining inside the host, pupae and larvae.

Questions 3.1

Which type of graph would you use to display the data? Include it here.

*Stacked barplot of all types (not including the total category).*

Which crosses display hybrid F2 breakdown? Does the number of offspring or the developmental speed differ between the crosses?

*All interspecific crosses (AD, AF, CB, CD, CH, ED, EF, GB, GF and GH) display F2 breakdown, but to varying degrees of severity.*

*The hybrid females have fewer offspring than the purebred females, with the exception of F1 females from the male N. longicornis (C) x female N. giraulti (D) and male N. giraulti (G) x female N. longicornis (F) crosses The offspring of purebred females develop faster than the offspring of hybrid females. Hybrids females will therefore have a greater proportion of larval and pupal offspring than adult offspring compared to the purebred females. Many of the offspring of F1 hybrid females will remain in the pupal stage especially those from male N. longicornis (C) x female N. giraulti (D) and male N. giraulti (G) x female N. longicornis (F) crosses.*

*The results of the three reciprocal crosses are not equal. F2 hybrids with Nasonia vitripennis grandmothers, and therefore N. vitripennnis type cytoplasm, have more offspring than hybrids with N. vitripennis grandfathers and the cytoplasm type of a non-N. vitripennis grandmother species. F1 females of N. longicornis (C) x female N. giraulti (D) and male N. giraulti (G) x female N. longicornis (F) crosses have roughly equal degrees of reproductive success. Post-zygotic isolation is strong in hybrid crosses with N. vitiripennis but less strong in N longicornis to N. giraulti crosses (regardless of the male-female combination). If it is taken into account that almost no wasps develop beyond the pupal stage in hybrid crosses, post-zygotic isolation is almost complete. Reciprocal crosses differ in the degree of post-zygotic isolation; it is stronger in N. vitripennis crosses wherein F1 hybrid females have a N. longicornis or N. giraulti cytoplasm type (i.e. hybrid crosses using N. vitripennis males results in greater hybrid breakdown than those using N. vitripennis females).*

Based on the hybrid F2 breakdown, which females and males do you think belong to the same species?

*Again it should be noticeable that A, B, E and H are the same species. The CD and GF crosses perform reasonably well and may lead the students to conclude that these could be the same species.*

How many species were present in the dataset, based on the observations of the postzygotic isolation experiment?

*3*

**Experiment 4 Phylogeny of *Nasonia***

The previous experiments have yielded data about the different isolation barriers between the wasps. However, it did not provide any information on the species identity. Some of the species occur sympatrically, so even their location of origin would not necessarily be informative. As the species differ in their host usage, data on host use could provide some clues, if you had no other way of identifying the exact species. An easier method would be genotyping the wasps and comparing their DNA sequence data to one another and to *Nasonia* data already available on Genbank.

The *Nasonia* strains were genotyped for the mitochondrial marker, cytochrome c oxidase subunit 1 (COI), which is often used to “barcode” species in identification programs (i.e. track the species identity of individuals). Here we provide 8 amplified products of COI for the *Nasonia* species (representing the A-H groups you used in the previous experiments), each with a chromatogram of the forward sequence and a chromatogram of the reverse sequence. We also provide a sequence for the outgroup species *Trichomalopsis sarcophagae,* An outgroup species is a related species that is known to be more distant relative than the experimental groups being analyzed for their unknown evolutionary relationships to each other, which acts as calibration point. Divide the *Nasonia* chromatogram files between you and your teammate. The chromatograms (DNA sequence files) that you will analyze are t(.ab1 files). These files can be opened with a chromatogram-viewer, Chromas.

Open the chromatogram-file in Chromas by double-clicking on it or by starting Chromas and clicking the ‘Open’ button on the top menu. Assess the peak pattern and exclude unreliable regions (containing overlapping peaks or high levels of background noise) by trimming the sequence on the left and the right side (Figure 6).

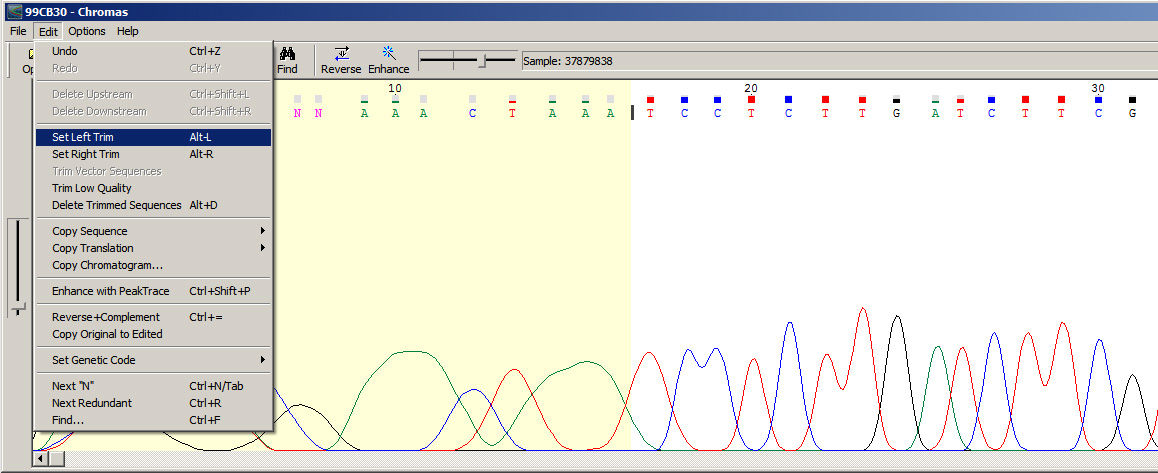


Figure 6. How to trim a chromatogram file in Chromas. This function was not available in Chromas lite.

Examine if there are any cases of multiple peaks or unclear nucleotide calls in the sequence. Replace these calls with a lower case “n” (Figure 7). The letter “N/n” indicates that a specific call (A/C/T/G) cannot be made, whereas the lower case allows you to distinguish between calls made by the sequencing software (capital letters) and adaptations you made.

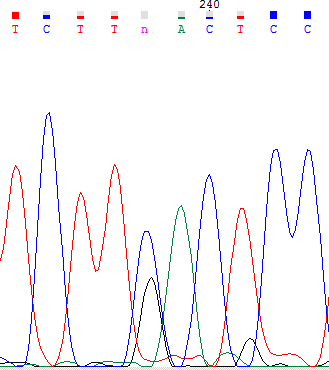


Figure 7. Example of a double peak requiring a manual change to a lower case “n”.

Export the sequence as a fasta-file (Figure 8.) This fasta file can be loaded into MEGA. Trim all chromatogram files and convert them into fasta files, before proceeding to MEGA.

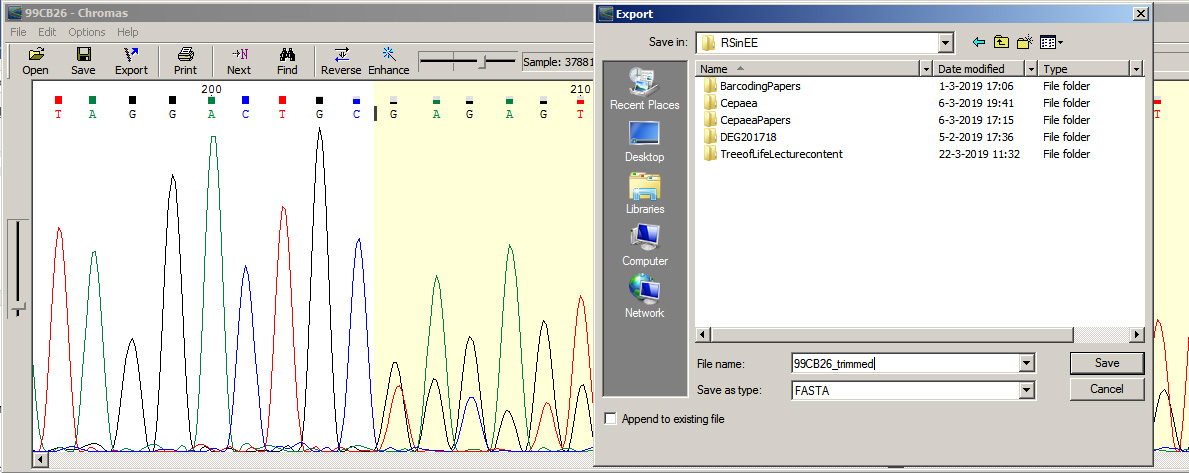


Figure 8. Exporting a trimmed sequence from Chromas. The trimmed portion of the sequence (yellow background) contains a clear example of overlapping peaks.

Open all 16 fasta-files of your marker into a single MEGA alignment session. Half of your sequences results from the reverse primer and need to be both reversed as well as complemented (because they are from the opposite strand). You can do this in MEGA for each sequence from a reverse primer (Figure 9).

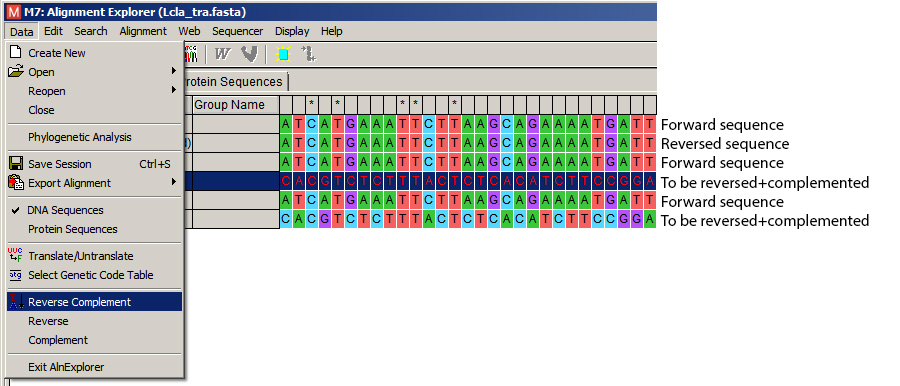


Figure 9. How to obtain the reverse complement of a specific sequence.

After all the sequences are in the same orientation, make an alignment. Leave the forward and reverse sequence separate. Are they identical? Check your alignment for any irregularities. Insert the outgroup COI sequence from *Trichomalopsis sarcophagae* and redo the alignment. Check the alignment again and produce a neighbor-joining tree rooted on the outgroup *Trichomalopsis*.

Additionally, take the sequences of all samples and run them through BLAST (explained below). This will yield a species identification based on previously uploaded sequences in Genbank. You can use this to answer question 4.1.

How to run online Blast:

* Go to the NCBI (National Centre for Biotechnological Information) BLAST website; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).
* Select “Nucleotide Blast”.
* Copy-paste a sequence from MEGA or upload a fasta-file.
* Select ‘nucleotide collection (nr/nt)’ as the database.
* (Optionally, select “Nasonia (taxid:7424)” in the Organism field to increase the speed of the BLAST search.)
* Also click ‘show results in a new window’.
* Now start the ‘BLAST’ search (blue button).

Questions 4.1 *(First prepare your phylogenetic tree!)*

Based on your analyses of the sequence data, which species did you perform these experiments with?

|  |  |  |
| --- | --- | --- |
| Vial | Sex | Species |
| A | Male | *Nasonia vitripennis* |
| B | Female | *Nasonia vitripennis* |
| C | Male | *Nasonia longicornis* |
| D | Female | *Nasonia giraulti* |
| E | Male | *Nasonia vitripennis* |
| F | Female | *Nasonia longicornis* |
| G | Male | *Naosnia giraulti* |
| H | Female | *Nasonia vitripennis* |

Discuss whether the strength of prezygotic and postzygotic isolation between the species coincides with the relatedness between the species.

C:\Users\p250307\Desktop\Nasonia_tree.tif

Pre- and post-zygotic barriers between *N. longicornis* and *N. giraulti* are not very strong, which matches the relatedness of these two species. According to the tree they split after splitting from *N. vitripennis*, thus the expectation would be that each species is equally discriminatory against *N. vitripennis* (especially as they occur sympatrically with *N. vitripennis*). However, *N. longicornis* demonstrates stronger female discrimination against *N. vitripennis.*

Notes on sequences: sequence E forward is too messy to use. The students should recognize this (it is why we are making them go through this by hand) and not use it in the alignment. If they take the readable sequences, trim them, and use them the same way (correct open reading frame (ORF) and invertebrate mitochondrial genetic code): the tree should look approximately as above.

Questions 4.2

What can you conclude about the strength of **pre**zygotic isolation in sympatry and allopatry?

*Pre-zygotic isolation is strongest in sympatric species (N. vitripennis and N. longiornis; N. vitripennis and N. giraulti), and less strong in the allopatric species*

*N. longicornis and N. giraulti.*

What can you conclude about the strength of **post**zygotic isolation in sympatry and allopatry?

*Postzygotic isolation is strongest in the sympatric species (N. vitripennis and N. longicornis; N. vitripennis and N. giraulti), and less strong in the allopatric species N. longicornis and N. giraulti. Interspecific mating could occur in nature, as the pre-zygotic isolation is not absolute. If natural hybrids have a low fitness, natural selection can cause the courting behavior of the three species to become more and more different.*

*The differences in reproductive isolation in Nasonia may also have nothing to do with allopatry versus sympatry, but with the amount of time since species divergence. Nasonia longicornis and N. girualti split most recently and therefore did not build up as many differences across their genomes compared to N. vitripennis, resulting in the lowest isolation between these two species. Thus postzygotic isolation may be mainly determined by time, and in turn reinforce prezygotic isolation.*