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REVIEW ARTICLE

Standard methods for molecular research in *Apis mellifera*

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Summary

From studies of behaviour, chemical communication, genomics and developmental biology, among many others, honey bees have long been a key organism for fundamental breakthroughs in biology. With a genome sequence in hand, and much improved genetic tools, honey bees are now an even more appealing target for answering the major questions of evolutionary biology, population structure, and social organization. At the same time, agricultural incentives to understand how honey bees fall prey to disease, or evade and survive their many pests and pathogens, have pushed for a genetic understanding of individual and social immunity in this species. Below we describe and reference tools for using modern molecular-biology techniques to understand bee behaviour, health, and other aspects of their biology. We focus on DNA and RNA techniques, largely because techniques for assessing bee proteins are covered in detail in Hartfelder *et al.* (2013). We cover practical needs for bee sampling, transport, and storage, and then discuss a range of current techniques for genetic analysis. We then provide a roadmap for genomic resources and methods for studying bees, followed by specific statistical protocols for population genetics, quantitative genetics, and phylogenetics. Finally, we end with three important tools for predicting gene regulation and function in honey bees: Fluorescence in situ hybridization (FISH), RNA interference (RNAi), and the estimation of chromosomal methylation and its role in epigenetic gene regulation.

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Métodos estándar para la investigación molecular en *Apis mellifera*

Resumen

Las abejas de miel han sido durante mucho tiempo un organismo clave para avances fundamentales en biología a partir de estudios de su comportamiento, comunicación química, genómica y de biología del desarrollo, entre otros muchos. Con la secuencia del genoma en la mano y herramientas genéticas mucho mejores, las abejas son ahora un blanco aún más atractivo para responder a las preguntas más importantes de la biología evolutiva, la estructura de las poblaciones y la organización social. Al mismo tiempo, los incentivos agrícolas para entender cómo las abejas caen enfermas, o evadir y sobrevivir a sus muchas plagas y patógenos, han presionado para comprender genéticamente la inmunidad individual y social en esta especie. A continuación se describen y se hace referencia a herramientas que hacen uso de modernas técnicas de biología molecular para entender el comportamiento de las abejas, su salud y otros aspectos de su biología. Nos centramos en las técnicas de ADN y ARN, en gran parte debido a que las técnicas de evaluación de las proteínas de la abeja se tratan en detalle en Hartfelder *et al.* (2013). Cubrimos las necesidades prácticas de toma de muestras de abejas, su transporte y almacenamiento, y luego se discuten una serie de técnicas actuales de análisis genético. A continuación, se proporciona una hoja de ruta para los recursos genómicos y métodos para estudiar las abejas, seguido de protocolos estadísticos específicos de la genética de poblaciones, la genética cuantitativa y la filogenia. Finalmente, se termina con tres herramientas importantes para predecir la regulación génica y la función en las abejas melíferas: la hibridación in situ fluorescente (FISH), la interferencia de ARN (iARN), y la estimación de la metilación cromosómica y su papel en la regulación epigenética de los genes.

西方蜜蜂分子研究的标准方法

摘要

通过行为、化学通讯、基因组和发育生物学等方面的研究，蜜蜂已经成为用于在生物学基础研究领域取得重大突破的一种重要模式生物。结合已有的基因组序列和多种改进的遗传学工具，蜜蜂已经越加成为回答进化生物学、种群结构和社会性结构等方面重大问题极具吸引力的研究目标。与此同时，农业上为了解蜜蜂如何困于病害或者避开和幸存于多种害虫和病原菌的危害，也促进了对这一物种个体和社会免疫的遗传学理解。以下我们介绍和引用了一些运用现代分子生物学技术研究蜜蜂行为、健康、以及其它方面生物学的工具。Hartfelder等2013已对研究蜜蜂蛋白做了详细的论述，因此我们将重点放在DNA和RNA技术上。本文也包含了在蜜蜂采样、运输和保存过程中的实际需要，并讨论了当前的一系列遗传分析技术。然后我们提供了研究蜜蜂时所需的基因组资源和方法的路线图，以及群体遗传学、数量遗传学和系统发生学研究中特定的统计学方法。最后，我们以预测蜜蜂基因调控和功能的三个重要工具收尾：荧光原位杂交（FISH）、RNA干扰（RNAi）和染色体甲基化及其在表观遗传基因调控中作用的估算。

Keywords: *Apis mellifera*, pollination, disease, development, genomic, Colony Collapse Disorder, population genetics, methylation, RNA interference, RNAi, Southern Blot, Northern Blot, *In situ* Hybridization, DNA extraction, Next-generation sequencing, mitochondrial DNA, microsatellite, quantitative PCR, COLOSS, *BEEBOOK*, honey bee

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1. Sample management

1.1. Introduction

In order to best reflect honey bee biology, data generated from molecular-genetic studies should reflect as closely as possible the state of honey bee tissues, entire bees, or colonies just prior to sampling. This fact places a premium on collecting and storing samples in a way that retains this state. Although technological developments in molecular biology allow for a great diversity of insights from collected bee samples, it is often forgotten how much these insights are hampered by errors in the collection, storage and processing of samples (Chernesky *et al.,* 2003). These problems are especially evident when data from different studies or laboratories are compared (Birch *et al.,* 2004). The only solution to this is optimization of collection, storage and primary processing protocols, so as to minimize the influence of sample degradation on the molecular analyses and the reliability of the data. As is often the case, cues can be taken from other areas of biology, notably the medical field, where such practices are widely adopted (Valentine-Thon *et al.,* 2001; Verkooyen *et al.,* 2003).

A secondary consideration is that a sample may be used for several different analyses; proteins, nucleic acids, fats and lipids, metabolites *etc.*, requiring a collection and processing protocol suitable for all compounds analysed. Usually this means that the sample management conditions follow the requirements for the least stable of the compounds, which for bee research is usually the RNA. RNA is highly sensitive to degradation by robust RNAse enzymes found in all cells, unless the sample is stabilized with RNAse-inhibiting additives and/or frozen as soon as possible. Given the necessity of RNA analyses for many questions related to bees and their parasites and pathogens (e.g., de Miranda *et al.* 2013), field-appropriate methods for stabilizing RNA are required.

1.2. Sample collection

The optimum strategy for collection and transport of bee samples depends partly on what type of sample is collected. Bees, pupae, larvae and eggs can be sampled whole or as field-dissected components, such as heads, thoraxes, abdomens, guts, endophalli, semen, ovaries *etc*. Many bee viruses are shed in large amounts in the guts, as are many bacterial and protozoan pathogens (Shimanuki, 1997; Fries, 1997). Faeces may therefore be a good marker for the infection status of the whole bee, although care has to be taken to distinguish between passively acquired/passaged microbes and true tissue infections. Faeces also allow bees to be sampled repeatedly, and non-destructively. It may therefore be useful for determining the virus status of queens (Hung, 2000), especially if these are a major source of infection of the worker population (Chen *et al.,* 2005b; Fievet *et al.,* 2006), or for following disease progression in individual bees.

Below are suggestions for the collection of different types of bee samples. In all cases a priori decisions are all needed with respect to the use of chemical stabilizers, collection cards and the temperatures during transport and storage.

1.2.1. Adult bees

1.2.1.1. Nurse bees

Inspect each frame in a colony and find a frame with sealed and unsealed brood which is covered by adhering nurse bees and then take the frame out of the colony.

1.2.1.2. Foraging bees

Block the hive entrance where foraging bees are accumulating and collect the returning foraging bees.

1.2.2. Pupae

1. Cut out a section of sealed brood, to be transported whole. Such a brood section can be sent through the post, although with the caveat that such transport away from the hive might affect bee or parasite gene activities.
2. Uncap brood cells, lift pupae by their neck by curling fine curved forceps underneath their heads and transfer to a suitable transport medium, either individual microcentrifuge tubes or collection cards (see section 1.3.5).

1.2.3. Larvae

1. Cut out a section of open brood and transport in a temperature -humidity controlled box, to prevent dehydration.
2. Remove larvae from the comb using either a blunt grafting needle (small larvae) or soft forceps (large larvae) and transfer to individual microcentrifuge tubes or collection cards.

1.2.4. Eggs

1. Cut out a section of comb with eggs and transport in a temperature-humidity controlled box, to prevent dehydration.
2. Remove eggs using a blunt needle and transfer individually or in bulk to microcentrifuge tubes or collection cards.

As an alternative for the rapid collection of massive amounts of eggs and early embryos:

1. Strike soundly a frame containing early-stage bees onto a sterile surface twice.

This releases over half of the eggs and embryos held by that frame,

2. Brush or lift into a new vessel.

While uncapped honey will drip via this method, if done at the right intensity, older uncapped larvae will remain in their cells.

1.2.5. Extracted guts

1. Grab the stinger and last integument of adult worker bees firmly with a pair of fine, straight forceps.
2. Pull backwards gently, removing the whole hindgut and midgut.
3. Transfer guts to individual microcentrifuge tubes or collection cards (see section 1.3.5.).

1.2.6. Drone endophallus and semen

1. Turn drone upside down and grip laterally between thumb and index finger.
2. With the other hand, gently but persistently squeeze the abdomen of the drone dorso-ventrally, exerting pressure backwards, until the endophallus is extruded from the drone.
3. Apply more severe pressure, again backwards, to avert the endophallus and, for mature drones, cause ejaculation of semen.
4. Cut off the entire endophallus with scissors, or collect the exposed semen (brown-red colour) and/or seminal fluid (translucent white) with a sterile micropipette.
5. Collect the material individually in microcentrifuge tubes or on collection cards.

1.2.7. Faeces

If destructive sampling is allowed:

1. Remove the whole gut from individual bees (see above) and expel faecal mass.

If repeated sampling is required:

1. Place adult bees into a Petri dish until defecation has occurred.
2. Collect faeces individually or pooled in microcentrifuge tubes or on collection cards.

1.2.8. Dead bee samples

Many bee disease experiments involve bee death as a parameter. Dead bee samples from such experiments are, of course, valid material for analysis. They should be treated like freshly killed material and frozen as soon as possible to minimize the effects of decay on RNA integrity, using the collection methods appropriate for the sample type, as given above. Dead bee traps attached to hives are suitable for collecting such bees and should be emptied daily to minimize the effects of decomposition.

Passive surveys also involve dead bee samples, in this case those sent in by beekeepers for *post-mortem* analysis of the cause of colony death. These bees will have been dead long enough for decomposition and drying to have severely affected the integrity of the RNA, including viral RNAs. Such degradation can severely affect the accuracy and reliability of detecting and quantifying individual RNAs (Bustin and Nolan, 2004; Fleige and Pfaffl, 2006; Becker *et al.,* 2010). This means that only positive results from such samples are informative, since negative results can be either due to the absence of virus or the degradation of the RNA.

It is possible to adjust for differential RNA degradation in the different samples with quantitative RT-qPCR techniques, by using host internal reference gene levels for normalizing the virus titers (Dainat *et al.,* 2011) and setting the threshold for template detection with the most degraded sample, so that all samples are evaluated by the same degradation criteria. How to determine the detection thresholds using RT-qPCR assays is covered in section 4.4.

1.3. Sample transport

Sample transport from the collection site to the laboratory is the most critical step in sample management, since this is where the integrity of the sample is most easily compromised (Chen *et al.,* 2007). Sample integrity can be preserved to different degrees with the following methods, given in order of effectiveness. The gold standard for sample collection and transport is to freeze on-site, but this is not always possible. All alternatives are basically aimed at getting the samples as quickly and conveniently as possible into a freezer, with minimum degradation. The most useful tool for transporting frozen material is a liquid nitrogen-based 'dry shipper', which is specifically developed and approved for international shipment of biological samples at ultra-low temperatures (-150^{o}C). The best can hold these temperatures for more than one week. Other options, for more local transport, are (dry) ice-boxes and portable/car freezers. Courier and mail services are less reliable, both with respect to the maintenance of temperature and the duration of transport.

1.3.1. Freezing with dry ice

- Samples: all.
- Conditions: freeze instantly; keep frozen throughout transport using blocks of dry ice in a cooler.
- Transport: restricted transport; dry ice must be replenished ca. every 48 hours.
- Processing: transfer samples to freezer.
- **Pros:** gold standard; fast.
- **Cons:** very expensive; complex field operation.

1.3.2. Freezing with 'wet' ice

Short-term field-storage on 'wet' (frozen water or ice packs) ice is cheap and very practical for many field-studies and surveys. The samples should be frozen as soon as possible, ideally within hours, and kept frozen continuously until RNA processing (a complete frozen transport chain). If a complete frozen transport chain cannot be guaranteed, then a chemical stabilizing agent (see section 1.3.4.) should be used to prevent degradation of the RNA by RNAses, until the samples enter a frozen transport chain. The most important rule for RNA preservation is to keep the samples as cold as possible, as

long as possible and to avoid thawing the sample after it has been frozen unless it is to extract RNA.

- Samples: all.
- Conditions: collect in freezer bags, store on wet ice.
- Transport: cold transport; wet-ice; < 12 hours.
- Processing: transfer samples to freezer.
- **Pros:** simple; fast; cheap field operation.
- **Cons:** heavy, expensive mail transport, leaks due to thawing.

1.3.3. Live transport

Bees can also be transported live, which allows them to be sent much more quickly, cheaply and reliably by post than frozen samples. One drawback is that the stress of live transport may affect the expression of host genes, and possibly virus replication, which should be taken into account when planning experiments.

1. Adult bees can be transported live 1) In a well-ventilated bee shipping box containing queen candy and a sponge soaked in water glued to the bottom of the box or 2) in units of 10-15 bees in commercial queen cages with queen candy. Such queen cages are readily available to most beekeepers.

- Samples: adults.
- Conditions: room temperature.
- Transport: < 48 hours.
- Processing: freeze on arrival.
- **Pros:** simple; fast; suitable for beekeepers.
- **Cons:** stress during transport.

2. Pupae can be transported live 1) as a section of capped brood in a well-ventilated bee shipping box, preferably in a warm environment to prevent chilling, 2) as queen cells for queen pupae in a specialized temperature-humidity controlled queen-cell transport container, available from beekeeping suppliers. Such cells should be handled with great care, as developing queen pupae are very sensitive to disturbance, or 3) as a whole frame in a specialized carrier box for frames, available from beekeeping suppliers, or in a swarm box/nucleus hive.

- Samples: pupae.
- Conditions: room temperature.
- Transport: < 48 hours.
- Processing: remove samples from comb and freeze.
- **Pros:** simple, fast.
- **Cons:** pupae may emerge during transport**.**

3. Larvae and eggs can be transported live 1) as a section of comb, in a temperature and humidity-controlled box or 2) as a whole frame in a specialized carrier box for frames, or in a swarm box/nuc.

- Samples: larvae; eggs.
- Conditions: controlled temperature and humidity.
- Transport: less than 48 hours.
- Processing: remove samples from comb and freeze.
- **Pros:** simple, fast.
- **Cons:** expensive by mail, unsealed larvae are subject to temperature stress and starvation.

1.3.4. Chemical stabilizers

There are a number of chemicals that can be used to help stabilize nucleic acids during transport. Their purpose is to inhibit nucleases, especially the resilient RNAses, and in doing so destroy all enzymatic activity in the sample. So, if the final assays include natural enzymatic activity, these stabilizers should be avoided. For similar reasons, many stabilizers are also incompatible with serological detection methods, such as ELISA.

A large excess (5-fold by weight) of stabilizer should be added to ensure a high enough concentration within the tissues for inhibiting RNAses. It is also essential that the solution penetrates the tissues completely to abolish all RNAse activity. This is a major difficulty for aqueous stabilizers, which cannot penetrate the hydrophobic insect exoskeleton. These are therefore only suitable for extracted tissues, eggs and small larvae, unless bodies are partially disrupted at the start. Organic preservatives, such as 100% ethanol, have much more effective penetration of the exoskeleton and are therefore better for stabilizing whole adult bee samples. Although 100% ethanol is suitable for preserving RNA destined for short-fragment RT-qPCR-based assays, storage in 70% ethanol has been shown to result in strong degradation (Chen *et al.,* 2007). However, recent data using a short amplicon (124 bp) diagnostic for Deformed wing virus (DWV) in a Taqman assay (Chantawannakul et al., 2006), showed no loss of DWV signal after adult bees were stored for 4 weeks in 70% EtOH at room temperature compared to snap frozen controls (G. Budge, unpublished data). RNA can also be stabilized by high concentration sulphate salt solutions (Mutter *et al.,* 2004), of which RNAlater® (Qiagen) is the best known. A generic version can be made as follows:

700 g	di-ammonium sulfate
40 ml	0.5M EDTA (pH 8.0)
25 ml	1M tri-sodium citrate (di-hydrate salt; 29.4 g/100 ml)
1l	sterile water
~1.3 l	total volume

Once stabilized, RNase activities will be inhibited and samples can be stored for up to 1 month at 4°C, and long-term at -20°C or -80ºC with minimal degradation. The stabilizer should be removed from the bee sample prior to homogenization and RNA extraction.

1. 100% ethanol
- Samples: whole adult bees; pupae; large larvae; tissues.
- Use: 5 volumes by weight.
- Storage: 1 month at room temperature or lower.
- Processing: Remove ethanol and process samples as normal.
- **Pros:** Cheap; effective penetration.
- **Cons:** Evaporation; possible transport restrictions; heavy; incompatible with serological assays .

2. RNAlater® & generic equivalent
- Samples: tissues; eggs; small larvae.
- Use: 5 volumes by weight.
- Storage: 1 month at room temperature, or lower.
- Processing: Remove stabilizer and process samples as normal.
- **Pros:** Non-hazardous; effective penetration.
- **Cons:** Expensive (except generic version); heavy.

It is possible to use RNAlater® for darkened pupae and adult bees, if they are crushed into a paste or cut into 5mm sections (Chen *et al.,* 2007). This is laborious and risks losing virus particles and RNA to the stabilizing solution, but may be required in certain circumstances. In such cases, the crushed bees should be centrifuged at 1,000 rpm for 5 minutes at 4°C before removing the stabilizer and processing the crushed bee tissues.

1.3.5. Sample collection cards

Samples can also be dried on filter paper-based collection cards. In this case the molecules are stabilized primarily through desiccation, rather than low temperature, so thorough drying during sampling and low humidity during storage is essential for this method. The FTA™-cards produced by Whatman are furthermore impregnated with chemicals to prevent bacterial or enzymatic degradation of nucleic acids (Becker *et al.,* 2004; Rensen *et al.,* 2005). The method is ideal for liquid samples (blood, urine, sputum *etc.*) but has also been used for insect samples (Harvey, 2005) including honey bee larvae, pupae, extracted tissues and mites. Such filter-dried samples can be analysed for all manner of compounds (Jansson *et al.,* 2003; Chamoles *et al.,* 2004; Li *et al.,* 2005; Zurfluh *et al.,* 2005) including RNA (Karlson *et al.,* 2003; Prado *et al.,* 2005). The major advantages are the ease and reduced costs of collection, transport, labelling and long-term storage at room temperature, reducing the requirements for freezer space, boxes and tubes (Kiatpathomchai *et al.,* 2004; Harvey, 2005; Karlson *et al.,* 2003; Rensen *et al.,* 2005; Prado *et al.,* 2005). The major disadvantages are the uneven distribution of target across the filter paper and the gradual loss of target during prolonged storage (Chaisomchit *et al.,* 2005). Samples collected on collection cards should therefore also be processed as soon as possible, by cutting out the entire dried sample and soaking this in an appropriate buffer, as recommended by the FTA™-card protocol, for the recovery of nucleic acids.

FTA™ collection cards
- Samples: Tissues; faeces; eggs; larvae; pupae; mites.
- Use: Squash sample on card and air-dry.
- Storage: At room temperature in dry container. Not in freezer.
- Processing: Cut out sample and soak directly in extraction buffer for 15 minutes. Proceed as for fresh samples.
- **Pros:** Excellent preservation; light; easy storage and indexing; versatile; preservation of faeces.
- **Cons:** Expensive; variable processing; uneven distribution across card; not suitable for adult bees, not suitable for bulk samples.

1.4. Long-term sample storage

The critical factors for long-term sample preservation, as with degradation in the weeks after collecting, are minimizing the activity of nucleases. This can be achieved by a combination of:

1.4.1. Freezing

Freezing at -80ºC is the gold-standard for long-term storage of bee samples intended for RNA analysis. Freezing at -20ºC also provides good storage for preserving the quality of bee samples. However, significant to complete degradation of RNA can occur within days in dead bees kept at 4°C (Chen *et al.,* 2007; Dainat *et al.,* 2011). It is therefore strongly recommended to transfer frozen bees to the -80°C freezer immediately after samples are brought back from the field to the laboratory, if analysis is not initiated immediately.

1.4.2. Drying

Apart from drying soft bee stages and tissues on collection cards, bulk samples of whole bees can also be freeze-dried, or lyophilized. Lyophilization is a convenient way to store samples long-term at room temperature and preserves the chemical integrity of most compounds, although some functional activity may well be lost. Freeze-drying/ lyophilization requires a specialized instrument that draws a vacuum while the samples are kept below the point where solid and liquid phases can co-exist (below -50°C), so that the ice sublimates, *i.e.* changes directly to vapour without melting first. Any biological sample can be lyophilized and the instructions for this come with the particular lyophilizing apparatus. It should be noted here that reconstituted dried tissue is fundamentally different from frozen wet tissue, with different and more variable recovery efficiencies for the different biomolecules than for fresh tissues. Lyophilized samples are stored at room temperature in a sealed box with desiccating packages, to prevent re-hydration.

1.4.3. Chemical stabilizers

There are several chemical agents that inhibit RNAses and thus reduce RNA degradation during handling and storage (see Section 4.4.4.). They do not provide any additional benefit to frozen samples,

but can be useful for storing samples temporarily at room temperature. Their effectiveness varies and they do not prevent degradation absolutely (Chen *et al.,* 2007) but they are useful if minor degradation can be tolerated and the samples can be processed within a few months of collection.

2. Sample processing

2.1. Introduction

The initial processing of a sample is another key step in ensuring the uniformity and reliability of an assay. Nevertheless, generally little attention is paid to optimizing this part of the protocol for both maximum recovery of the target molecule(s) and for reducing variability. In general, the shorter and faster the protocol the better, since each additional step will contribute to the overall error and reduce the recovery efficiency, both of which compromise results. Here we will describe generalities of sample processing before independent chapters describing RNA and DNA extraction from samples.

2.2. Sample homogenisation

A highly variable step in sample processing is sample homogenization, not only between different homogenization options but also between different samples using the same protocol. The choice of homogenization method depends on the sample type and number of bees per sample. There are numerous options outlined below.

2.2.1. Bead-mill homogenizers

These are the best option for uniform and reproducible homogenization of small (individual) bee samples. The samples are mixed with glass, ceramic or steel 1-3 mm beads and extraction buffer in sturdy disposable plastic tubes and shaken at high velocity in a machine. They also provide consistent cell wall disruption of bacteria and other microbes for parasite/pathogen or microbiome surveys.

- **Pros:** Low-medium volume; rapid; uniform; no cross-contamination.
- **Cons:** Generally only suitable for small bee samples (1-10 bees).

1. Place single bee in a 2 ml screw-cap microcentrifuge tube.
2. Add four 2 mm glass beads.
3. Add 500 µl ice-cold buffer.

For medium-large volume beadmills, increase the number of bees, beads and buffer in proportion to the maximum allowable volume of the disposable container.

4. Make sure that the bead mill is balanced, if this is a requirement.
5. Shake for 5-10 minutes at the highest setting.

2.2.2. Blender

An excellent, cheap alternative to the beadmills, especially for large volumes is homogenisation with a blender.

- **Pros:** Large volume; rapid; uniform.
- **Cons:** Cross-contamination risk due to re-use of blender; incompatible with organic solvents; corrosion of blender due to salts.

1. Add between 30-200 frozen bees to blender.
2. Add 500 µl ice-cold buffer per bee.
3. Homogenise by gradually raising the blender settings, for about 5 minutes total homogenization.

2.2.3. Paint shaker

A paint shaker (e.g. Automix shaker; Merris Engineering ltd) is a surprisingly efficient method of grinding bulk samples which has been used for various matrices including soil , grains, rice, wheat, honey, and bees (Woodhall *et al.*, 2012; Budge, unpublished data). The method is completely scalable ranging using polypropylene wide-mouth environmental bottles (Nalgene) ranging in size from 60 ml to 2000 ml.

- **Pros:** Large volume; easy; cheap; no cross-contamination; high throughput.
- **Cons:** Large piece of equipment required.

1. Place 30-1000 frozen bees in an appropriately sized bottle (Nalgene) containing 5 x 25.4 mm stainless steel ball bearings.
2. Dry grind on the paint shaker for 8 minutes until the sample is sufficiently disrupted.
3. Add the required volume of extraction buffer depending on the protocol.
4. The addition of 1% Antifoam B (Sigma) to GITC, GHCl or CTAB extraction buffers can aide buffer recovery and reduce cross contamination.
5. Wet grind for a further 4 minutes.
6. Spin at 6000 g for 5 mins.
7. Recover supernatant.

2.2.4. Mortar and pestle

Traditional manual method for pulverizing samples.

- **Pros:** Medium volumes; cheap; low maintenance.
- **Cons:** Cross-contamination risk; time consuming; lack of uniformity.

1. Place 1-30 bees in a pre-frozen mortar of appropriate size.
2. Add liquid nitrogen to cool samples to well below freezing.

3. Grind the bees to a powder using an appropriate sized, pre-frozen pestle.
4. Transfer the powder to a plastic tube or bottle.
5. Add 500 µl extraction buffer per bee.
6. Shake tube until the powder has suspended fully in the buffer.

2.2.5. Mesh bags

Mesh bags are sturdy plastic bags with a small pore fine mesh inside. The sample is placed on one side of the mesh, ground from the outside and the homogenate is collected from the other side of the mesh, filtering out large particles.

- **Pros:** Medium-large volume; easy; cheap; no cross-contamination.
- **Cons:** Lack of uniformity; split bags.

1. Place up to 30 frozen bees in a disposable mesh-bag (e.g., www.bioreba.com; #430100).
2. Add 500 µl buffer per bee.
3. Flash-freeze the entire bag in liquid nitrogen.
4. Remove from liquid nitrogen.
5. Wait 30 seconds.
6. Pulverize contents by grinding the bag with a large pestle for 2 minutes on a hard surface, taking care not to damage the bag.
7. Massage the bag until completely thawed.
8. Remove one (or more) 100 µl aliquots of homogenate.
9. As an alternate method, described in section 4.3.2. samples can be crushed in disposable mesh bags using a heavy rolling pin.

2.2.6. Micropestle

You will need individual disposable micro pestles that fit microcentrifuge tubes. These can be bought or made by heating a 1000 µl blue tip in a flame and moulding it into a disposable pestle in a microcentrifuge tube while it cools.

- **Pros:** Single bees; cheap; low maintenance.
- **Cons:** Time consuming; lack of uniformity.

1. Grind a frozen bee tissue or larval sample with the micropestle in a microcentrifuge tube.
2. Discard pestle.
3. Add 500 µl buffer per bee.
4. Mix with a vortex.

2.2.7. Robotic extraction

Companies produce robotic extraction stations to facilitate high-throughput analysis of samples. Comparisons between several such systems, or between automated and manual extraction, generally find little difference in terms of assay sensitivity and reproducibility (Rimmer *et al.,* 2012; Bruun-Rasmussen *et al.,* 2009; Agüero *et al.,* 2007; Petrich *et al.,* 2006; Knepp *et al.,* 2003, but see Schuurman *et al.,* 2005). Such systems are generally only suitable for easily disrupted, soft tissues or samples.

- **Pros:** Single bees; rapid; high throughput; uniform; low cross contamination risk.
- **Cons:** Expensive; inflexible protocols; soft tissues only.

Follow manufacturers' protocol for sample processing.

3. DNA extraction and analysis

3.1. Introduction

Isolating and analysing an organism's DNA is key for developing insights into species or strain identification, for uncovering variants useful in breeding or a more thorough understanding of biology, and for discovering the microbes carried by individuals. DNA extraction methods must be robust for small amounts of starting material even if that material has become degraded. They must deliver extracted DNA of sufficient quality, purity, and quantity for downstream efforts ranging from target identification (e.g., via the Polymerase Chain Reaction, PCR, below in section 6.3.1.), sequence analysis, and cloning, among others. Below are tested protocols for common DNA analyses of diverse bee samples, starting with the isolation and purification of DNA. Isolating DNA from tissues can be accomplished using a variety of commercial kits, or via procedures built on standard disrupting and separating agents as below. Here we describe protocols made from primary ingredients, since this is illustrative of the critical components in these and pre-made extraction protocols.

3.2. Genomic DNA extraction from adult bees

3.2.1. DNA extraction using CTAB

This protocol is for the extraction of DNA from bee abdomens and/or the thorax, using a lysis buffer containing CTAB, a compound that is able to separate polysaccharides from other cell materials. The choice of tissues avoids eye contaminants such as pigments, which can inhibit PCR and other downstream applications. The method can be scaled down for the extraction of *Varroa destructor* mites (see the *BEEBOOK* paper on varroa (Dietemann *et al.*, 2013) for details on sampling) or bee embryos and up for larger larvae and pupae (see section 1.2. for their collection). Volumes should be adjusted accordingly based on sample volume (i.e. initial grinding in 5X sample volume of buffer, ca.25-> 200 µl). The subsequent two extraction protocols are simpler, but the CTAB procedure is excellent for problematic samples and is flexible in terms of tissue disruption, separation, and rescue of nucleic acids.

1. Extract only the abdomen and/or thorax if possible. If a whole animal is extracted, use a Qiagen or similar column following manufacturer's protocol for final purification of extracted DNA in order to reduce pigments that can inhibit genetic assays.
2. Put tissue from a single bee in a 1.5 ml microcentrifuge tube.
3. Add 500 µl of CTAB + 2 µl 2-mercaptoethanol (0.2%).

CTAB buffer:

- 100 mM Tris-HCl, pH 8.0
- 1.4 M NaCl
- 20 mM EDTA
- 2% w/v hexadecyl-trimethyl-ammonium bromide (CTAB)

This buffer both stabilizes nucleic acids and aids in the separation of organic molecules. See MSDS as CTAB is a potential acute hazard.

4. Homogenize with pestle.
5. Add 50 µg proteinase K and 25 µl of RNase cocktail.

While this step is optional, proteinase K improves yields by disrupting cell and organelle boundaries and is critical for extraction of DNA from many microbes.

6. Vortex briefly to mix.
7. Incubate at 55-65°C from several hours to overnight. Invert occasionally during incubation (e.g. once every 30 minutes for the first two hours).
8. Centrifuge for 1 min at maximum speed (~14,000 rpm).

Unwanted tissue debris will form a pellet at the bottom of the microcentrifuge tube.

9. Transfer liquid to fresh tube, leaving tissue debris pellet behind.
10. Add equal volume phenol:chloroform:isoamyl alcohol (25:24:1).
11. Invert several times (10-20 times) to mix then put on ice for 2 min.
12. Spin at full speed (~14,000 rpms) for 15 min at 4°C.
13. Transfer upper phase to fresh tube.
14. Add 500µl cold isopropanol + 50 µl 3 M NaOAc.
15. Vortex to mix, then incubate at 4°C > 30 min.

Samples can be stored at ambient temperature at this point for several days if needed for transport or timing, otherwise 4°C is best.

16. Spin at full speed (~14,000 rpms) for 30 min at 4°C.
17. Carefully decant liquid from DNA pellet.
18. Add 1 ml 4°C 75% EtOH. Tap vortex briefly to loosen pellet.
19. Spin at full speed for 3 min at 4°C.
20. Decant liquid from pellet.
21. Air dry pellet about 10 minutes to evaporate all residual traces of alcohol.

Do not over dry pellet, as it will be hard to resuspend.

22. Resuspend in 50-100 µl nuclease-free water (overnight at 4°C).
23. Check DNA quantity and integrity on an agarose gel.
24. First, prepare TBE gel buffer (an aqueous solution with a final working concentration of 45 mM Tris-borate and 1 mM EDTA). This is often prepared first as a '5x' concentration comprised of 4 g Tris base (FW = 121.14) and 27.5 g boric acid (FW = 61.83) dissolved into approximately 900 ml deionized water. Add 20 ml of 0.5 M EDTA (pH 8.0) to this solution and adjust the solution to a final volume of 1l.

Confusingly, the 'working solution' of this buffer for most uses is as 0.5x = a 1/10 dilution of the stock buffer.

25. For a 1.5% agarose gel on a large-format gel rig, add 3 g of sterile agarose to 200 ml TBE buffer in a 500 ml or larger Erlenmayer flask, microwave at high heat for ca. 45 s (without boiling). For smaller gel rigs the volume of the gel can be from 50 to 100 ml. Take flask out and swirl, then heat in the microwave again until at full boil for 45 seconds, monitoring to avoid spillover. The agarose must fully dissolve so the liquid is perfectly clear
26. Let the solution cool while swirling every minute until the flask can be held for several seconds without unbearable heat
27. While hot, pipette in 10 µl ethidium bromide solution (EtBr, 0.5 mg/ml, used with caution as EtBr is a carcinogen and mutagen) and swirl until mixed
28. Pour into a horizontal gel rig and insert plastic combs holding ca. 10 µl of sample each
29. Let the gel solidify fully; gels can be wrapped in plastic wrap for longterm storage (overnight in place or for days at 4°C).
30. Mix 5 µl of the extraction solution with 2 µl of a 40% weight/ volume sucrose load buffer (made as 4 g sucrose and 25 mg bromophenol blue in 10 ml distilled water)
31. Submerge gel in a rig containing 0.5 x TBE, remove gel comb and load the 7 µl of sample/dye mix in separate wells using DNA molecular weight standards (e.g., 500 bp molecular ruler, www.biorad.com)
32. Draw the DNA across the gel toward the anode/positive charge at ca. 100 V depending on the gel rig size and specifications.
33. Monitor via the blue bromophenol blue stain movement (which tracks a DNA size fragment of ca. 300 bp in a 1.5% gel), stopping the gel and visualizing the DNA using ultraviolet light when it has progressed enough.
34. DNA can also be quantified via a spectrophotometer such as the Nanodrop (www.nanodrop.com), following manufacturer's protocol: Briefly, after calibration 1 µl of nucleic acid solution is placed onto a cleaned pedestal, the lid is closed and a reading is taken prior to cleaning by wiping the pedestal in preparation for the next sample. The machine will estimate concentration using the equation dsDNA: A^{260} 1.0 = 50 ng/µl.
35. Store at -20°C or below.

3.2.2 DNA extraction using Qiagen Blood and Tissue DNA kits

This is a reliable extraction method using a commercial kit sold by Qiagen (www.quiagen.com), it is suitable for honey bee guts, small larvae or tissues from larger larvae or adults (avoid using the compound eyes).

1. Place 50 mg honey bee material in a centrifuge tube and mince thoroughly on ice with a mini pestle
2. Add 180 µl Buffer ATL and 20 µl Proteinase K at the provided concentration
3. Vortex 30 seconds and incubate at 56°C for 1 hour, vortexing for 30 seconds after 30 min
4. Premix equal volumes of Buffer AL and ethanol (96-100%), mixing enough to provide 400 µl per sample plus 10% extra
5. Vortex samples 30 seconds and add 400 µl AL/EtOH mix each, vortex again 30 seconds
6. Pipette all into DNeasy Mini spin column nested in a 2 ml collection tube.
7. Centrifuge at $\geq$ 8000 rpm in a microcentrifuge (6k g). Discard flow-through and collection tube
8. Place spin column in new 2 ml collection tube, add 500 µl Buffer AW1, centrifuge 1 min at $\geq$ 8000 rpm. Discard flow-through and collection tube
9. Place spin column in new 2 ml collection tube, add 500 µl Buffer AW2, centrifuge 3 min at $\geq$ 14000 rpm. Discard flow-through and collection tube
10. Remove spin column, checking to be sure ethanol is gone and place into a clean 1.7 ml centrifuge tube
11. Add 200 µl Buffer AE to the centre of the membrane, incubate at room temperature and then centrifuge for 1 min at $\geq$ 8000 rpm. Eluted DNA will be in tube. Check quantity by Nanodrop or agarose gel as in section 3.2.1 above.

3.2.3. DNA extraction using Chelex

The Chelex method (Walsh *et al.*, 1991) provides a very rapid way to protect DNA from degradative enzymes and from some of the potential contaminants that might inhibit experiments. In principle, the Chelex resin will trap salts needed by degradative enzymes, leaving DNA in solution. In practice, Chelex extractions can be prone to degradation, and should be kept in the freezer when not in use, or these extractions should be used within 24 hours of extraction. If the extracted tissues contain pigments and other inhibitors for downstream experiments, it is often successful to dilute the Chelex extraction 1:9 with distilled water before use. Finally, when drawing from these extractions it is important to pipette from the top of the aqueous layer, avoiding the resin itself. Below is a recipe that works well for legs from adult bees or beetles, for whole varroa mites, or for other tissues of about that size.

1. Add two posterior legs into Eppendorf tubes.
2. Allow them to dry until the EtOH evaporates.
3. Transfer to each tube:

- 100 µl of Chelex® (5% solution in water),
- 5 µl of proteinase K (10 mg/ml).

4. Incubate the samples in a thermocycler with the following program:

- 1 h at 55°C,
- 15 min at 99°C,
- 1 min at 37°C,
- 15 min at 99°C,
- Pause at 15°C.

3.3. DNA detection using southern blots with DIG labelling

Southern blotting was invented by Edward M Southern as a means for detecting specific nucleotide sequences in a complex mixture and determining the size of the restriction fragments, which are complementary to a probe. Southern blotting combines transfer of restriction-enzyme-digested and then electrophoresis-separated DNA fragments from a gel to a membrane and subsequent detection by probe hybridization. A variety of non-radioactive methods have been developed to label probes for detection of specific nucleic acids. The Roche Applied Science DIG system is a simple adaptation of enzymatic labelling and offers a non-radioactive approach for the safe and efficient labelling of probes for hybridization reactions.

3.3.1. Restriction enzyme digestion and agarose gel electrophoresis

This step is carried out in order to array chromosomal sections across a one-dimensional space so that unique sections can be probed for matches to a query sequence. In principle, the targeted gene will be embedded in a single chromosomal segment flanked by specific sequences that match the restriction enzyme used.

1. Digest 5-10 µg of genomic DNA in a volume of 30 µl with an appropriate restriction enzyme by setting up reaction as follows:

- 3 µl 10X buffer,
- 0.3 µl of BSA if needed (this will be on the restriction enzyme label),
- 3 µl enzyme (10U/µl),
- 5-10 µg genomic DNA,

Add sterile water to reach a total volume of 30 µl.

Generally, enzymes that cut frequently in the target genome are used here (e.g., 'four-cutters' that cut at a specific four-base-pair sequence, an event expected to occur ca. once every several hundred base-pairs).

2. Allow the digestive reaction to go for overnight at 37°C (or temperature appropriate to your specific enzyme).
3. Run the full 30 µl of reaction mixture with 3 µl 6X loading dye on 1% agarose gel (see section 3.2.1) containing ethidium bromide (1 µg/ml) for 2 hours at 100 volts. Include one lane of a DIG-labelled DNA Molecular Weight Marker at the appropriate level.
4. Take a picture of the digestion.
5. Depurinate the agarose gel for exactly 10 min in 0.25 M HCl if DNA fragment > 4 kb.
6. Denature the gel in freshly made denaturing solution (0.5M NaOH, 1.5 M NaCl) for 2 x 15 min at RT, slowly shaking on rotating shaker.

Denaturation of the DNA into single strands allows hybridization with a probe possible.

7. Rinse the gel with sterile water.
8. Neutralize the gel in neutralizing solution (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl) for 2 x 15 min, slowly shaking on rotating shaker.
9. Equilibrate gel in 20X SSC for 10 min.

3.3.2. Assembly of the transfer setup and transfer of DNA from gel to membrane

DNA is here pulled from the gel into a nylon membrane by capillary action pulled by the positive charge of the membrane. Once in contact with the membrane, DNA is attached using high-voltage cross-linking.

1. Set up capillary transfer using 20X SSC as a transfer agent: Inside a baking glass dish filled with 20X SSC, place a glass plate that is elevated by four rubber stoppers that is slightly larger than the gel.
2. Cover the glass plate with a piece of wick-blotting paper that has to be long enough so that it is in contact with the 20X SSC transfer solution.

The buffer flows up the wick-blotting paper by capillary action, then through the gel to the membrane.

3. Smooth out the air bubbles between the glass and the blotting paper by gently rolling with a glass pipette.
4. Place the gel facing down on the wet blotting paper.
5. Cut a small triangular piece from the top left-hand corner to simplify orientation.
6. Smooth out the air bubbles.
7. Cut one piece of positively charged nylon membrane to match size of the gel.
8. Soak the membrane in water for 2-3 min to wet and then float in 20X SSC.
9. Gently place the membrane on the top of the gel.
10. Mark well positions on the membrane.
11. Smooth out the air bubbles.
12. Cut 4-5 sheets of Whatman 3MM paper to the same size as the gel and place on top of the membrane.
13. Place a stack of paper towels on top of the Whatman 3MM papers.
14. Add a 200-400 g weight to hold everything in place.
15. Allow the DNA to transfer for 10-16 hours.
16. After transfer, rinse the membrane briefly in 6X SSC.
17. Immobilize DNA to the membrane by UV cross-linking (120,000 microjoules per cm^2). Membrane is now ready for labelling (section 3.3.3.).

3.3.3. Synthesis of DIG-labelled DNA probe

DIG-labelled probes offer a method to identify where probes have attached on the membrane (e.g.., the location of their targeted DNA match). These DIG probes are an alternative to highly regulated and more dangerous radio isotopic probes.

1. Mix the DIG-labelled PCR reaction components from the Roche Applied Science PCR DIG Labelling Mix with the probe template as follows:

- 5 µl PCR Buffer (10X),
- 5 µl PCR DIG Labelling Mix,
- 0.5 µl Upstream Primer (25 µM),
- 0.5 µl Downstream Primer (25 µM),
- 0.5-1 µl Template (plasmid DNA, 10-100 pg, or genomic DNA, 1-50 ng),
- 0.75 µl Enzyme Mix,
- Add sterile water until total reaction volume is equal to 50 µl.

2. Set the annealing temperature of PCR reaction to reflect the predicted annealing temperature of the primers, also reported at the time of purchase.
3. The kit contains a post-hoc check for probe labelling efficiency that is recommended.

3.3.4. Hybridizing the DIG-labelled DNA Probe to DNA on the Blot

This procedure relies on the Roche Applied Science DIG Easy® Hyb, DIG Wash and Block Buffer Set, (with the fluorescent reporter CSPD®).

1. Pre-warm an appropriate volume of DIG Easy® Hyb solution® to the hybridization temperature.
2. Pre-hybridize membrane in a small volume of pre-warmed DIG Easy® Hyb solution (20 ml if in a 200 ml hybridization tube).
3. While the membrane is pre-hybridizing, denature 10 µl of DIG -labelled DNA by boiling for 5 min.
4. Rapidly cool on ice.
5. Add appropriate amount of denatured probe to give you (25 ng/ml) into DIG Easy® Hyb solution.

6. Incubate with agitation in a hybrid oven at 55-58°C for overnight.
7. Wash membrane in 25-50 ml Washing Solution-1 (2X SSC, 0.1% SDS) 2X for 5 min at room temperature under constant agitation.
8. Wash membrane in 25-50 ml Washing Solution-2 (0.1% SSC, 0.1% SDS) 2X for 5 min at 68°C under constant agitation.
9. Wash membranes briefly (1-5 min) in 25 ml of 1X Washing Buffer provided in DIG Wash kit.
10. Incubate membranes for 30 min in 1X Blocking Solution diluted in maleic acid buffer (supplied in the kit).
11. Incubate membrane in Anti-body solution for 30 min.

To make anti-body solution, add 1 µl anti-body to 20 ml 1X blocking solution.

12. Wash membrane in 1X Washing buffer 2X for 15 min.

Make sure membrane is immersed in the Washing buffer.

13. Prepare 20 ml 1X Detection Buffer.
14. Equilibrate membrane in 20 ml 1X Detection Buffer for 2-5 min.
15. Transfer the membrane with DNA side facing up to a Plastic wrap that is at least twice the size of the membrane.
16. Apply 1 ml of CSPD®, ready-to-use (about 20-25 drops) to the membrane.
17. Quickly cover the membrane with the plastic wrap.
18. Incubate for 5 min at RT.
19. Drain off excess buffer by gently brushing across the top of the membrane covered by plastic wrap with a paper towel.
20. Tape the membrane into a film cassette.
21. Close the cassette and incubate at 37°C for 10 min to enhance the luminescent reaction.
22. Remove the film for development using a standard x-ray film developer.

4. RNA methods

4.1. Introduction

Analyses based on RNA have two major advantages over DNA analyses. First, they are by definition restricted to a step in the expression of proteins from an organism's genome. This means that RNA pools are generally far less complex than are pools of DNA representative of the organism's entire genome, and that a quantitative estimate of different RNA's can provide a useful surrogate for the proteins produced at that time point for a specific organism or tissue within an organism. Second, since nearly all of the recognized viral threats to honey bee exist without a DNA stage, these threats are only visible via RNA analyses. These arguments make RNA the resource of choice for many honey bee analyses; despite greater concerns over storage and preservation of tissues.

A common strategy is to extract total nucleic acids directly in strongly denaturing buffers, so as to inactivate RNAses immediately during homogenisation. RNAses have numerous disulphide bridges. This makes them very stable in a very wide range of conditions, such that strong denaturants are required to permanently inactivate them. Heat, detergents (sodium dodecyl sulphate), organic solvents (phenol), proteinases, chaotropic salts (guanidine isothiocyanate), reducing agents (β-mercaptoethanol; dithiothreitol) and nucleic acid protecting compounds (CTAB; cetyl trimethylammonium bromide) are some of the more common methods used to inactivate RNAses. The nucleic acids can be purified from other compounds with affinity columns, magnetic bead-linked nucleic acid binding agents or by precipitation with alcohol or lithium chloride. The most common, quickest and most reliable combination is a chaotropic salt/β-mercaptoethanol extraction buffer, followed by purification on disposable affinity columns (Verheyden *et al.,* 2003). The main disadvantage of RNA precipitation (with 2 volumes ethanol, 1 volume isopropanol or with 6M LiCl) is that many undesirable compounds often co-precipitate with the nucleic acid, requiring further precipitations or washes to clean the sample. There are many excellent commercial RNA extraction kits available, based on one or more of these principles. However, their performance in comparative tests varies greatly, depending on the organism, tissue type and nucleic acid extracted (Konomi *et al.,* 2002; Knepp *et al.,* 2003; Wilson *et al.,* 2004; Schuurman *et al.,* 2005; Labayru *et al.,* 2005). Below are two protocols, representing the most common approaches to RNA extraction.

4.2. Affinity column purification

The processing consists of making a primary homogenate from 1-30 bees, purifying RNA from one (or more) aliquots of the homogenate using affinity columns, and measuring the RNA concentration. β-mercaptoethanol is toxic so processing should be done in a fume hood. Prepare all the buffers and tubes before starting.

The protocol below is based on the columns marketed by Qiagen or generic equivalents. The maximum recommended amount of tissue per column is 20 mg. More than 20 mg tissue causes the column to bind too much protein, reducing the yield and quality of the nucleic acid. Bees, pupae and large larvae weigh between 100-180 mg each, and so need to be homogenised first in a primary extract, from which a volume equivalent to 20 mg tissue is then processed on the affinity columns. A suitable denaturing buffer for this primary extract is a Guanidine Iso-Thio Cyanate (GITC) buffer, which has similar properties to the Qiagen RLT buffer:

1. Mix the GITC buffer:

- 5.25 M guanidinium thiocyanate (guanidine isothiocyanate),
- 50 mM TRIS.Cl(pH 6.4),
- 20 mM EDTA,

- 1.3% Triton X-100,
- 1% β-mercaptoethanol.

2. Place exact, pre-determined number of frozen bees in the homogenizer of choice.
3. Per bee, add the following amount of GITC buffer:

Bee	Weight	Buffer	Total volume
Worker bee	120 mg	500 µl	600 µl
Drone	180 mg	700 µl	900 µl
Worker pupa	160 mg	650 µl	800 µl
Drone pupa	240 mg	1000 µl	1200 µl

With these extract volumes, 100 µl extract is approximately 20 mg bee tissue

4. Mix:

- 100 µl bee extract,
- 350 µl RLT buffer + 1% β-mercaptoethanol.

5. Proceed according to the Plant RNA extraction protocol (see Qiagen instructions booklet).

Inclusion of the Qia-shredder column step is not required, but significantly increases yield and purity of nucleic acid.

6. Elute in 100 µl nuclease-free water.
7. Determine nucleic acid concentration and purity (see section 8.4.; "Nucleic Acid Quality Assessment").
8. Store as two separate 50 µl aliquots at -80°C, one for working with and one for storage.
9. Include a 'blank' extraction (*i.e.* an extraction of purified water) after every 24 bee samples, to make sure none of the extraction reagents have become contaminated.

4.3. Acid phenol RNA extraction from adult bees

The below recipes use an acid-phenol phase separation for isolating RNA from DNA and other tissue components. The TRIzol® (Invitrogen™) protocol is the most commonly used, and widely available, method of acid-phenol extraction of RNAs. However, using a generic lysis and acid-phenol buffer (e.g. section 4.3.2) provides a cost effective alternative than TRIzol®, and allows a great reduction in the use of the caustic chemical phenol for pooled samples. We use honey bee abdomens because they provide representation of the microbes and immune components of the honey bee, while avoiding pigments in the eye which can inhibit downstream enzymatic reactions. The procedure is also appropriate for larvae, whole adult bees and pupal RNA extractions, if volumes are scaled upward, i.e. doubled, to reflect the volume of the sample, for the latter two.

4.3.1 TRIzol® extraction

Advanced preparation: You will need RNase-free bench, pipettes, barrier tips, pestles and 1.5 ml microcentrifuge tubes. Bench tops and other glass and plastic surfaces can be treated to remove RNAse contamination by application of RNAse Zap (Ambion), following manufacturer's protocol. Disposable tips, pestles, and microcentrifuge tubes should be purchased RNase–free. You will need cold 75-80% ethanol and 100% isopropanol, both nuclease-free; and a pre-chilled centrifuge (at 4°C for 30 min) for Step 9. Have ready at room temperature, the TRIzol® and other reagents needed. It is recommended to use a vented fume hood for safety when working with TRIzol® and chloroform. It is also very important to work quickly with bee tissue, as it is possible that RNA will degrade if bees thaw for ten or more minutes (Dainat *et al.*, 2011).

In a very sterile (RNAase-free) environment:

1. Add 500 µl of TRIzol® to frozen bee abdomens in 1.5 ml tubes.
2. Mash the tissue until completely homogenized with a pestle and shaking.

All soft tissues should be disrupted completely. Remove pestle and scrape it off along the rim of the microcentrifuge tube. Sample should be viscous.

3. Centrifuge at 5,000 rpm for 1 min to pellet debris.
4. Transfer the TRIzol® suspension to a fresh tube, leaving out the chitinous debris pellet.
5. Add another 500 µl TRIzol® and invert several times to mix.
6. Add 200 µl chloroform.
7. Shake vigorously for 15 sec.

Do not vortex! This may increase DNA contamination in your sample.

8. Incubate at RT for 2-3 min.
9. Spin at 4°C for 15 min at ~14,000 rpm.

NOTE: Be especially diligent about avoiding RNases from this point on!

10. Label a fresh set of RNase-free microcentrifuge tubes.
11. Carefully remove tubes from centrifuge.
12. Use a 1 ml pipette tip with pipettor set at 550 µl to draw off the upper phase and transfer it to a fresh tube.

Carefully avoid the interface (one product that ensures a clean break between phases is the Phaselock gel (5 Prime Inc.) and could be used here).

13. Add 500 µl 100% Isopropanol.
14. Invert 3-5 times to mix gently.
15. Incubate at RT for 10 min.
16. Centrifuge at 4°C for 10 min at full speed (~12,500rpm), placing all tubes in the same rotation (e.g., hinge facing away from arc) so pellet location will be consistent.

17. Carefully siphon off liquid using a 1 ml pipette tip.

Observe the pellet (white) so you do not inadvertently aspirate it into the tip! Be cautious as it may dislodge and float.

18. Add 1 ml of cold 75-80% nuclease-free EtOH.
19. Invert several times to mix.
20. Spin at 4°C for 5 min at full speed.
21. Carefully decant liquid using a 1 ml pipette tip, avoiding the pellet and tilting the tube so no alcohol remains at the bottom of the tube covering the pellet.
22. Let tubes air dry in a clean area just until the EtOH has evaporated (~20-30 min).
23. Resuspend RNA pellet in 100 µl of RNase-free water.
24. Incubate at 55°-60°C for 10 min in water bath, ideally with shaking or flicking tubes for 10 seconds once during this time.
25. Quantify and validate RNA integrity using spectrophotometer (Nanodrop, section 3.2.1), following manufacturer's protocols, or run a small amount on 1-2% agarose gel (see section 3.2.1) to verify RNA quality. This can be accomplished by looking for degradation products migrating as a diffuse smear below the sharp 28S and 18S ribosomal RNA bands, which migrate at an analogous rate to ca. 1.75 and 2 kb double-stranded DNA markers. Alternatively, an Agilent 2100 RNA chip will provide both an accurate quantification and a measure of RNA integrity.
26. Freeze for storage at -80°C for long term storage, -20°C if you plan to use the RNA within 24 hrs.
27. Yields should be at least 100 µg (1 µg/µl) total RNA.

4.3.2. Bulk extraction of RNA from 50-100 whole bees using the acid-phenol method

For colony-level surveys of bee microbes, including pathogens, it is often important to analyse a bulk sample of bees in order to ensure a more accurate view of colony loads (most parasites and pathogens are not found uniformly across all bees in the hive, see section 4. 'Obtaining adult workers for laboratory experiments' of the *BEEBOOK* paper on maintaining adult workers in vitro laboratory conditions (Williams *et al.*, 2013) and the *BEEBOOK* paper on statistics (Pirk *et al.*, 2013) for details on how to sample bees). Similarly, if a colony-level genetic or phenotypic (gene-expression) trait is desired it is often better to generate an estimate that is the average across many colony members rather than a few selected bees. Extractions from a sample of tens of bees can be costly since volumes of reagents must be scaled up. The below protocol greatly reduces the most costly (and hazardous) ingredient used in RNA extractions, acid-phenol, and otherwise generates equivalent yields and purity to the TRIzol® extraction described above.

1. Put whole frozen bees (stored at -80°C since death) into a disposable extraction bag (e.g. www.Bioreba.ch) and add 500 µl lysis/stabilization solution (section 4.3.3) per bee (i.e. for 50 bees add 25 ml solution).
2. Mash until homogenized using a rolling pin, leaving the bag partly open initially to allow air to escape.
3. Allow to settle ~10 min so bubbles go down.

You can mash 10 or so bags consecutively at a time. By the time #10 is finished, the bubbles in #1 have subsided. Keep pending bags on ice in bucket.

4. Transfer 620 µl of extraction liquid into a pre-labelled 1.5 ml micro tube.

Note: It is advisable to save subsamples of the lysed tissues as a reserve (Store at-80°C).

5. Add 380 µl acid phenol.
6. Vortex 30 sec to mix well.
7. Incubate 10 min in a 95°C hot block.

Place weight on top of tubes to prevent lids from popping open.

8. Wearing goggles and a lab coat carefully remove weight and then transfer the tubes from hot block to pre-chilled rack in ice.

It is best to keep hot block in hood to contain the phenol.

9. Incubate on ice for 20 min.
10. Bring to RT.
11. Add 200 µl chloroform.
12. Shake vigorously for 1 sec.
13. Incubate at RT 3 min.
14. Centrifuge at 14,000 rpm for 15 min at 4°C.
15. Transfer 500 µl upper phase to fresh tube.
16. Add equal volume of isopropanol (100%).
17. Invert ten times to mix.
18. Incubate at RT 15 min.
19. Centrifuge at 10,000 rpm for 10 min at 4°C.
20. Carefully decant liquid from pellet.
21. Wash w/ 1 ml of cold 75% EtOH.
22. Centrifuge at 10,000 rpms for 2 min at 4°C.
23. Carefully decant liquid from pellet.
24. Spin 1 min.
25. Remove excess alcohol with pipette tip.
26. Air dry completely.
27. Resuspend in 200 µl nuclease-free H_2O.
28. Solubilize for 10 min at 55°C.
29. Store at -80°C.
30. Yields should be higher than 200 µg (1 µg/µl) total RNA, and extractions should be stable for > 5 years. RNA degradation can be checked using an Agilent Bioanalyzer or by 2% agarose gels looking for the co-migrating large ribosomal RNA's as a sign of largely intact RNA. If extractions are to be shipped or kept at temperatures above -50°C for more than 48 hours, RNA should first be precipitated in an equal volume of isopropyl alcohol, shipped in that state, then suspended starting at step 22 above.

4.3.3. RNA lysis/stabilization buffer

1. Fill a 1l beaker with 300 ml of nuclease-free water and insert a large magnetic stir bar.
2. Following safety procedures (http://www.sciencelab.com/msds.php?msdsId=9927539) add:
   94.53 g guanidine thiocyanate ($CH_5N_3 \cdot CHNS$; MW = 118.16) (Sigma #50981), 30.45 g ammonium thiocyanate (CH_4N_2S; MW = 76.12) (Sigma #43135), 33.4 ml of 3M sodium acetate (NaOAc), pH 5.5 ml ultrapure molecular biology-grade (USB # 75897 or Sigma #71196).
3. Stir until completely dissolved.
4. Pour into 1l graduated cylinder and bring up to 550 ml with nuclease-free water.
5. Pour from graduated cylinder into autoclave-safe desired 1l bottle.
6. Add: 50 ml glycerol ($C_3H_8O_3$; MW=92.09 g/mol) (Sigma # G6279) and 20 ml Triton-X 100 (Sigma #T8787).
7. Autoclave on liquid cycle for 15 min with slow exhaust.
8. Remove from autoclave immediately, cool and store at 4°C.

This makes a total volume of 620 ml.

4.4. RNA quality assessment

The next step is to determine the condition of the RNA sample, prior to any assay. The three critical parameters are quantity, quality and integrity (i.e. absence of degradation). Quantity and quality are usually assessed by spectrophotometry (Green and Sambrook, 2012), by comparing the peak absorbance at 260 nm (nucleic acids), 280 nm (proteins) and 230 nm (phenolic metabolites). A number of companies now market spectrophotometers and fluorometers that provide a complete UV absorbance profile from 1 µl of sample, from which the concentration of the nucleic acid can be determined, as well as its purity with respect to protein and metabolite contaminants. However, nucleic acid integrity can only be determined by running an electrophoretic trace profile, and assessing the degree of degradation by comparison of different nucleic acid size classes. The most comprehensive RNA quality analysis is through a chip-based microelectrophoresis system that provides a complete electrophoretic trace of the RNA sample which is used to quantify the integrity of the RNA, as well as the amount and purity (Bustin, 2000). Agilent, Qiagen, Invitrogen and BioRad market such systems. However, for fresh samples or those preserved with stabilizers or in a frozen transport chain, with little expected degradation, a simple UV absorbance spectrum is usually sufficient.

- Read the absorbance of an RNA sample at 230 nm, 260 nm and 280 nm.
- A^{260} of 1.0 = 40 ng/µl ssRNA
  = 37 ng/µl ssDNA
  = 50 ng/µl dsDNA
- A^{260}/A^{280} < 2.0 indicates contamination with proteins.
- A^{260}/A^{230} < 2.0 indicates contamination with phenolics.

4.5. cDNA synthesis from total RNA

Most downstream measurements of RNA traits rely on the complementary DNA (cDNA) generated by back-transcribing RNA using a commercially available reverse transcriptase such as 'Superscript' (Invitrogen). Reverse transcription is the most delicate step in RT-PCR. This step is very sensitive to inhibitors and contaminants in the sample (Ståhlberg *et al.,* 2004b) such that the efficiency can vary between 0.5% and 95%. This efficiency is furthermore also strongly affected by both the absolute and relative amounts of target RNA in a sample, especially at very low levels of target (Ståhlberg *et al.,* 2004a; 2004b), and by a variety of reaction conditions (Singh *et al.,* 2000).

To minimize this variability, the RNA concentrations should be measured accurately by spectrophotometry (Qubit; Invitrogen), and a constant amount added to the cDNA reactions. If the RNA concentration is very low (< 10 ng/µl), 100 ng neutral carrier tRNA can be added to the reaction prior to addition for cDNA synthesis stabilize reverse transcription and detection reliability. The final major parameter to optimise is the cDNA primer. Different target-specific cDNA primers (such as used in One-step RT-qPCR reactions), can have significantly different reverse transcription reaction efficiencies, which will affect the quantitative estimation of the targets in the sample (Bustin, 2000). A useful, practical approach is therefore to first prepare a fully representative cDNA 'copy' of the entire RNA population, using random 'hexamer' (6-nucleotide) primers. Such a complete cDNA population will have much less quantitative biases between different targets due to variable reverse transcriptase reaction efficiencies, allowing for more accurate quantitative comparison and normalisation between different targets. However, cDNA prepared with random primers can sometimes overestimate the original amount of target RNA (Zhang and Byrne, 1999). Another commonly used technique for sampling RNA pools is to use poly-dT primers targeting the polyadenylated stretch found at the 3' end of most messenger RNAs and also on most of the honey bee viruses.

4.5.1 Reverse Transcription of RNA

The following is a robust reverse-transcription protocol for generating cDNA that is fully representative of the original RNA population:

1. Mix:
- 0.5 µg sample RNA template,
- 1 ng exogenous reference RNA (*e.g.* Ambion RNA250),
- 1 µl 50 ng/µl random hexamers,
- 1 µl 10 mM dNTP,
- up to 12 µl RNAse free water.

2. Heat the mixture to 65°C for 5 min and chill quickly on ice.
3. Add:
- 4 µl 5X First-Strand Buffer,
- 2 µl 0.1 M DTT,
- 1 µl (200 units) of M-MLV RT.
4. Mix by pipetting gently up and down.
5. Centrifuge briefly to collect the contents at the bottom of the tube.
6. Incubate 10 min at 25°C.
7. Incubate 50 min at 37°C.
8. Inactivate the reaction by heating 15 min at 70°C.
9. Dilute the cDNA solution ten-fold with nuclease-free water before using in PCR assays.

4.6. Qualitative RT-PCR for honey bee and pathogen targets

Detection by PCR can be "qualitative", *i.e.* recording only the presence or absence of the target cDNA, by analysing the accumulated "end-point" PCR products after the PCR is completed. The sensitivity of the assay can be raised or lowered as desired by, respectively, increasing or decreasing the number of amplification cycles. Usually PCR does not exceed 40 cycles, which is theoretically sufficient to detect a single molecule of the target DNA in the original template, when analysing the end products by agarose gel electrophoresis. Consider the following rough calculation:

- Assuming perfect doubling with each amplification cycle.
- 2^0 molecules (*i.e.* 1 molecule) prior to PCR = 2^{40} molecules after 40 cycles of PCR.
- 2^{40} molecules of a 100 bp DNA fragment (mw ~ 61,700 g/mol)

= 1.1×10^{12} molecules x 1 mol/6.0221415×10^{23} molecules
= 1.8×10^{-12} mol x 61,700 g/mol
= 1.1×10^{-7} g = 110 ng DNA

Normally, 20 ng DNA is easily visible as a single band on an ethidium bromide-stained agarose gel. Even when allowing for imperfections in the amplification, 40 cycles are therefore theoretically more than sufficient to detect a single molecule in a reaction.

However, such extreme sensitivity is rarely required in practical or even most experimental settings. Furthermore, by aiming for absolute detection at the level of a single molecule of target DNA, the detection system becomes axiomatically susceptible to high rates of detection error: both false positives (accidental amplification of contaminating molecules) and false negatives (non-detection of a single molecule due to amplification insufficiencies).

By raising the detection threshold a few orders of magnitude, to around 1,000 molecules per reaction (~2^{10} molecules prior to PCR) it is possible to produce detectable amounts of target DNA (~2^{40} molecules) with 30 cycles of amplification (2^{10+30} molecules), again assuming perfect doubling each cycle. This avoids most of the risk of both types of detection errors, since chance contamination events of singular molecules (false positive results) are now below the detection threshold and there is sufficient initial target DNA in the reaction to avoid accidental non-detection (false negative results). A few more cycles beyond 30 can be added to compensate for the imperfections in the PCR efficiency. This means that 35 amplification cycles should be the upper limit for most practical applications. Beyond 35 cycles, the rapidly increasing risk of detection errors outweighs the marginal gains in sensitivity.

4.7. Quantitative RT-PCR for honey bee and pathogen targets

Detection of specific PCR products can also be made continuously as the PCR proceeds (*i.e.* in 'real time'). In this case the cycle number at which the accumulated PCR products reach a fluorescence detection threshold, read after each cycle by laser optics, can be very accurately related to the initial amount of target in the reaction, through the use of exponential algorithms and internal and external quantitation standards (Bustin *et al.,* 2009; 2010). This is the basis for real-time quantitative PCR (qPCR). The great advantage of real-time qPCR, apart from the accurate quantitation of the initial amount of target DNA in the reaction, is that the diagnostic threshold for qualitative detection can be set after the reactions have taken place, or at a number of different levels, from the same data set. This is useful if different diagnostic sensitivities are required for different experimental or reporting purposes, or for quality control management purposes.

There are numerous methods for qPCR in the literature, and this approach has been used for measuring gene activity in honey bees and all of their major parasites and microbial associates. The primary difference in those cases will come in the specific primers used for amplification and in some cases in changes to the chemistry or thermal conditions. One main decision point is between using SYBR green or another non-specific fluorescent marker that measures (amplified) DNA non-discriminately versus reporters that target specific amplified products directly such as TaqMan probes (Applied Biosystems; e.g. Chen *et al.*, 2004). There is considerable debate over the merits of each approach. Assays using Taqman® chemistry and other internal probe methodologies are inherently more specific than those using Sybr chemistry, due to the additional match required in the probe sequence. Therefore, Taqman® assays are more prone to Type II errors (false negative), where a negative result is returned despite the sample being positive (perhaps due to slight modification in the probe region within the sample). Sybr-based assays are more likely to return a Type I error (false positive), due to difficulties in distinguishing between low positive signal at the threshold of detection and non-specific binding. The errors for both methods can be minimized after careful preparatory work.

4.7.1. One-Step versus Two-Step RT-PCR

The buffer conditions for reverse transcription and PCR are largely compatible, which means that the two steps can be coupled in a single tube reaction, with the incubation conditions favouring first the reverse transcription, and then the PCR. Such 'One-Step' RT-PCR kits reduce the number of manipulations and associated errors, both qualitative and quantitative. The disadvantage is that they use up the sample RNA at a much higher rate than 'Two-Step' RT-PCR, where the cDNA is produced independently in a separate reaction. One-Step RT-PCR is also generally less sensitive than Two-Step RT-PCR, since the reaction conditions are not optimised exclusively for reverse transcription, and cannot account easily for variable reverse transcription efficiencies between different assays/primers (Bustin, 2000; Bustin *et al.,* 2009). The main disadvantage of Two-Step RT-PCR is that the additives included in the reverse transcription buffer to enhance primer binding and reaction efficiency, can also encourage the production of non-specific PCR products during PCR, which affects the quantitation accuracy. To minimize such effects, cDNA should be diluted ten-fold with water before being used for Two-Step RT-PCR.

Commercial One-Step or Two-Step RT-qPCR kits have proprietary reagent mixtures that are optimised for the corresponding recommended cycling profiles. Different kits therefore perform differently with particular primers and cycling profiles (Grabensteiner *et al.,* 2001), and the choice of RT-PCR kit is therefore also part of the optimization procedure. To take maximum advantage of such pre-optimized systems, the most practical approach is to design the assays and primers to fit these optimized recommendations, whenever this is possible.

4.7.2. One-Step RT-qPCR

The following is a robust, standard One-Step RT-qPCR protocol for amplifying and quantifying targets <400bp in length, using SYBR-green detection chemistry, and starting with an RNA template:

1. Mix:
- 3 µl 5 ng/ µl RNA,
- 0.4 µl 10 µM Forward primer (0.2 µM final concentration),
- 0.4 µl 10 µM Forward primer (0.2 µM final concentration),
- 0.4 µl* 10 mM dNTP* (0.2 mM final concentration*),
- x µl OneStep Buffer + SYBR-green (as per manufacturer),
- y µl nuclease-free water,
- r µl reverse transcriptase (as per manufacturer),
- z µl Taq polymerase (as per manufacturer),
- 20 µl total volume.

(* dNTPs are often included in the optimized buffer)

2. Incubate in real-time thermocycler:
- 95°C 5 min,
- 35 cycles of:
  95°C 10 sec,
  58°C 30 sec *read for qPCR.
3. For Melting Curve analysis of the products, incubate:
- 95°C 1 min,
- 55°C 1 min,
- +0.5°C increments for 5 sec, with reads from 55°C to 95°C.

In addition, DNA sequencing of the amplified products is recommended.

4.7.3. Two-Step RT-qPCR

The following is a robust, standard qPCR protocol for amplifying and quantifying targets <400bp in length, using SYBR-green detection chemistry, and starting with a cDNA template:

1. Mix:
- 3 µl cDNA (pre-diluted 1/10, in nuclease-free water),
- 0.4 µl 10 µM Forward primer (0.2 µM final concentration),
- 0.4 µl 10 µM Forward primer (0.2 µM final concentration),
- 0.4 µl* 10 mM dNTP* (0.2 mM final concentration*),
- x µl Buffer + SYBR-green (as per manufacturer),
- y µl nuclease-free water,
- z µlTaq polymerase (as per manufacturer),
- 20 µl total volume.

(* dNTPs are often included in the optimized buffer)

2. Incubate in real-time thermocycler:
- 95°C for 5 min,
- 35 cycles of:
  95°C for 10 sec,
  58°C for 30 sec* read (qPCR),
3. For Melting Curve analysis of the products, incubate:
- 95°C for 1 min,
- 55°C for 1 min,
- +0.5°C increments for 5 sec, with reads from 55°C to 95°C.

4.7.4. Two-step Quantitative PCR for high-throughput assays

The below variant of qPCR is for a 96-well plate format on the CFX96 real time system (BioRad) or related machines, and works for both bee transcripts and pathogen targets. The primary difference over the prior protocol is that this one is initiated with cDNA generated in a non-specific way, rather than from *de novo* reverse-transcription for each viral and/or host test and control (as shown in the previous section).

1. Mix 1x SsoFast EvaGreen® supermix (BioRad) with 3 mM of each forward and reverse primer for a given target (final volume 4 µl).
2. Add 1 µl (~8 ng) of cDNA template to specific wells.
3. Use the following cycling conditions:
- 97°C for 1 min,
- 45 (maximum 50) cycles of:
  95°C for 2 sec,
  60°C for 5 sec,

Melt curve from 65-95°C at +0.5°C/5 sec increments.

4. Verify amplicon melting points for every positive sample. Amplicons from positive controls and initial samples should be cloned into pGEM-T Easy vector (Promega) to verify sequence.
5. Run four distinct no-template controls on the plate to monitor for contamination and non-specific amplification.
6. Standard curves should be run using a recombinant plasmid dilution series of the primer targets from 10^1 to 10^8 copies, providing a linear equation to calculate the copy number in each sample using $10^{Cq-b/m}$, where Cq = quantification cycle, b = y-intercept, and m = slope.

4.7.5. Multiplex RT-(q)PCR

Often there is a need to amplify several target RNAs from a single sample. This can be done in several parallel 'uniplex' reactions, or in a single 'multiplex' reaction containing the primer pairs for all different targets (Williams *et al.,* 1999; Wetzl *et al.,* 2002; Syrmis *et al.,* 2004; Szemes *et al.,* 2002). Detection of the different amplicons is usually by size difference and electrophoresis for qualitative PCR, or by target -specific labelled probes in real-time quantitative PCR (Mackay *et al.,* 2003). A number of such qualitative multiplex PCR protocols have been designed for honey bee viruses as well (Chen *et al.,* 2004b; Topley *et al.,* 2005; Grabensteiner *et al.,* 2007; Weinstein-Texiera *et al.,* 2008; Meeus *et al.,* 2010). Real-time qPCR can also be multiplexed, by using a range of different fluorophores and excitation-reading laser channels. This is useful for minimizing between-reaction variability, if both target and internal reference standards can be amplified simultaneously, in the same reaction. Other uses are to distinguish between variants of the same gene or pathogen. Currently, up to four different targets can be detected and quantified simultaneously in qPCR.

However, there are many serious disadvantages of multiplexed PCR methods that may ultimately outweigh the advantages of consolidation and efficiency:

- Multiplex RT-PCR is considerably less sensitive than uniplex RT-PCR (the reagents will be exhausted by several targets instead of just one), as much as several orders of magnitude depending on the number of targets (Herrmann *et al.,* 2004).
- Optimization of multiplex (q)PCR assays is considerably more complicated than uniplex (q)PCR, due to the large number of primers and probes that need to be optimized simultaneously for absence of undesired interactions. An alternative to multiplex RT-(q)PCR that avoids many of the assay optimization problems due to the complex primer mixes is the Multiplex Ligation Probe Amplification method. .
- The PCR products need to be resolved on size or by fluorophore, before they can be quantified, nullifying many of the gains in efficiency and cost.
- Amplification (and thus quantification) of one target can be strongly affected by the prior amplification of more abundant targets in the reaction, either through competition for a limited pool of reagents, or through inhibition of the PCR reaction at later stages by the PCR products produced during earlier cycles, which sequester most of the polymerase (Santa Lucia, 2007).

For these reasons, it is often much more practical and simple to use uniplex RT-PCR, even for large volume and throughput projects.

4.8. Primer and probe design

There are numerous primer design software packages around to help design primers and, if appropriate, TaqMan® probes for the amplified regions (Yuryev, 2007). Such software generally recommends using very short amplicons (< 100 nucleotides), which shortens the cycling times, avoids incomplete amplicons and saves reagents, avoiding competition even at late cycles. However, longer amplicons (up to 500 base pairs) provide much greater flexibility in designing an internal (e.g., TaqMan®) probe for the target. The probe should as much as possible be devoid of secondary structures (stem-loops) and have a T^m slightly higher than that of the amplification primers, so that it anneals to the denatured target molecules before any primer-driven polymerisation takes place. 'G' bases should be avoided at the 5' end, where the fluorophore usually resides, since they quench fluorescence, even after cleavage (Bustin, 2000).

4.8.1. Primer length, melting temperature and composition

Both amplification primers should ideally be the same length (around 20 nucleotides) with similar melting temperature (T^m) between 55°C-60°C, giving enough room for experimental annealing temperature optimization and long enough to avoid non-specific amplifications. It is useful to design all assays and primers around the same annealing temperature, so that a single cycling program can be used for all assays, and that different assays can be run concurrently with the same program, on the same plate. 56°C is a good, standard, robust target for the *in silico* estimated T^m for primers. The primer sequences should be evenly balanced between A/T and G/C nucleotides and avoid long homopolymeric stretches (*i.e.* runs of more than 4 of the same nucleotide).

4.8.2. Annealing temperature

The annealing temperature for the assay should be optimized experimentally, with a temperature gradient, which can be generated by most modern thermocyclers in a single run. Set the annealing temperature at 1-2°C below the highest temperature that still generates a signal/band, to make sure the assay is both specific and robust. For primers with a T^m of 56°C, the optimized assay annealing temperature is usually around 58°C and the maximum annealing temperature still generating a (weak) signal around 60°C.

4.8.3. Cycling parameters

The default incubation times recommended for particular kits have been optimized for the reaction components and should be followed unless there are compelling reasons not to. Typical for PCR products < 400 bp is 10 seconds denaturation at 95°C; 15 seconds annealing-extension at 58-60°C. Longer products require an additional incubation of 60 seconds per 1,000 bp at 72°C.

4.9. Assay optimization

Each assay should be optimized experimentally since the various components can significantly affect the reaction dynamics (Caetano-Anolles, 1998). The criteria for optimization can be sensitivity, specificity or reproducibility, and for qPCR also reaction efficiency. Higher primer concentrations and lower annealing temperatures increase sensitivity, but reduce specificity. Optimising for reproducibility usually means identifying the highest annealing temperature, the lowest primer concentrations and the shortest incubation times that consistently generate the right product, without secondary products, at a consistent amplification cycle.

4.9.1. Primer-dimers and other PCR artefacts

PCR is susceptible to qualitative and quantitative errors caused by the accidental, and highly efficient, amplification of short non-target PCR templates, especially when there is little target template available. Such artefactual amplifications arise from fleeting, partial complementarity of the primers with non-target templates, or among the primers themselves (SantaLucia, 2007). The latter version is called 'primer-dimer' and is formed through (self)-complementarity at the 3' end of the amplification primers. For example, if one primer ends in N_{16}AC-3' and another primer in N_{16}GT-3', the two primers can form a short template through the pairing of these two 3' base-pairs (Fig. 1A). If a primer ends in complementary bases (N_{16}GC-3' or N_{16}AT-3') it could even create a 2-bp overlap with itself (Fig. 1B), generating a short amplifiable fragment. The risk of primer-dimer increases with the number of unique primers in a reaction, such as in multiplex PCR (see section 4.7.5.; "Multiplex RT-(q)PCR"). Primer-dimer is identified if a product is produced in a template-free reaction. If PCR artefacts are only produced in samples, but not template-free controls, then the cause is less clear, involving most likely other nucleic acids molecules present in the samples. In both cases, the easiest solution is to design new primers and test these experimentally (SantaLucia, 2007).

4.9.2. Primer concentration

Primer concentration can be conveniently optimised at the same time as annealing temperature (Topley *et al.,* 2005; Todd *et al.,* 2007). A useful starting point is 0.2 µM reaction concentration for each primer. Higher concentrations tend to increase sensitivity but also non-specific products, which interfere with accurate quantification. Lower concentrations reduce sensitivity and accurate quantification.

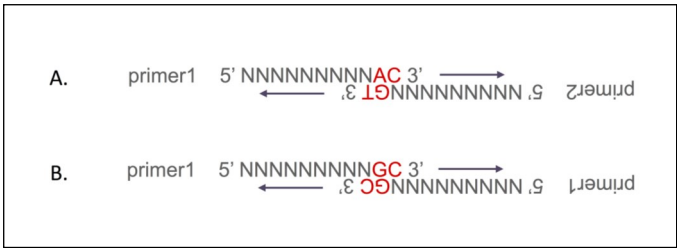

Fig. 1. Formation of primer-dimer through complementarity between the 3' ends of two primers (A) or self-complementarity of the 3' end of a single primer (B).

4.9.3. Magnesium concentration

Magnesium is an essential ion required for most nucleic-acid processing enzymes, particularly polymerases. The use of ion-chelating agents, such as EDTA, to stop or inhibit polymerases and nucleases attests to the importance of Mg^{+2} in nucleic acid reactions (Green and Sambrook, 2012). Most commercial buffers contain optimized concentrations of Mg^{+2}, so that currently there is little need for further Mg^{+2} optimization. Above a certain minimum concentration, magnesium has only marginal influence on reaction efficiency and almost none on reaction specificity.

4.10. Quantitation Controls

In order to accurately quantify the amounts of target in individual samples a number of different controls are used. These can be broadly divided into external reference standards, which are used to quantify the targets, and internal reference standards, which are used to correct the quantitative data for differences between individual samples in overall RNA quality and quantity.

4.10.1. External reference standards

The classic way to relate indirect measurements to absolute amounts of target is through external reference standards. These are established by running the RT-qPCR assay on a dilution series of a known amount of target (external standard), using the resulting data to calculate the relationship between the absolute amount of target and cycle number, and then using this equation to convert the sample data to absolute amounts (Bustin, 2000). All modern real-time PCR thermocyclers have this function automatically included in their software, requiring as only input the absolute concentrations of the external standards. Such curves are also extremely useful during optimization of the RT-qPCR reaction conditions, particularly for determining the reaction efficiency (Bustin *et al.,* 2009).

External reference dilution standards should be prepared for all targets, including the internal reference standards. This is done, in short, by amplifying the appropriate fragment with PCR, purifying and cloning this fragment in a plasmid and preparing purified, well quantified plasmid DNA. This plasmid DNA can be either used directly to prepare DNA-based external standard series, or be used to

synthesize RNA transcripts which in turn are quantified accurately and used to prepare RNA-based external standard series. DNA-based standards tend to be more sensitive and reproducible but RNA-based standards are more realistic and also take the cDNA reaction efficiency into account. The professional literature is divided on the issue, with good arguments for both approaches (Pfaffl and Hageleit, 2001). Both curves still require several positive control RNA samples per run, to normalize between runs for differences in reagent mixtures and, in the case of the DNA curve, to account for the reverse transcription step as well.

Reference standards from PCR products:

1. Amplify the target fragment by RT-PCR.
2. Confirm the amplification and absence of secondary products with electrophoresis.
3. If there are secondary products, excise the correct fragment under low-intensity UV light.
4. Purify the fragment using a commercial DNA affinity purification column.

The purified PCR fragments can be used directly to prepare an external reference standard, as follows:

1. Estimate the DNA concentration of the fragment in ng/µl, using spectrophotometry (e.g. Nanodrop, section 3.2.1) or fluorimetry (e.g. Qubit®; www.inVitrogen.com).
2. Estimate the molecular weight of your fragment.

This can be done exactly, based either on actual sequence or on fragment length, in the tools tab at www.currentprotocols.com. An approximate estimate for fragments within the 100-1000 bp range is:

MW^{dsDNA} = bp x 617 ng/nmol

3. Convert the DNA concentration to copies/µl as follows:

copies/µl = [ng/µl]/[MW^{dsDNA}] x [6.0221415 x 10^{14} copies/nmol]

4. Store the undiluted DNA fragment in aliquots at -80°C.
5. Prepare a working quantification standards series by serial ten -fold dilution of the DNA fragment, ranging from 10^{12} – 10^{0} copies/µl, in 10 ng/µl yeast or *E. coli* tRNA (Bustin *et al.,* 2009), to minimize loss of standard DNA due to adsorption to the microcentrifuge tube walls.

Whether or not the PCR products are used directly for preparing external reference standards, they should also be cloned: for confirmation of the fragment by sequencing, for long-term preservation of a positive DNA control and for the synthesis of RNA-based external reference standards. The fragment should be cloned into a T/A plasmid cloning vector that has T7 and T3 RNA promoters either side of the cloning site. Many molecular supply companies market such T/A cloning vectors, which are specially prepared for cloning PCR fragments.

1. Clone PCR fragments.

Protocols for cloning PCR fragments are beyond the scope of this paper. For this, the reader is referred to the product manuals provided by commercial suppliers of T/A cloning kits, and specialist manuals, such as the outstanding and long-established "Molecular cloning: a laboratory manual", by Green and Sambrook (2012).

2. Confirm candidate bacterial clones by colony PCR. This is a conventional 20 µl PCR reaction using the primers and amplification profile appropriate for the target, containing a small smudge of primary bacterial colony as template.
3. Run the colony-PCR products on an agarose gel (Green and Sambrook, 2012, see section 3.2.1).
4. Identify those colonies containing a plasmid with a cloned target.
5. Prepare small-scale liquid cultures of positive bacterial clones (Green and Sambrook, 2012).
6. Mix 0.5 ml of liquid bacterial culture with 0.5 ml 50% sterile glycerol and store this at -20°C (glycerol stocks).
7. Prepare plasmid DNA from the remaining liquid bacterial culture, using either a commercial plasmid purification kit or home-made reagents recommended in a molecular laboratory manual (Green and Sambrook, 2012).

Make sure that the protocol includes an RNAse step, to digest any bacterial RNA.

8. Purify the plasmid DNA on a commercial DNA affinity purification column.
9. Sequence the plasmid, using universal plasmid-based primers.

This is best done at specialist commercial facilities.

10. Confirm the presence of the insert in the plasmid from the sequence data, and the orientation of the insert in the plasmid.
11. Estimate the DNA concentration of the plasmid in ng/µl, using spectrophotometry (e.g. Nanodrop, section 3.2.1) or fluorimetry (e.g. Qubit®; www.InVitrogen.com). dsDNA A^{260} 1,0 = 50 ng/µl
12. Estimate the molecular weight of the plasmid + insert, by combining their lengths in bp and converting either exactly at www.currentprotocols.com or approximately as follows:

$MW^{dsDNA} = (bp^{plasmid} + bp^{insert}) \times 607.4 + 157.9$ ng/nmol

13. Convert the DNA concentration to copies/ul as follows:

copies/µl = [ng/µl]/[MW^{dsDNA}] x [6.0221415 x 10^{14} copies/nmol]

14. Store the undiluted plasmid in aliquots at -80°C.
15. Prepare a working quantification standards series by serial ten -fold dilution of the plasmid, ranging from 10^{12} – 10^{0} copies/µl, in 10 ng/µl yeast or *E. coli* tRNA (Bustin *et al.,* 2009).

RNA-based external reference standards

1. Transcribe RNA from purified plasmid DNA, using either the T7 or the T3 promoter, depending on the orientation of the insert and the desired strand polarity of the RNA.
2. Linearize the plasmid with a restriction enzyme that digests right after the cloned fragment, in the desired orientation.

This ensures that the RNA transcripts have a defined length.

3. Transcribe the digested plasmid with a specific commercial T3/ T7 RNA transcription kit.

Follow the corresponding instructions. Alternatively, detailed protocols with home-made reagents can be found in Green and Sambrook (2012).

4. Digest the synthetic, transcribed RNA with DNAse, as recommended by the kit manufacturer.

This is to remove contaminating plasmid DNA which may co-amplify and thus interfere with correct quantification.

5. Purify the DNAse-treated RNA on RNA affinity purification columns.
6. Estimate the RNA concentration in ng/µl, using spectrophotometry (e.g. Nanodrop, section 3.2.1) or fluorimetry (e.g. Qubit®; InVitrogen). ssRNA A^{260} 1,0 = 40 ng/µl
7. Calculate the insert size (number of bases from the T3/T7 promoter site to the restriction enzyme site on the other side of the insert used for digesting the plasmid).
8. Estimate the molecular weight of the RNA transcript either exactly at www.currentprotocols.com or approximately as follows:

   $$MW^{ssRNA} = nt \times 320.5 + 159.0 \text{ ng/nmol}$$

9. Convert the concentration of the synthetic RNA to copies/µl as follows:

   copies/µl = [ng/µl]/[MW^{ssRNA}] x [6.0221415 x 10^{14} copies/ nmol]

10. Store the undiluted RNA in aliquots at -80°C.
11. Prepare a working quantification standards series by serial ten -fold dilution of the RNA, ranging from 10^{12} – 10^{0} copies/µl.

Do not use an RNA carrier for preparing the dilution series, since this carrier RNA will participate in the reverse transcriptase reaction and thereby significantly affect quantification!! Instead, dilute either in nuclease-free water or in 10 ng/µl of a neutral DNA carrier, obtained from a commercial source.

4.10.2. Internal reference standards

Unfortunately, external standards cannot correct for factors unique to each sample that affect the RT and/or PCR reactions, such as RNA quality and quantity, enzyme inhibitors, sample degradation, internal fluorescence *etc.* To correct for these factors, internal reference standards are used. These come in two forms:

4.10.2.1. Exogenous internal reference standards

Exogenously added internal reference standards are a pure, unrelated RNA of known concentration and integrity that is added to each sample prior to analysis. Such RNAs can be bought commercially (for example, Ambion's RNA250) and can be used to correct the data for the presence of enzyme inhibitors in individual RNA samples (Tentcheva *et al.,* 2006). The amount added per reaction should be low; < 1% of the amount of sample RNA, so as not to affect the RT-qPCR reaction efficiencies.

4.10.2.2. Internal reference standards

Endogenous internal reference standards (commonly called 'housekeeping genes') are relatively invariant host mRNA targets present in every sample that can be used to normalize quantitative data for minor variations between samples in RNA quality and quantity (Bustin *et al.,* 2009; Radonić *et al.,* 2004). The problem is that it is impossible to prove categorically that the expression of any candidate 'invariant' gene is not affected by the expression of the target gene (Radonić *et al.,* 2004). For this reason it is currently recommended to use a battery of 3 or 4 internal controls, from different classes of genes (metabolic enzymes, structural proteins, transcription factors, ribosomal proteins *etc.*) and construct a control-gene index, with which to normalise between samples (Bustin, 2000). Common internal reference standards for honey bee research are β-actin (Chen *et al.,* 2005a; Shen *et al.,* 2005a; 2005b; Locke *et al.,* 2012), rRNA (Chantawannakul *et al.,* 2006), microsomal glutathione-S transferase (Evans and Wheeler, 2000; Gregory *et al.,* 2005); ribosomal proteins RP-S5 (Evans, 2004; 2006; Wheeler *et al.,* 2006), RP49 (Corona *et al.,* 2005; Yañez *et al.,* 2012), RP-S8 (Kucharski and Maleszka, 2002), and transcription factors eIF3-S8 (Grozinger *et al.,* 2003) and eF1α (Toma *et al.,* 2000; Yamazaki *et al.,* 2006).

One technical difficulty with endogenous internal reference standards is the presence of contaminating genomic DNA in a sample, which could be amplified instead of the mRNA. There are two solutions to this:

- Digest the RNA sample with DNAse prior to RT-PCR. Many RNA purification kits come with this option.
- Design the RT-PCR assay such that the primers are separated by a (large) intron in the genomic copy of the gene.

Only the spliced mRNA will be amplified by the assay (Bustin, 2000). Such intron-spanning primers have been designed for the honey bee RP49 mRNA and B-actin-isoform-2 mRNA (de Miranda and Fries, 2008; Yañez *et al.,* 2012; Locke *et al.,* 2012).

As stated above, all internal reference standards also require their own external standards for accurate quantification. The inclusion of internal reference standards obviously greatly increases the cost of a project. The inclusion of internal controls is therefore one of several

parameters to be decided on when starting a project, based on the projects' objectives, requirement for quantitative precision and available finances. Generally, the need for internal controls is greater for fully quantitative experiments with highly detailed analysis of relatively few samples. The need is much less for semi-quantitative survey-type studies, with fewer specific analyses and large numbers of samples.

4.10.2.3. External standard for viral target quantification

1. Extract RNA (Qiagen RNeasy® Mini Kit and QiaShredder®, according to manufacturer´s protocol) of bees with an RNA target (in this example DWV).
2. Generate an external standard by amplifying a DWV genomic fragment of 1520 bp via RT-PCR, using the primers Fstd (5´-GGACCATCCTTCCAGTCTACGAT-3´) and Bstd (5´-CTGTAGGTTGTGCTCCTGATGAAGA-3´) and the one-step RT-PCR kit from Qiagen.
3. This fragment contains the 354 bp fragment, which can be amplified by the primer pair F1/B1 (Genersch, 2005), for quantification.
4. Quantify the number of PCR-fragments via photometric analysis at 260 nm wavelength (Nanodrop, section 3.2.1).
5. Prepare a dilution series from the initial concentration through three orders (10-fold dilutions) of concentrated solutions.

This set of fixed dilutions will be used to ensure that PCR efficiency is maintained and to identify the precise predicted copy number for a particular C_q threshold.

4.11. Microarrays

A microarray is a powerful multiplex detection technology consisting of an ordered array of hundreds of molecular probes specific for different target RNAs bound to a solid support, usually a slide. The target sequences in an RNA sample are hybridized to these probes and these hybridization events are detected by a variety of, usually optical, detection chemistries (de Miranda, 2008). The power of the technology lies in the massive multiplexing potential where the relative and absolute amounts of hundreds of different targets can be determined simultaneously (Cheadle *et al.,* 2003; Gentry *et al.,* 2006). As molecular biology, pathology and diagnostics moves away from single organism/gene effects to surveying interactions among pathogens and (host) genes, microarray-based diagnostics will become increasingly relevant. Microarray printing technology is becoming cheaper and more reliable, and single-use disposable microarrays for specific multi-target diagnosis are increasingly available (Yuen *et al.,* 2003; Lieberfarb *et al.,* 2003; Noerholm *et al.,* 2004; Lin *et al.,* 2004; Perreten *et al.,* 2005; Fiorini *et al.,* 2005). Uniformity of hybridisation across the microarray, important for reliability in quantitation, is maximized with a range of nano-technological innovations (Yuen *et al.,* 2003; Noerholm *et al.,* 2004; Fiorini and Chiu, 2005), improved oligonucleotide design (Rouillard *et al.,* 2003) and with replication of the spots or even whole arrays (an array of arrays) across the slide. The probe-target hybridisation can be detected through FRET-based probes, SYBR-green-I dye, or labelling of the nucleic acid sample containing the target sequences. Microarray technology can also be combined with quantitative RT-PCR, multiplex (pyro)sequencing and label-free electronic or optical detection technologies to increase the speed, accuracy, specificity or information content of the diagnosis (Weidenhammer *et al.,* 2002; Erali *et al.,* 2003; Gharizadeh *et al.,* 2003; Fixe *et al.,* 2004).

Numerous honey bee arrays have already been designed for different research purposes (Whitfield *et al.,* 2002; Evans and Wheeler, 2000; 2001; Robinson *et al.,* 2006). A microarray has also been developed for the semi-quantitative detection of honey bee viruses (Table 5 in Glover *et al.,* 2011) which will be developed further for diagnostic purposes.

Microarrays can also be developed for serology-based detection of proteins (Sage, 2004), using a similar approach as the sandwich ELISA (Enzyme-Linked ImmunoSorbent Assay: see de Miranda *et al.*, 2013). The probe-target recognition events are visualized and detected using similar detection chemistries as for nucleic acid-based microarrays.

4.12. Northern blots using DIG labelling

The primary advantage of using Northern blot analyses for identifying specific predicted RNA's, versus a PCR-based method, comes in the ability to predict the size of the entire transcript that is targeted using standard gel size markers. This is key especially when transcripts are subjected to editing (splice variants or enzymatic cutting as for small RNAs) and editing must be validated using a technique other than PCR. In addition, since probe binding is more permissive of nucleotide changes, Northern blots can be used to verify transcripts that might have mutations at primer sites used for PCR. In addition, this approach has somewhat lower vulnerability to point mutations that might cause a specific primer pair to fail to amplify a predicted target. The disadvantage to using Northern blots versus a PCR method as above is in time and expense and in a somewhat reduced ability to quantify transcript abundance. The below protocol avoids the use of radio-isotopic nucleotides as probes.

4.12.1. Agarose /formaldehyde gel electrophoresis

What follows is a standard protocol for denaturing gels suitable for linear separation of RNA strands:

1. Be RNase free!! Use gel apparatus designated for RNA. Wipe apparatus with "RNaseAway" and rinse thoroughly with RNAse -free water.
2. Prepare 100 ml of 1% agarose/formaldehyde gel:
    1. Dissolve 1 g agarose in 72 ml DEPC-treated water in a 250 ml glass flask.

2. Cool to 60°C in a water bath.
3. Add 10 ml of 5X MOPS running buffer (200 mM MOPS buffer, 50 mM Sodium acetate, 20 mM EDTA, pH 7.0) and 18 ml of 37% formaldehyde.
   Precautions: Formaldehyde vapours are toxic. Prepare the gel in a fume hood.

3. Pour the gel to the gel tank and allow it to set.
4. Add sufficient 1X MOP running buffer to fill the tank in order to cover the gel and remove the comb carefully.
5. To prepare samples for gel electrophoresis, mix:
   - 11 µl of each RNA sample (0.5-1 µg/µl),
   - 5 µl 5X MOPS running buffer,
   - 9 µl 37% formaldehyde,
   - 25 µl of 50% formamide.
6. Heat the sample at 65°C for 15 min.
7. Cool on ice for 2 min.
8. Add 3 µl loading dye mix and 2 µl ethidium bromide (0.5 mg/ml).
9. Run the gel immediately after loading samples.
10. When the gel dye bands have separated and migrated at least 2-3 cm into the gel, or as far as 2/3 the length of the gel, visualize under UV light and take picture.

The 28s and 18sribosomal RNA (rRNA) should appear as sharp bands on the gel with no apparent smearing from degradation. The 28S rRNA band should be approximately twice as intense as the 18S.

4.12.2. Assembly of the transfer setup and transfer of RNA from gel to membrane

1. To prepare a gel for transfer, rinse the gel in DEPC-treated water twice for 20 min to remove the formaldehyde, which will otherwise interfere with transfer of RNA from gel to the membrane.
2. Soak the gel in RNase-free 20X SSC (3.0 M NaCl and 0.3 M sodium citrate, pH 7.0) for 45 min before proceed to setting up the transfer.
3. Cut uncharged nitrocellulose membrane to size of gel.
4. Soak the membrane in water for 2-3 min to wet .
5. Float in 20X SSC.

The transfer is conducted by the capillary method (Fig. 2).

1. Place a piece of thick blotting paper on the top of a glass plate that is elevated by four rubber stoppers placed near each corner of a baking glass dish.
2. Drape the ends of the wick blotting paper over the edges of the plate.
3. Fill the glass dish with RNase-free 20X SSC until the wick blotting paper on the top of glass plate is completely wet.
4. Squeeze out all air bubbles by rolling with a glass rod or pipette.
5. Place the gel facing down on the wet blotting paper.
6. Squeeze out air bubbles by rolling a glass pipette.
7. Cut a small triangular piece from the top left-hand corner to simplify orientation.
8. Place the wetted membrane on the surface of the gel by aligning the cut corners.
9. Get rid of any air bubbles under the membrane by rolling a glass pipette.
10. Cut 4-5 sheets of Whatman 3MM paper to the same size as membrane.
11. Place on top of the membrane.
12. Place a stack of paper towels on top of the Whatman 3MM papers.
13. Add a 200-500 g weight to hold everything in place.
14. Allow the transfer of RNA to proceed by capillary action overnight.
15. Disassemble the transfer stack at the next day.
16. Rinse the membrane briefly in 6X SSC.
17. Immobilize RNA to the blot by UV cross linking while the membrane is still damp.

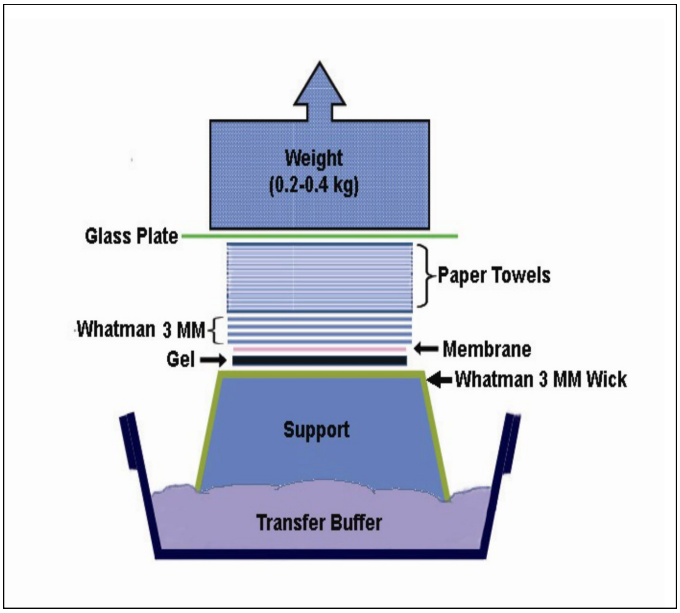

Fig. 2. Schematic diagram of the process used to transfer nucleic acids from a gel onto a binding membrane.

4.12.3. Preparation of DIG labelling (non-radioactive) probe

While DNA probes can also be used to detect RNA targets, a DIG-labeled RNA probe is ideal for detecting RNA on a Northern blot because RNA probes (riboprobes) that are transcribed in vitro are able to withstand more rigorous washing steps preventing some of the background noise. RNA probes give better sensitivity for detecting low amounts of RNA target than DNA probes. The following protocol is based on the Roche DIG RNA Labelling Kit, SP6/T7.

1. Linearize the recombinant plasmid DNAs with the target insert by cutting a restriction enzyme cleavage site downstream from the cloned insert using a restriction enzyme that creates 5' overhangs (the choice of this enzyme will depend on the sequence of both the plasmid and insert).
2. After restriction digestion, purify the DNA by spin column purification or via phenol/chloroform/isoamyl alcohol extraction and ethanol.

This is commonly referred to as the 'plasmid mini-prep' and there are numerous commercial and home-made recipes for doing so that all work well.

3. Add the 2 µg purified template DNA to the following transcription reaction mixture to make 26 µl probe as follows:

- 4 µl 10X NTP labelling mixture,
- 4 µl 10X Transcription buffer,
- 2 µl Protector RNase Inhibitor,
- 4 µl RNA Polymerase SP6/or T7.

Adjust the volume with additional water until a final volume of 26 µl.

4. Place transcription reaction in the 37°C incubator for 2 hours. Longer incubations do not increase the yield of labeled RNA.
5. Stop reaction with 2µl 0.2M EDTA (pH 8.0).

Labeled probes are stable for at least one year at -15 to -25°C.

4.12.4. Hybridization analysis

(Roche Applied Science DIG Easy® Hyb, DIG Wash and Block Buffer Set, CSPD®Ready-to-use protocol)

1. Pre-hybridize the blot with pre-warmed DIG Easy® Hyb (10– 15 ml per 100 cm^2) in a specialized hybridization bag or any sealable container:
    1. Incubate the blot for 30 min at 65°C.
    2. Agitate gently during the pre-hybridization step.
2. Pipette the desired volume of probe (50-100 ng probe per ml hybridization buffer) into the hybridization bag.
3. Continue to incubate with rotation at 65°C for 10-16 hours.
4. After the hybridization is complete, wash the blot in a tray containing Low Stringency Buffer (2x SSC containing 0.1% SDS) twice by incubating the tray at RT for 5 min with gentle agitation.
5. Transfer the blot to preheated High Stringency Buffer (0.1x SSC containing 0.1% SDS).
6. Incubate the blot twice (2 x 15 min, with shaking) in High Stringency Buffer at 65°C.
7. After last wash, pull out the blot out of the hybridization container.
8. Place it between two Whatman paper sheets.

Do not allow the membrane get too dry so the membrane can be stripped and reused for hybridization.

9. Place blot onto a piece of Plastic wrap that is at least twice the size of the membrane.
10. Add 1 ml detection reagent (anti-digoxigenin-AP conjugate and the premixed stock solution of CSPD® ready-to-use) to stain the membrane and leave for 5 min.
11. Completely wrap up the blot with the plastic wrap.
12. Put it in a film cassette for chemiluminescent detection of hybridization signals.

4.13. *In situ* hybridization

4.13.1. Tissue fixation

1. Dissect out individual tissues.
2. Wash tissues with cold PBS 2-3 times.
3. Fix tissues in freshly made 4% paraformaldehyde in 100mM PBS (pH 7.0) overnight at 4°C.
4. Rinse in nuclease-free water three times.
5. Store tissues in 70% ethanol at 4°C until further use.
6. For tissue dehydration, carry out successive incubation in ethanol (70%, 95% and 100%) and xylol (2 x 5 min each).
7. Embed in paraffin.
8. Cut Paraffin sections into 2-5 micron thick segments.
9. Mount on poly-L-lysinated slides which are to be stored at 4°C overnight.
10. To rehydrate the sections prior to hybridization:
    1. Carry out descending concentration of ethanol (100%, 95% and 70%).
    2. Dewax in xylol.
    3. Treat with proteinase K (10 ug/ml) for 30 min.
    4. Acetylate with 0.33% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min.

4.13.2. Preparation of DIG labelling (non-radioactive) probe

Using Roche DIG RNA Labelling Kit, SP6/T7. The same procedures as for Northern blot (see section 4.12.).

4.13.3. Hybridization Analysis

1. Pre-hybridize the sections in pre-hybridization solution (50% formamide, 5X SSC, 40 µg/ml salmon sperm) at 58°C for two hours.
2. Incubate in hybridization buffer with Dig-labeled TARGET probe solution to a concentration of 100-200 ng/ml of probe in pre-hybridization solution at 58°C overnight.
3. After hybridization, wash the sections twice in low stringency wash solution (2X SSC, 0.1% SDS) at room temperature for five minutes.
4. Wash twice in high stringency wash solution (0.1 × SSC, 0.1% SDS) at 52°C for 15 min.

Note: The hybridization signals are detected with Alkaline phosphatase (AP)-labeled sheep anti-DIG antibody conjugate (Roche Applied Science).

5. Add the conjugate solution to the dry sections.

6. Incubate at 4°C for two hours in a chamber in which humidity is maintained at > 70% relative humidity.
7. Rinse the slides three times with washing buffers.
8. Perform the colour development by adding the buffer solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) on the tissue sections.
9. Incubate for three to six hours at RT protected from bright light.
10. Stop the colour reaction by a 5 min wash in Tris/EDTA (0.1 mM, pH 8.0).
11. Remove the non-specific staining in 95% EtOH overnight.
12. Rehydrate the sections through:
    1. Successive incubation in ethanol (70%, 95%, and 100%).
    2. Incubation in xylol (2 X15 min each).
13. Mount in Eukitt® resin (Sigma).

Note: Negative control reactions include regular dUTP instead of DIG-labeled TARGET probe.

14. Observe and photograph *In Situ* hybridization slides under a light microscope.

The hybridization signals are shown by dark blue sites where the DIG-labeled probe bound directly to the viral RNA. The section of negative control will stain pink only with the nuclear fast red.

5. Proteomic methods

5.1. Introduction

Proteins are the ultimate functional product of most gene expression so optimally one would prefer to look at proteins when trying to understand the mechanisms an organism uses to respond to a given condition. As with any other biomolecule, tools for identifying and quantifying proteins are a prerequisite to their successful study. Single proteins are typically detected using antibodies but very few antibodies have been generated against bee proteins and none have been commercialized. Of all the analytical methods available for studying proteins, mass spectrometry is the most sensitive, most accurate and least biased. Proteins can be identified by mass spectrometry by first hydrolyzing them with a specific protease such as trypsin. The masses and fragmentation patterns of the resulting peptides can then be determined and used to identify the peptides individually and the protein(s) they came from (i.e. proteomics). This process works best when all possible proteins that might be present are known and is only really successful when an organism's genome has been sequenced. To this end, in recent years proteomics has begun to be applied in bees towards understanding a range of paradigms.

Where is the future of proteomics research in bees heading? Mapping protein expression across all tissues and castes in adult bees is the logical next step after sequencing the bee genome. The genome helps to determine which proteins may be present but where are those proteins expressed? The protein expression atlas in bees will tell that and will mark a significant step forward for bees as a model system as this would be the first such comprehensive atlas in any multicellular organism. Additional protein-based methods (protein extraction and immunochemical assays for protein abundance) are covered in further detail in the *BEEBOOK* paper on physiology and biochemistry (Hartfelder *et al.*, 2013).

6. Population genetics

6.1. Introduction

Measuring the current variation in genetic traits within and across populations can give insights into past movement of individuals, population size, and the association of specific genetic histories with honey bee biological traits such as behaviour, disease resistance, and colony life histories. Honey bees, thanks to human transport and breeding, are made up of numerous and often entwined genetic lineages and one aim of population-genetic analyses are to resolve these connections.

6.2. Mitochondrial DNA analysis

In principle, one honey bee (worker or drone) per colony is enough to determine the maternal origin of the whole colony given the maternal inheritance of mitochondrial DNA (mtDNA), i.e. all the daughter workers and son drones from one honey bee queen share the same molecule. Due to the risk of drifting between colonies, it is ideal, and in some cases essential, to make this one collected individual of a life stage where host colony is unambiguous (e.g., a developing bee or one observed exiting from a brood cell). In cases where this is not possible, pre-flight worker bees could be substituted, although it is arguably worth sampling more than one individual to avoid mistakes in assigning colony heritage.

The most widely used mitochondrial region for population genetic studies is the intergenic region located between the $tRNA^{leu}$ and cox2 (subunit 2 of the cytochrome oxidase) genes. This region shows length and sequence variation that allows discrimination of honey bee evolutionary lineages (Garnery *et al.*, 1993). It is composed of two types of sequences: P and Q. The sequence P can be absent (lineage C from east Europe) or present in four different forms: P (lineage M from west Europe), P_0 (lineages A from Africa and O from Near East), P_1 (Atlantic African sub-lineage) and P_2 (restricted to the Y lineage from Ethiopia; De la Rúa *et al.*, 2009). The number of Q sequences and the sequence variation developed through a RFLP test with the restriction enzyme *Dra*I (Garnery *et al.*, 1993) can be used to determine the haplotype within each lineage. Below is the protocol to determine the mitochondrial haplotype, modified from Garnery *et al.* (1993) by including a new thermal regime for PCR and optimizing the

chemistry of PCR reactions. A full description of how this locus can discriminate among honey bee ecotypes is presented in the *BEEBOOK* paper on characterizing subspecies and ecotypes by Meixner *et al.* (2013).

1. Honey bee samples are immediately transferred into tubes with absolute EtOH and preserved at -20°C until DNA extraction. A single or two legs from one individual are enough to extract total DNA following the Chelex®-based (Biorad, Inc.) protocol (Walsh *et al.*, 1991; see section 3.2.3.).
2. PCR amplify the intergenic region with the primers E2 (5'-GGCAGAATAAGTGACATTG-3') located at the 5' end of the gene $tRNA^{leu}$ and H2 (5'-CAATATCATTGATGAACC-3') located close to the 5' end of the gene cox2 (Garnery *et al.*, 1993).

This amplification can be performed by using Ready-To-Go TM PCR Beads (product code 27-9557-01, GE Healthcare), that are pre-mixed and pre-dispensed reactions for PCR featuring, therefore reducing the pipetting steps and the chances to handling error. They contain all the necessary reagents for a 25 µl reaction volume.

3. Add 20.2 µl of PCR-quality water to each tube.
4. Mix by gently flicking it with the fingers.
5. Add 0.4 µl of each primer (10 mM) and vortex and centrifuge the mix to get all the components at the bottom of the tubes.
6. Add 4 µl of DNA extraction solution and mix.
7. Place the reaction mixtures in a thermo cycler with the following amplification program:
   Denaturation at 94 °C for 5 min,
   Followed by 35 cycles of:
   94 °C for 45 sec,
   48 °C for 45 sec,
   62 °C for 2 min,
   Final elongation step of 20 min at 65 °C.
8. To identify successful amplicons, 2 µl of the PCR product of each sample are electrophoresed in a 1.5% agarose gel (see section 3.2.1) with ethidium bromide included and photographed over a UV light screen.
9. Aliquots of the PCR product are then digested with the endonuclease *Dra*I (recognition site 5'-TTTAAA-3') by adding:
   10X endonuclease buffer to a final concentration of 1X,
   0.06U of *Dra*I/,
   10 µL of PCR product,
   Incubate at 37°C overnight.
10. To determine RFLP patterns, the digested products of each sample are electrophoresed in a 4% agarose Nusieve® or Metaphor® (Lonza Biosciences) gel at 100 volts for ca. 1 hour 30 min and photographed over a UV light screen.
11. At least one sample with a characteristic RFLP pattern should be directly sequenced using the same primers as for the amplification.
12. Prior to sequencing, purify PCR products:
    Either with QIAquick® PCR Purification Kit (Qiagen).
    Alternatively, PCR products can be purified with isopropanol and 5 M ammonium acetate as follows:
    1. To 10 µl of PCR product add:
       7 µl of 5 M ammonium acetate,
       17 µl of isopropanol.
    2. Leave 10 min at room temperature.
    3. Centrifuge 30 min to 13,500 rpm.
    4. Discard the supernatant.
    5. Add 500 µl of cold 70% EtOH.
    6. Centrifuge 5 min at 13,500 rpm.
    7. Remove supernatant and allow to dry overnight.
    8. Re-suspend in 30 µl of water.

6.3. Nuclear DNA analysis

Nuclear markers are biparentally inherited and allow genotyping of workers to obtain information from both the mother queen and the drones she has mated with. Nuclear analyses of *A. mellifera* involve widely used microsatellites and more recently, single nuclear polymorphisms or SNPs.

6.3.1. Microsatellites

Microsatellites consist of short motifs (one to six nucleotides) that are repeated from four to 20+ times at points scattered across all eukaryotic genomes. They are useful as markers for genetic structure since the number of repeats at any given locus is unstable and new repeat variants are constantly arising by mutation and being lost by drift and other population-level events. Strategies to screen a total of 550 polymorphic microsatellite loci have been described in *A. mellifera* (Solignac *et al.*, 2003), and many thousands more are found in the complete honey bee genome (Honey Bee Genome Sequencing Consortium, 2006). The protocol described here has been used to analyse the temporal genetic variation of island honey bee populations (Muñoz *et al.*, 2012), the mating frequency of the Iberian honey bee (Hernández-García *et al.*, 2009) and the population genetic structure of European honey bees (Muñoz *et al.*, 2009; see also the *BEEBOOK* paper on characterizing subspecies and ecotypes by Meixner *et al.* (2013) for a full review of the use of microsatellites in determining honey bee ecotypes). It takes advantage of multiplexing, whereby multiple loci are screened in a single PCR reaction and size assay. These loci are widely used, and it is subsequently possible to compare allelic counts and genotypes across different studies.

6.3.1.1 Microsatellite reaction mix

To prepare the reaction mix, add:

- 1X reaction buffer (provided as a 10x solution with Taq polymerase),
- 1.2 mM $MgCl_2$,

- 0.3 mM of each dNTPs,
- 0.4 µM of each primer,
- 1.5 U Taq polymerase,
- > 5 ng DNA (provided in 2µl DNA solution).

6.3.1.2 Primers for multiplexed honey bee microsatellite loci

A113-F-(FAM)	CTC GAA TCG TGG CGT CC
A113-R	CCT GTA TTT TGC AAC CTC GC
A007-F-(NED)	GTT AGT GCC CTC CTC TTG C
A007-R	CCC TTC CTC TTT CAT CTT CC
AP043-F-(VIC)	GGC GTG CAC AGC TTA TTC C
AP043-R	CGA AGG TGG TTT CAG GCC
AP055-F-(PET)	GAT CAC TTC GTT TCA ACC GT
AP055-R	CAT TCG GTA TGG TAC GAC CT
B124-F-(FAM)	GCA ACA GGT CGG GTT AGA G
B124-R	CAG GAT AGG GTA GGT AAG CAG

6.3.1.3 Thermal cycling conditions for multiplex PCR

Incubate the samples as follows:

5 min at 95°C,
Followed by 30 cycles:
95°C for 30 sec,
54°C for 30 sec,
72°C for 30 sec,
Final extension is 30 min at 72°C.

6.3.1.4 Size estimation of PCR products

PCR products are visualized by capillary electrophoresis and sized with an internal size-standard (e.g., using the Applied Biosystems or MegaBACE systems, both of which have extensive use for microsatellite scoring). Alleles are subsequently scored using GeneMapper v3.7 software (Applied Biosystems™). It is also possible to measure microsatellite size variants using large denaturing polyacrylamide gels (e.g. Evans, 1993) although this method has fallen from favour do to the hazards of polyacrylamide and difficulties in manually scoring allele sizes.

Once genotypes of samples have been established, microsatellite data are well suited for assessing parentage of nestmates (Evans, 1993), for standard population-genetic statistics including ecotype determination (Estoup *et al.*, 1993; reviewed by Meixner *et al.*, 2013), and for genome mapping (Solignac *et al.,* 2003), among other uses.

6.3.2. Single-nucleotide polymorphisms (SNPs)

In the honey bee and other species for which extensive data have been gathered on genomic sequence variants, it is possible to use SNPs to reconstruct past migration events, and to separate races and populations. A SNP is any validated nucleotide change between the genomes of two or more samples, and SNP's can occur both within the coding regions (exons) of genes and in the vast regions that separate genes or lie in non-coding parts of the genome. SNP analyses are standard in human, veterinary, and agricultural systems, and this approach will continue to increase as a viable option for the study of honey bees. Unfortunately, high-throughput SNP genotyping remains an expensive endeavour that requires cutting edge technologies and the expertise often only available in a core laboratory facility or at larger institutions. In addition, prior to genotyping the honey bee sample of interest, a SNP assay must be developed (or purchased, if commercially available) from sequence data relevant for the study population. At present, there are only two SNP assays developed and published for honey bees. The first one (Whitfield *et al.*, 2006), which consisted of 1536 SNP loci that were selected mainly based on spacing criteria, was developed for genotyping using the Illumina GoldenGate™ assay and is not commercially available, although a honey bee SNP database (over 1 million SNPs) is available at NCBI (http:// www.ncbi.nlm.nih.gov/snp/) and this resource could be exploited to establish a system for genotyping. More recently, Spötter *et al.* (2011), published a 44,000 SNP assay designed for analysis of varroa-specific defence behaviour in honey bees. This assay uses Affymetrix™ technology, and it is now commercially available via AROS Applied Biotechnology AS.

As illustrated in Spötter *et al.* (2011), development of a SNP assay is a time and resource intensive undertaking, yet it can be designed to address a specific objective (e.g., to investigate varroa-specific defence behaviour). Once the design stage is accomplished, the assay can then be used to genotype honey bee samples at hundreds to thousands of loci via high-throughput technologies. Illumina® technologies, for example, offer a number of options for high throughput genotyping depending on the number of SNPs to be interrogated. The GoldenGate assay, employed by Whitfield *et al.* (2006), interrogates 96, or from 384 to 1,536 SNP loci simultaneously (plex levels can be 384, 768, or 1,536). For genotyping a number of SNPs larger than 6,000 up to 2,500,000 the Infinium assay (also a product from Illumina) is required.

Both the GoldenGate assay and the Infinium assay take three days for completion and require reasonable quality and accurately quantified genomic DNA. DNA concentrations should be 50 ng/µl, quantified a fluorometric assay (e.g., Picogreen) or spectrophotometry (e.g., Nanodrop, section 3.2.1). DNA can be extracted from the thoraces of honey bees that had been stored at -80°C or in absolute EtOH. The GoldenGate assay involves several steps including DNA activation for binding to paramagnetic particles, hybridization of activated DNA with assay oligonucleotides, washing, extension, ligation, PCR, hybridization onto the BeadChip, and finally analysis of the fluorescence signal on BeadChip by the iScan System.

Unlike in the GoldenGate assay, where universal primers are used to amplify SNP-reactive DNA fragments, in the Infinium assay genomic targets hybridize directly to array-bound sequences. Following hybridization onto the BeadChip, samples are extended and

fluorescently stained. As for the GoldenGate assay, the last step consists of analysis of scanned BeadChips using the iScan System. Genotype data generated by both assays using the iScan System (and other systems), are then analysed using the GenomeStudio Genotyping (GT) Module. The calls are automated but can be manually verified and edited if necessary (e.g., if there are signs of unequal proportions of an expected biallellic marker). Finally, summary statistics and results are exported for further analyses using standard population genetics software packages such as STRUCTURE (http://pritch.bsd.uchicago.edu/structure.html).

With increasingly affordable sequencing costs allowed by next generation technologies (e.g., 100 bp or shorter), it will be feasible to carry out population-genetic and strain-identifying projects via whole-genome sequencing. This technique involves a scan (usually 3-fold sequencing depth or more, i.e., > 750 million sequenced bases for the honey bee) of a genome or population of interest followed by an alignment of those short reads to a reference genome (for the honey bee this would be the genome assembly from HGSC, 2006). It is relatively straightforward, using free programs available for download (e.g., http://bioinformatics.igm.jhmi.edu/salzberg/Salzberg/Software.html) to identify and in some cases quantify SNPs that differ among samples. There are also public sites at which one can import data and benefit from a maintained supercomputer dedicated to such genomic analyses (e.g. https://main.g2.bx.psu.edu/). SNP analyses derived from sequencing data have yet to make an impact on honey bee science but they are expected to in the next few years.

7. Phylogenetic analysis of sequence data

7.1. Introduction

The goal of this protocol is to provide the reader with an easy to use, reliable, and technically appropriate method to choose, align and analyse sequence data for phylogenetic analyses of taxa or genes of interest. Analysis of highly conserved loci (i.e. rRNA, cytochrome oxidase I) or population genetic studies from one species, require nucleotide level data to achieve necessary resolution in tree topology. Amino acid sequences are typically used when reconstructing phylogenies from an encoded protein across a large evolutionary distance, which can make alignment at the nucleotide level difficult. Over time, one develops their preferred approach and programs to use in this process, of which there are many. While the following protocol reflects preferences of the authors, it is appropriate for a wide variety of applications, user skill levels, and relies on freely available programs with graphical user interface (GUI)-based options. Detailed information on use is available from each of the program sites, given below. As a disclaimer, concatenation of sequence data, while appropriate and employed for taxonomy classification, is a more specific approach some users may wish to use but will not be discussed here. Additionally, though PAUP is also widely used in phylogenetic analyses, it requires a small fee and therefore is not discussed here, though labs with frequent phylogenetic needs may wish to purchase this program. MEGA and other software free to the public can invoke many or all of the same phylogenetic analyses as PAUP.

The steps to perform a phylogenetic analysis are:

1. Obtain and format sequences of interest.
2. Format sequence data in FASTA format.
3. Align sequence data.
4. Trim aligned sequence data to equal length.
5. Perform phylogenetic analyses.

Each step is described below in detail.

7.2. Obtaining and formatting sequences of interest for phylogenetics

Once you have obtained DNA sequence data for your study, you may wish to add accessioned sequence data to your analyses. This will be particularly important if you want to root your phylogeny and provide an outgroup (sequence(s) to which all of your sequences are distantly related) to strengthen comparative interpretation of your data. Accessions from nucleotide sequence data banks can be searched, using a keyword(s) or via a BLAST search algorithm (i.e. blastn, megablast, etc.), to identify homologues to your sequence of interest. These include GenBank (via NCBI; (http://www.ncbi.nlm.nih.gov/), EMBL-Bank (via EBI; http://www.ebi.ac.uk/embl/), and DNA Data Bank of Japan (DDBJ; http://ddbj.sakura.ne.jp/). If using rRNA sequence data, SILVA rRNA database (http://www.arb-silva.de/) can be used to retrieve reference sequences that are quality-scored (Pruesse *et al.*, 2007).

7.3. Sequence data in FASTA format

For compatibility in downstream analyses, sequence data should be in a single file and FASTA formatted. Sequence databases include FASTA as an option for output format. An example of FASTA formatted sequences retrieved from GenBank (abbreviated in length for the sake of space):

>gi|21747902|gb|AY114459.1| *Apis mellifera mellifera* isolate melli4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product
CCCCGAATAAATAATGTTAGATTTTGATTACTTCCTCCCTCATTAT
TAATACTTTTATTAAGAAATTTATTTTACCCAAGACCAGGAACTG
GATGAACAGTATATCC

>gi|14193071|gb|AF153104.1| *Apis cerana* haplotype 4 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial gene for mitochondrial product
TTTCTAATTGGAGGTTTTGGAAATTGATTAATTCCTTTAATATTA
GGATCTCCAGATATAGCATTTCCTCGAATAAATAATATTAGATTC
TGATTACTCCCTCCTTC
>gi|67626085|gb|DQ016070.1| *Apis dorsata* haplotype 7 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial
TTTTTAATTGGAGGATTTGGAAATTGATTAATCCCTTTAATATTA
GGGTCTCCAGATATAGCATTTCCTCGAATAAATAATATTAGATTT
TGATTATTACCTCCTT

The sequence identifier (e.g. accession number) and title for each entry is preceded by a carrot symbol ">" and ends with a hard return. The immediate next line below this is the sequence information and should contain no spaces. The end of the sequence is determined by a hard return. You will want to abbreviate the title of your sequence entries now, prior to alignment, using the all-important accession number or perhaps just the species name. The number of characters allowed in the sequence title is limited, to varying degrees, by alignment programs but are typically 30 characters or less. Only letters, numbers, underscores "_", and pipes "|" are typically allowed. The above sequence entries are prepared for alignment like this:

>AY114459_A_mellifera
CCCCGAATAAATAATGTTAGATTTTGATTACTTCCTCCCTCATTAT
TAATACTTTTATTAAGAAATTTATTTTACCCAAGACCAGGAACTG
GATGAACAGTATATCC
>AF153104_A_cerana
TTTCTAATTGGAGGTTTTGGAAATTGATTAATTCCTTTAATATTA
GGATCTCCAGATATAGCATTTCCTCGAATAAATAATATTAGATTC
TGATTACTCCCTCCTTC
>DQ016070_A_dorsata
TTTTTAATTGGAGGATTTGGAAATTGATTAATCCCTTTAATATTA
GGGTCTCCAGATATAGCATTTCCTCGAATAAATAATATTAGATTT
TGATTATTACCTCCTT

Note that you may want to keep two copies of your sequence data files: one with all the original information pertaining to the sequences and a second with just the abbreviated titles prepared for alignment analysis.

7.4. Alignment of sequence data

The alignment quality of sequences is critically important to achieving a strong phylogenetic reconstruction. There are a variety of multiple sequence alignment programs available, with varying capacity for the number of sequences input and user-determined parameter adjustments. Additionally, some aligners may be specific for protein sequence data vs. nucleotide data. Two alignment programs that are available for all computing platforms (Mac OSX, Windows, and Unix/Linux), accessible at an off-site server via the web if installing locally is not desired, and known for robust alignment algorithms are discussed here.

7.4.1. Clustal

Clustal (Thompson *et al.*, 1994) is a commonly used alignment program that will handle protein, DNA, or RNA sequence data and is actively maintained (http://www.clustal.org/). The version ClustalW, currently in version 2.1 (Larkin *et al.*, 2007), can be installed locally and run in command-line or it can be run remotely at an off-site server where it is already installed (i.e. at EMBL-EBI; http://www.ebi.ac.uk/Tools/msa/clustalw2/ or at GenomeNet; http://www.genome.jp/tools/clustalw/). ClustalW allows the user to specify certain parameters of the alignment algorithm. Users who do not have the knowledge to make parameter specifications may choose general purpose, default settings (as at the EMBL-EBI site). ClustalX, a graphical version and Clustal Omega, specifically for large sets of protein sequence data are also available.

7.4.2. MUSCLE

Though less commonly used than Clustal, MUltiple SequenCe aLignmEnt (MUSCLE; http://www.drive5.com/muscle/) is another easy to use, good option for sequence alignment (currently limited to 500 sequences/1MB of data).

7.5. Trimming aligned sequence data to equal length

To properly compute phylogenetic analyses on a sequence data set, the number of positions in each sequence should be equal. This includes gaps and insertions/deletions (indels) in the aligned data set, not the actual number of nucleotides or amino acids. Use your sequence alignment editor to trim the aligned files to equal size or to the size of the region you are interested in analysing (i.e. a specific domain encoded within your gene).

7.6. Performing phylogenetic analyses

Again, there are a number of options for users to perform phylogenetic analyses, but only two will be discussed here: MEGA and SATé.

7.6.1. Using MEGA

The program MEGA (Molecular Evolutionary Genetics Analysis; http://megasoftware.net) (Tamura *et al.*, 2011), currently available as version 5.05, is continually being updated and improved. Note: use of MEGA will require the user to download and install the freely available software to their computer. MEGA 5 can be used as a platform to complete all of the above steps (building your sequence data file,

alignment using Clustal or MUSCLE, and trimming sequence data). It gives the user the ability to construct phylogenies using distance based (i.e. Neighbour Joining) and character based (i.e. Maximum Likelihood) methods (see Table 1) and test them using bootstrapping. It also includes a tree viewer that allows for some editing of the final output tree and many additional features not covered here.

7.6.1.1. Converting data to MEGA format

Before phylogenetic tests can be run on your sequence data file, it must be converted into a format that MEGA can read, the *.meg file format.

1. From the 'File' menu in MEGA, select 'Convert File Format to MEGA…', browse to your alignment file, select 'FASTA format' from the 'Data Format' pull down menu, then select 'OK'.
2. A window will open asking for you to specify a name and location for the newly created *.meg file.

The new file will be created and opened under a tab in the same window next to your open FASTA format file.

3. MEGA will warn you to check the file for any errors and adjustments to your *.meg file can be made in this window and saved.

Details about the *.meg format are available in MEGA.

7.6.1.2. Constructing and testing phylogenetic trees

Described below is a basic, distance based Neighbour-Joining analysis using bootstrap statistical tests for robustness (Felsenstein, 1985).

1. From the main MEGA window, open the 'Phylogeny' pull down tab and select 'Construct/Test Neighbour-Joining Tree'.
2. Browse to and open the .meg file you just created. Select the appropriate data type (nucleotide or protein sequence data).

Note the defaults for missing data, alignment gaps and identity and make any changes if necessary then select 'OK'.

3. A window will open asking you to identify your sequence data as protein-coding or not.
4. Another window will open asking for genetic code selection.

For the example, provided here, using the cytochrome oxidase I (COI) gene, select 'Invertebrate Mitochondrial'.

5. A third window opens and allows you to select a number of parameters for your analysis.

A minimum of 100 and typically 1,000 iterations of bootstrapping are used to test the robustness of your phylogeny. For now, we will accept the default parameters for our simple analysis.

6. A progress window will open for you until the test is completed.
7. When complete, a window opens with two tabs showing the 'Original tree' generated, as well as a 'Bootstrap consensus tree', which is the tree you should refer to.

Bootstrap support values show the percentage of iterations supporting the shown topology.

8. The tree image can be saved as a pdf for good resolution for presentation (Fig. 3A), can be saved as a mts Tree Session File for future viewing in MEGA, or exported and saved as a more general Newick (.nwk) format file that is readable by a variety of other tree viewing programs (e.g. FigTree http://tree.bio.ed.ac.uk/software/figtree/ ; TreeDyn http://www.treedyn.org/).

For comparison, a Maximum Likelihood (ML) analysis of the same alignment was performed in a similar manner and is shown in Fig. 3B.

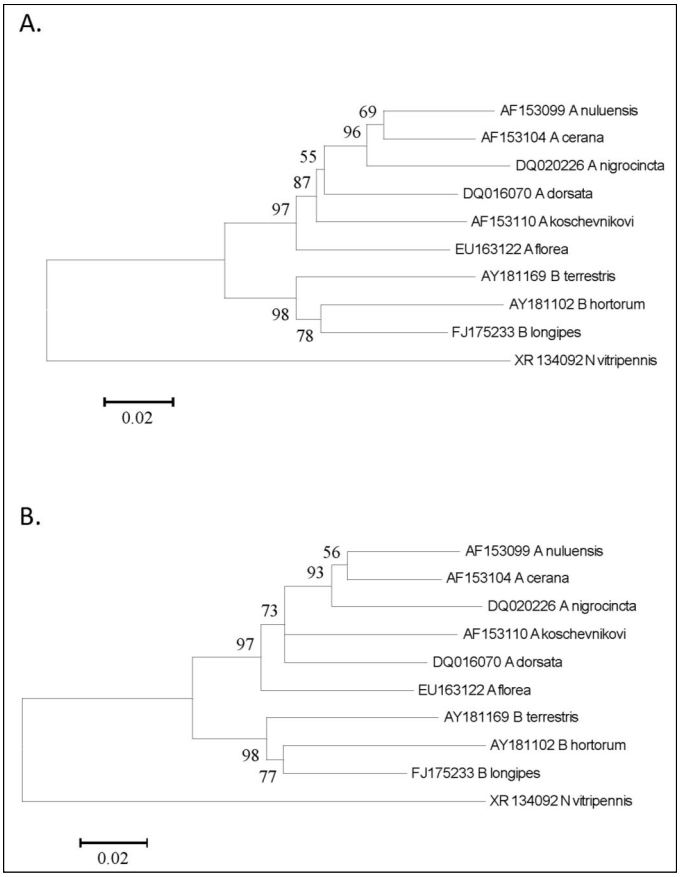

Fig. 3. Phylogenetic reconstruction of cytochrome oxidase I gene from *Apis* and *Bombus* species (Hymenoptera; Apidae) using Neighbour-Joining method (A) and Maximum Likelihood (B). Topology of each was tested with 1,000 bootstrap iterations (consensus tree is shown) using *Nasonia vitripennis* (Hymenoptera; Pteromalidae) as outgroup. Scale represents the substitution rate per site from a total of 981 positions. A) was computed using Maximum Composite Likelihood (Tamura *et al.*, 2004) with uniform rates among sites and pairwise gap deletion. B) was computed using Tamura-Nei model (Tamura and Nei, 1993) with uniform rates at all sites.

7.6.2. Using SATé

An alternative software package to MEGA for ML analyses is SATé (Simultaneous Alignment and Tree estimation; Liu *et al.*, 2012), which infers sequence alignment and tree building concurrently as an iterative process using the ML method. This program must also be downloaded for use, and is currently freely available as SATé-II at http://phylo.bio.ku.edu/software/state/sate.html. The user experience for SATé is still being improved, including recommendations for how to parse phylogenetic runs. A strength of SATé is that it accepts up to 1,000 sequences in the FASTA format as described in section 7.3., and claims speed and precision in phylogenetic analyses. Through changes in subproblem size parameters (below) it is possible to run SATé on desktop machines, but using this software on high-memory and high-CPU clusters will be simpler since those parameters will be less likely to affect performance. Several alignment programs are bundled with the download, including Clustal and MUSCLE. If an alignment is already prepared, SATé will use Randomized a(x)-elerated Maximum Likelihood (RaxML) (Satmatakis, 2006) to infer an initial tree for phylogeny reconstruction.

From the main SATé window, select the desired analysis criteria in the following sections:

7.6.2.1. External tools

SATé breaks the tree topology down into subproblems during each round of analysis and realigns the data for each subset, merges the alignments into a full alignment and re-estimates the tree for full alignment.

1. 'Aligner' is used to select the multiple sequence alignment tool to produce the initial full alignment.
2. 'Merger' is used to select the multiple sequence alignment tool to merge the alignments of subproblems into a bigger and final multiple sequence alignment.
3. 'Tree estimator' uses RAxML for tree estimation with the chosen evolutionary 'Model'.

7.6.2.2. Sequence import and tree building

1. Click 'Sequence file…' to upload your sequence alignment file in FASTA format (Note: the file MUST have the extension *.fas or *.fasta to be read by SATé; see section 7.3.).
2. Select the appropriate 'Data Type' (nucleotide or amino acid).
3. If you have previously generated a 'Tree file', you can upload it as the initial guide for SATé, if appropriate.

7.6.2.3. Job Settings

1. Specify the 'Job Name' for identifying output files created by SATé.
2. Select the folder/directory for storing the created outfile files using 'Output Dir.'

7.6.2.4. SATé Settings

This section allows users to control the details of the algorithm. In each iteration, the dataset will be breaking down into non-overlapping sequence subproblems and these subproblems are given to the chosen alignment tool.

1. There are options under 'Quick Set' to allow a more or less intensive search during the SATé iterative process.
2. 'Max. Subproblem' is used to control the largest dataset that are aligned during the iterative process.
3. Use the 'Fraction' option to express the maximum problem size as a percentage of the total number of taxa in the full dataset.

This value will be limited by available computational power.

4. Use the 'Size' option for size cutoff in absolute number of sequences.
5. Select 'Decomposition' to choose how the process should be broken to create subproblems.
6. 'Apply Stop Rule' is used to control how SATé should be finished.

The decision to stop can be done based on number of iterations (one may be sufficient), the amount of time in hours or 'Blind Mode Enabled' meaning that SATé will terminate if it ever completes one iteration without improving the ML score.

7. Click 'Start' to run the SATé analysis.

There will be five files created in the selected directory after SATé is completed. An alignment file (*.aln), tree file (*.tre), best ML score file (*.score), error file (*.err) and history file (*.out). Unlike MEGA, SATé does not have bundled tree viewing or alignment viewing programs, so the user will need to open the tree file using one of the tree viewing programs described above and the alignment file using Clustal or a similar alignment viewing program. The other files can be opened with a text editor (i.e. Notepad, TextEdit). In addition, SATé does not utilize bootstrap testing to support inferred tree topology. Rather, a similarity score from 0-1 (0 is most similar and 1 is least similar) is placed on the branches to aid in topology interpretation. Hence, other reconstruction methods should be compared to confirm the output.

7.6.3. Building trees using distance and character based methods

To assess the reliability of the tree topology, users should be aware of the phylogenetic tree construction and tree analysis methods according to the data and algorithmic strategy used. Each method has different assumptions that may or may not be valid for the evolutionary process of the given sequence data. For example, the distance based method UPGMA (Unweighted Pair Group Method with Arithmetic mean) assumes a neutral mutation rate proportional to time (a molecular clock). Therefore, it is important to be aware of this

fact when evaluating tree topology generated by each method. It is encouraged that one run a variety of distance based methods (Neighbour-Joining, UPGMA, Minimum Evolution) that calculate evolutionary distance between sequences, and character based methods (Maximum Parsimony, Maximum Likelihood, Bayesian) that determine the most probable evolutionary event history between sequences (Table 1).

Statistical testing of topology should also be performed where possible, i.e. bootstrapping analysis. Altering the substitution model, rates and patterns, and treatment of gaps/missing data may also be warranted, though the varying justifications for each of these tests is beyond the detail provided here. Low branch support for any topology shown in the final tree or conflicts in topology determined by multiple testing should be addressed when presenting any phylogenetic data.

Table 1. Classification of phylogenetic analysis methods and strategies.

Tree building strategy	**Method**	
	Distance based	Character based
Clustering algorithm	UPGMA Neighbour Joining	n/a
Optimality criterion	Minimum Evolution	Maximum Parsimony Maximum Likelihood Bayesian Analysis

8. Genomic resources and tools

8.1. Introduction

Genomic analyses take several forms. At one level, any study that draws inferences for protein-coding genes or other genetic traits in the context of their neighbors on chromosomes is 'genomic'. More recently, genomic studies are those that use massive DNA sequencing strategies to describe and piece together entire sections of the targeted genome, without using PCR or other selective techniques to target specific short regions. Ultimately, genomic studies hope to assemble chromosome-length stretches of an organism's genetic blueprint, and then annotate or describe the functionality of specific regions within chromosomes. The field of genomics is driven by technological advances, including huge cost reductions for the sequencing of samples and advances in both statistical methods and computational resources for analysing the obligatory large datasets. Current estimates indicate that entire pipelines (sets of routines needed for an output analysis) are viable for only six months before becoming obsolete. One helpful review of modern techniques is given by Desai *et al.* (2012) and there are numerous advances and tutorials available via the forums Seqanswers.com and the GALAXY wiki (http://wiki.galaxyproject.org/FrontPage). Researchers are advised to consult these sources while planning genomic, transcriptomic, and metagenomic projects, as the standards and possible analyses are improving constantly.

8.2. Honey bee genome project

After a multi-year international project, the honey bee genome was described in fall, 2006, in a main overview paper (Honey bee Genome Sequencing Consortium, 2006) and > 30 satellite genome-enabled companion papers (primarily in the journals *Insect Molecular Biology* and *Genome Research*). Sequence data, generated using dideoxy sequencing was assembled into ca. 10,000 contigs (blocks of overlapping sequence reads) spanning *ca.* 238 million base pairs. These contigs are in many cases linked together by scaffolding (a strategy whereby long strands of DNA are sequenced from each end and linked via informatics) leading to an assembly that was > 95% complete for the non-repetitive genome. Honey bee genes and various genomic features are predicted based on homology to other organisms, evidence from RNA expression studies, and evidence for open reading frames. The current genome assembly along with a consensus ("GLEAN") gene set and other resources are available at "Beebase" (www.beebase.org/, Christine Elsik, Univ. Missouri) and at the U.S. National Institutes of Health National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genome?term=apis%20mellifera). Both sites allow for downloading sequences as well as searching the genome via the BLAST family of search algorithms, while the Beebase site also provides the chance to 'browse' the genome visually. Efforts are continuing to improve the primary *Apis mellifera* genome data while adding sequence data from different honey bee strains.

8.3. Honey bee parasite and pathogen genomes

Most of the named RNA viruses for honey bees have been sequenced and published. These genomes are relatively small and tend to be placed into the NCBI databases upon publication (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=439488). Genome sequences for several parasites and pathogens with larger genomes (e.g., *Paenibacillus larvae, Ascosphaera apis*, *Nosema ceranae*, and the mite *Varroa destructor*; (Qin *et al.*, 2006; Cornman *et al.*, 2009; Cornman *et al.*, 2010; Chan *et al.*, 2011) are held at the NCBI as well as at Beebase and can be queried there alongside the above honey bee genome data.

8.4. Comparative genomics

Currently, assembled genome sequences exist for over 30 insects and other arthropods, and that number is soon to increase dramatically (http://arthropodgenomes.org/wiki/i5K). Resources that have proven useful for comparing honey bee genes to those found in other insects (e.g., to *Drosophila* and other insects for which gene function is firmly

decided) include the NIH-NCBI and the OrthoDB database run from the University of Geneva (http://cegg.unige.ch/orthodb5). In addition, as each incarnation of the honey bee genome is published, annotations based on presence/absence and functional similarity to other insects is simultaneously added. With projects on four other *Apis* species underway, along with social and solitary apoid bees, strategies for comparative genomics will be in flux for some time. The orthoDB database, along with flybase (www.flybase.org) are good places to start for insights from comparative genomics, and each site has hosts the requisite tools (gene searching/alignment/retrieval) for carrying out comparative analyses.

8.5. Second-generation sequencing

Initial genome projects, from small viruses through the human and honey bee project, all relied on 'Sanger' dideoxy sequencing, a relatively expensive but accurate protocol developed in the 1980's that generates sequences (often drawn from random cloned fragments for the popular 'shotgun' sequence method) of several hundred to 1000 base pairs. Since 2000, there has been a great economization of sequencing, such that current technologies are more than ten-fold less expensive than Sanger sequencing. Nevertheless, Sanger sequencing persists and is often the right strategy when compared to newer technologies (which currently give either quite short or quite inaccurate sequences). Readers should consider ILLUMINA/SOLEXA sequencing (summarized in the methylation section 11. below), 454 pyrosequencing, SOLiD sequencing or the Ion Torrent platform (all platforms are reviewed by Metzger, 2010), and the final decision might rest on local availability along with different strengths of each platform. As of 2012, most DNA and RNA sequencing efforts include at least some component of ILLUMINA sequencing, as that technology is viewed as being most cost-effective.

8.6. Genomic sequence assembly

Standards and tools for genome sequence assembly and analysis are constantly improving and the best strategy for carrying out a genome project is often through collaboration or through mimicking the protocols and computational strategies used by a recent genome project of similar scope (genome size, budget for sequencing and informatics, etc.). Accordingly, we will not list specific pipelines for these processes, but can direct researchers to sites such as http://www.broadinstitute.org/scientific-community/science/programs/genome-sequencing-and-analysis/computational-rd/computational-, http://bioinformatics.igm.jhmi.edu/salzberg/Salzberg/Software.html, and http://soap.genomics.org.cn/soapdenovo.html. Of these options, whole-genome assemblies using the ALLPATHS-LG method (the first link above for the Broad Institute) have been highly successful for both microbes and higher organisms, and this method is arguably the tool of choice currently.

8.7. Transcriptomic analyses ("RNASeq")

Transcriptomic analyses are helpful for seeing trends in honey bee gene expression as well as changes due to experimental conditions, and bee researchers have carried out such studies for many years, adapting as new methods arise. Two recent papers have used the ILLUMINA platform for studying gene regulation in response to nutrition (Alaux *et al.*, 2011) and responsiveness to varroa mites and viruses (Nazzi *et al.*, 2012), respectively. Analyzing RNASeq data will depend on the sequencing platform as well as developments in software and public or personal computational resources, all of which are under constant renewal. Generally, RNASeq experiments rely on differential gene expression (DGE) between categories of one factor (e.g., bees exposed to mites versus controls) and the statistical analysis identifies which regions are up- or down-regulated in the context of this factor. Nazzi *et al.*, 2012 used a technique prescribed by Mortazavi *et al.*, (2008) that, like all current methods, first develops a model for how often a particular expressed region *should* be seen in a sequencing effort, and then uses the number of times that sequence *was* sampled to determine whether it was up-or down regulated compared to an expected level. There are now numerous such methods and both methods and strategies to trim the computational resources for their use are being improved monthly. Public platform with video tutorials for RNASeq analysis that promises to remain current is described at the Galaxy site (https://main.g2.bx.psu.edu/).

8.8. Metagenomics

Metagenomic approaches began as an attempt to study the functional significance of all organisms in a habitat, and the term was first used to describe soil microbes and their collective proteins (Handelsman *et al.*, 1998). In honey bees, usage has so far focused on identifying pathogen taxa (Cox-Foster *et al.*, 2007; Runckel *et al.*, 2011, Cornman *et al.*, 2012) and, more recently, on targeted surveys of bacterial associates in honey bee guts (Martinson *et al.*, 2011; Mattila *et al.*, 2012). There are six key decision points in metagenomic surveys and, rather than choose any specific recipe, we will instead list those decisions and their outcomes:

8.8.1. RNA versus DNA sampling

RNA pools contain those genomes that are actively producing proteins AND genomes of the key RNA viruses in bees. RNA sampling is often used for assessing bee pathogens. DNA sampling is preferable when samples are poorly preserved (given the higher stability of DNA) and as a means of reducing the often overwhelmingly high frequency of ribosomal RNA's (75-80%) in most sequenced RNA samples.

8.8.2. Sample preparation

Both RNA and DNA destined for metagenomic surveys can be extracted using the means described in sections 3 and 4 (e.g. Trizol® extractions for RNA and CTAB extractions for DNA). For more recalcitrant samples (e.g. spore stages, or samples of organisms with impermeable coats, common to bacterial species and fungi) it is important to use mechanical or enzymatic rupturing of the cell coat via proteinase K, as described in the above CTAB DNA extraction protocol (section 3.2.1.), or prolonged shaking with a suspension of low-affinity silica particles or other inert solids.

8.8.3. Amplicon-based or shotgun sequencing

Given the low costs of sequencing, it is feasible now to simply survey all nucleic acids in a sample and then assign them to taxa in various kingdoms via searches of local or online databases. Nevertheless, targeted deep sequencing of specific taxonomic groups can benefit from a selection of specific regions via PCR-based amplification prior to generating the sequencing libraries. This has been done most frequently with the 454 sequencing platform since relatively long read lengths on this platform (> 400 bp) enable the capture of sequence data for a substantial section of the targeted species. Several studies have now used amplicon-based sequencing to describe bacterial populations carried by honey bees. As with any PCR protocol, this approach will under sample taxa with mismatches to the initial primer sequences since no PCR primers are truly 'universal' to a targeted group. Nevertheless, there are many examples of primers that amplify broadly across all of the major bacterial taxonomic groups, and amplicon-based 454 sequencing has appears to provide a consistent and accurate view of bacterial communities in bees. The software environment Qiime (http://qiime.org/) is widely used to match amplicon-based sequences to microbial databases in order to identify and quantify taxa.

8.8.4. Assembly of shotgun sequences vs. read mapping

For sequences generated by shotgun sequencing, it is generally desirable to assemble all sequencing reads into contigs (aggregates of nearly identical sequences from the same region and species) prior to statistical analysis, since this can reduce computational needs greatly while retaining vital statistics including the number of reads per contig. Once the computationally intensive assembly of contigs has taken place (using for example the Metavelvet routine, http://metavelvet.dna.bio.keio.ac.jp/) datasets can be reduced by many orders of magnitude. This is critical if online or 'cloud' databases are searched for microbial matches since the data transfer speeds alone for such searches can be measured in days when using raw sequence reads. In addition, contigs are by definition longer than any individual read and therefore also can provide a more secure match to distant taxa. The count data for sequenced reads per contig provides the measure of depth that, once scaled to contig length, allows estimates of microbial frequency. Once metagenomic sequences have been assembled, moderate experiments can often be enacted without cost to the user at public resources such as GALAXY (https://main.g2.bx.psu.edu/). As with any complicated statistical procedure it is highly possible to get erroneous matches and statistical results, and researchers are advised to enlist the help of colleagues with current expertise here.

In practice, metagenomic analyses are also carried out by mapping (aligning with high probability) individual sequence reads to members of a reference database, and algorithms (including Tophat, http://genomics.jhu.edu/software.html) have been developed that are extremely efficient at doing so. For diagnostic regions with highly conserved sequences (e.g., parts of the rRNA operons) both assembling and mapping are problematic and query sequences often cannot be placed securely to even family-level matches. In this case, it is best to bin sequences at a higher taxonomic level (even Order) rather than force matches into a possibly erroneous taxon. Nevertheless, as genome sequencing of microbial species is increasing exponentially, even rare and distant taxa tend to have a fully sequenced family member in the public databases, as described below in section 8.8.5.

8.8.5. Databases for metagenomics

Several sites have emerged for mapping metagenomic sequence reads and amplicons, including the longstanding Ribosomal Database Project for bacterial and archael 16S alignments (http://rdp.cme.msu.edu/), the SILVA databases for rRNA's generally (http://www.arb-silva.de/), and MEGAN (ab.inf.uni-tuebingen.de/software/megan/), which aspires to map to targets across the tree of life. Each site allows for limited web searching, and for downloading relevant databases for more efficient local searches.

8.8.6. Post-assignment statistics

Quantifying differences between two or more samples in the taxa to which reads or contigs map is the ultimate goal for many metagenomic experiments. While not the only option, MG-RAST (metagenomics.anl.gov/) provides an example of statistical comparisons using read mapping. Assuming read accounts are normalized by size of their target (various methods have been used for this), and then the count frequencies themselves can be used with a variety of standard statistics. Similarly, Qiime, mentioned above in section 8.8.3, provides an effective way for mapping reads to microbial taxa.

9. Fluorescence *In Situ* Hybridization (FISH) analysis of tissues and cultured cells

9.1. Introduction

Fluorescence *in situ* Hybridization (FISH) is a sensitive and specific method for localizing expressed genes or microbes within tissues of the honey bee. In general, a probe matching a specific DNA or RNA sequence is exposed to prepared tissues. This probe can then be localized using fluorescent tags, pointing the researcher to the precise location of a desired target. To date this method has been used successfully to show the locations of bacterial associates of bees (Martinson *et al.*, 2012, Yue *et al.*, 2008, and below).

9.2. Tissue fixation and tissue sectioning exemplified with gut tissue

1. Immobilize about 20 bees with CO_2.
2. Cut of the head.
3. Fix the abdomen on a separation plate with micro pins.
4. Remove carefully the alimentary tract of each bee with forceps.
5. Transfer the hindgut and the midgut into one well of a 24-well microtiter plate.
6. Fix tissues in 4% formalin (Roth) for 24 hours at 4°C by shaking.

The further embedding and blocking procedure using e.g. Technovit 8100 and Technovit 3040 kits (Heraeus-Kulzer) should be performed as given in the manufacturer´s protocols (Heraeus-Kulzer, T8100 embedding kit).

7. Wash the alimentary tracts with 6.8% sucrose in 1xphosphate buffered saline (1xPBS, pH 7.0) for 24 hours at 4°C.
8. For dehydration transfer the tissue in 100% acetone for one hour.
9. Pre-infiltrate the organs with T8100 basic-solution and 100% acetone (mixed 1:1) for two hours.
10. Prepare the infiltration solution (0.6 g hardener I in 100 ml T8100 basic solution).
11. Transfer the organs into the infiltration solution.
12. Incubate at 4°C for at least 24 hours and up to one week, depending on tissue size.
13. Apply careful shaking for better infiltration results.
14. Prepare the embedding solution (mix 0.5 ml hardener II with 15 ml infiltrating solution).
15. Fill the mould of a Teflon-embedding form (pre-cooled at -20°C) with the embedding solution.
16. Transfer the tissue and orientate it in the mould.
17. Close the well immediately with a plastic strip.
18. Incubate at 4°C for 3 hours to allow polymerization.
19. Finally, block the polymerized probes with histoblocs and with the Technovit 3040 kit (both from Heraeus-Kulzer) using the manufacturer´s protocol.
20. Prepare semi thin-sections (2-4 µm) with a rotation microtome (Leica).

Use a knife with a hard metal edge (Tungsten).

21. For fluorescence *in situ*-hybridization transfer the tissue sections on Polysine™-covered glass slides (Fisher Scientific, Menzel-Gläser).

9.3. Fixation of cultured cells grown in suspension

1. Transfer 100 µl of cultured cells into a cell funnel-chamber of a cell spin (Tharmac).
2. Centrifuge the cells with 600 rpm (54xg) gently for 5 minutes on a glass slide (VWR).
3. Remove the cell funnel-chamber.
4. Let the medium air dry.
5. Fix the cells in 4% formalin (Roth) at 4°C for 24 hours.

9.4. FISH-analysis of tissue sections and fixed insect cells

1. Wash the slides (tissue sections and fixed insect cells) twice in 1xPBS.
2. For further processing, transfer each slide into a 10 ml dish.
3. Add 10 ml of 1 µg ml^{-1} Proteinase K in Proteinase K-buffer (0.2 M Tris-HCl, pH 7.5).
4. Transfer the dish into a humid chamber (Eppendorf, Thermostat Plus) for 5 min at 37°C.
5. Remove the Prot K and wash each slide with 10 ml of 1xPBS.
6. For post-fixation add 10 ml of 4% formalin (Roth).
7. Incubate at RT for 20 min.
8. Aspirate the formalin.
9. Remove remaining solution by washing the slides three times with 1xPBS-buffer.
10. Prepare hybridization buffer:
    - 200 µl of 100% formamide,
    - 180 µl 5 M NaCl,
    - 20 µl 1 M Tris/HCl,
    - 1 µl 10% SDS,
    - 599 µl DEPC-H_2O, pre-warm to 46°C in a heating block.
11. Add the probes to 37.5 µl pre-warmed (46°C) hybridization buffer:

    7.5 µl species-specific probe annealing to a region of the 16S rRNA or another species-specific genomic region of the pathogen to be detected labelled with fluorescein isothiocyanate-FITC with a final concentration of 15 ng $µl^{-1}$.

Sequence of *Nosema* spp.-probes: Gisder *et al.* (2011); sequence of DWV-probe: Möckel *et al.* (2011); sequence of *P. larvae*-probe: Yue *et al.* (2008)

5 µl Euk516-probe (5'-ACCAGACTTGCCCTCC-3', universally detecting eukaryotic ribosomes by hybridizing to a universal conserved sequence of the eukaryotic 18S rRNA) labelled with sulforhodamine 101 acid chloride-Texas Red® with a final concentration of 10 ng μl^{-1}.

12. Continue incubation in the heating block which is now covered with a lid (i.e. incubation at 46°C in the dark).
13. Cover the slides with LifterSlips (VWR).
14. Pipette 50 µl of hybridization buffer to each slide (tissue sections or fixed cells).
15. Transfer the slide into a hybridization chamber (Corning, Corning chamber), drop 15 µl H_2O into the given wells to preserve the humidity.
16. Close the chamber tightly.
17. Put the corning chamber in a 46°C water bath for overnight hybridization.
18. Open the hybridization chamber and remove the cover slips in 1xPBS.
19. Wash the slides three times with 1xPBS.
20. Let them air dry.
21. Stain the nuclei with 50 µl 4', 6-Diamidin-2-phenylindol- (DAPI, 1 µg ml^{-1} in 99% methanol) solution for 10 min in the dark.
22. Wash the slides again three times with 1xPBS.
23. Let them air dry.
24. Cover the slides with the ProLong Gold antifade reagent (Invitrogen) and a cover slip (Roth).
25. Analyse the tissue sections and the cells under an inverse fluorescence microscope (e.g. Nikon, Ti-Eclipse) at 100-fold and 600-fold magnification using consecutively a FITC-, TexasRed- and DAPI-filter.

10. RNA interference

10.1. Introduction

RNA interference (RNAi) is a cellular mechanism leading to a knock-down of gene expression mediated by target specific double-stranded RNA (dsRNA) molecules (Fire *et al.*, 1998). Understanding the mechanism of mRNA destruction by these dsRNA molecules dramatically increased the possibilities of functional genomics studies during the last decade especially in organisms where the recovery of mutants is not feasible. Thus RNAi has become a dominant reverse genetic method for the study of gene functions and furthermore, plays an increasing role in therapeutics and in pest control (Maori *et al.*, 2009; Liu *et al.*, 2010; Hunter *et al.*, 2010).

Up to now a dozen studies report on the successful usage of RNAi in honey bees. But the application methods and also the choice of RNAi effective molecules are very diverse. Several studies report on the application of dsRNA to eggs and larvae whether by injection (Aronstein and Salivar, 2005; Beye *et al.*, 2002; Maleszka *et al.*, 2007) or ingestion (Aronstein *et al.*, 2006; Patel *et al.*, 2007; Kucharski *et al.*, 2008; Nunes and Simoes, 2009; Liu *et al.*, 2010). Others report on a successful manipulation of adult bees (Amdam *et al.*, 2003; Farooqui *et al.*, 2004; Seehuus *et al.*, 2006; Schlüns and Crozier, 2007; Maori *et al.* 2009; Paldi *et al.*, 2010; Mustard *et al.*, 2010; Jarosch and Moritz, 2011; Jarosch *et al.*, 2011).

This section aims at a collection of RNAi protocols successfully applied in honey bees beforehand. The well-established protocols for producing dsRNA as well as siRNA (short interfering RNAs, the products of dsRNA once the enzyme Dicer and its partners have processed them) molecules are presented. Moreover, the two application methods feeding and injection are presented and compared to each other. In conclusion, we summarize five important factors that may decrease the effectiveness of target gene expression knock-down.

10.2. Production of RNA interfering molecules

10.2.1. siRNA design and synthesis

So far most bee scientists have used dsRNA rather than siRNA for RNAi experiments. Although dsRNA molecules have advantages in handling, off-target effects (Jarosch *et al.*, 2012) have been reported in honey bees. Therefore the usage of siRNAs is recommended where feasible. This allows the selection of one or a few short sequences to initiate RNAi, rather than the many tens of possible permutations generated by a typical dsRNA construct, any of which might cause effects away from the desired target.

1. Design 3-6 siRNAs for your target gene in order to find an optimal siRNA.

General guidelines for siRNA design:

- siRNA targeted sequence is usually 21 nt in length.
- Avoid regions within 50-100 bp of the start codon and the termination codon.
- Avoid intron regions.
- Avoid stretches of 4 or more bases such as AAAA, CCCC.
- Avoid regions with GC content < 30% or > 60%.
- Avoid repeats and low complex sequence.
- Avoid single nucleotide polymorphism (SNP) sites.
- Perform BLAST homology search to avoid off-target effects on other genes or sequences (16- to 18-nt–long stretches of homology are suggested as the maximum acceptable length in RNAi studies per Ambion siRNA design guidelines).

- Design negative controls by scrambling the target siRNA sequence. This control RNA has the same length and nucleotide composition as the target specific siRNA but in a different order. Make sure that the scrambled siRNA does not show homologies for any known bee gene.

Several web based programs for appropriate siRNA design, which implement the actual siRNA design algorithms, are available (e.g. siRNA target designer version 1.6 (Promega); siDesign center (Dharmacon, Inc); Block-iT™ RNAi Designer (Invitrogen).

2. Use T7 Ribomax™ Express RNAi System (Promega) for siRNA production.
    1. Follow the manufacturers´ instructions.
    2. Incubation time may be increased in order to increase the siRNA yield (A time-course experiment has to be performed beforehand in order to find the optimal incubation time).
3. Assess the quality and quantity by photometric measurements (OD260) and by capillary gel electrophoresis (alternatively agarose gel electrophoresis, see section 3.2.1).

10.2.2. Production of dsRNA

Since dsRNAs can cause off-target effects, you need to be careful in designing them. Nevertheless, RNAi efforts using dsRNA constructs have proven effective in honey bees. To avoid targets that might interfere with other honey bee genes, you need to compare your sequence with the honey bee genome during the design process using the Basic Local Alignment Tool (www.ncbi.nlm.nih.gov). Make sure none of the designed dsRNAs has 20-bp segments identical to any known bee sequence. As dsRNAs are processed by the dicer complex into a cocktail of siRNAs 19–21 nt in length, the absence of 20-nt stretches of homology minimizes the possibility of off-target effects.

1. Use the E-RNAi web application (Horn and Boutros, 2010) for optimal dsRNA design.

Design of dsRNA sequences has to be stringent in order to avoid/ minimize off-target effects.

2. Set up appropriate negative controls.

Note: be careful using GFP; this sequence might cause off-target effects in some cases (GenBank ID: U17997, Clontech; Jarosch and Moritz, 2012).

Other possible negative controls: e.g., Q-marker (Beye *et al.*, 2002).

3. Amplify the chosen target fragment by using target specific T7 (TAA TAC GAC TCA CTA TAG GGC GAT) added primer in optimized PCRs using approximately 100-ng genomic DNA obtained by chloroform– phenol extraction (e.g. Maniatis *et al.*, 1982).
4. Clone the amplified fragments into pGem-T easy vectors (Promega) according to the manufacturer's instructions. (Cloning eliminates the possibility of a dsRNA mixture due to a polymorphism of the PCR product).
5. Transform your plasmids into JM109 competent cells (Promega) following the instructions from the manufacturer.
6. Prepare the plasmids according to Del Sal (1988).
7. Analyse the identity of the cloned sequence by Sanger sequencing.
8. Once the right clone has been identified its insert needs to be amplified to serve as a template for dsRNA production by standard PCR using again T7 tailed primers.

    8.1. E.g. use Biotherm DNA Polymerase (Genecraft); chemicals:
    - 0.2 mM dNTPs,
    - 0.3 µM of T7-promotor added primer,
    - 5 U Taq Polymerase,
    - in a total reaction volume of 100 µl.

    8.2. PCR protocol:
    - 5 min DNA denaturation, and Taq activation, at 95°C,
    - 40 cycles of:
      95°C for 30 sec,
      x°C (primer specific annealing temperature) 30 sec,
      72°C for 1 min.
    - A final extension of 20 min at 72°C completes the protocol.
9. Purify the PCR-products with the QIAquick® PCR Purification Kit (Qiagen).
10. Use the T7 RibomaxTM Express RNAi System (Promega) for dsRNA production.

Time course experiments and experiments for optimizing the incubation temperature have to be conducted beforehand (e.g. Jarosch *et al.*, 2011 used an extended transcription time of 5 h at 32°C).

11. Purify the dsRNA by a Trizol® (Invitrogen) - chloroform-treatment following the manufacturers´ instructions.
12. Resolve the pellet in nuclease free water.
13. Assess the dsRNA quality and quantity photometrically and by agarose gels or capillary gel electrophoresis.

The photometric measurement of the OD260/OD280 ratio should be between 1.8 and 2. A lower ratio indicates contamination with proteins. As a contamination with DNA or dsRNA degradation cannot be detected by photometry, visualization of the dsRNA product is necessary. For this 1.5% agarose gels can be used, see section 3.2.1). A single distinct band should be visible.

14. Adjust dsRNA concentrations to 5 µg/µl by diluting with insect ringer (54 mM NaCl; 24 mM KCl; 7 mM $CaCl_2$ x $2H_2O$) right before the injection.

10.3. RNAi Applications

10.3.1. RNAi in adult honey bees via feeding

1. Take newly emerged bees (1-2 d old) from one colony from one brood frame.
2. Set up at least two controls:
    1. Bees fed with 50% sugar water alone.
    2. Bees fed with scrambled siRNA (siRNA with exactly the same nucleotides as the target siRNA but in an altered order lacking any similarity with other known bee genes).
3. Take a mixture of two siRNAs specific for the target gene.

Note: in previous experiments a mixture of two siRNAs was more effective than single siRNAs (Jarosch *et al.*, 2011).

4. Put 35-40 newly emerged bees in wooden cages (see the *BEEBOOK* paper on maintaining adult honey bees in vitro under laboratory conditions (Williams *et al.*, 2013)) supplied with a small comb and pollen *ad libitum*.
5. Put cages in temperature controlled incubators (see the *BEEBOOK* paper on maintaining adult honey bees in vitro under laboratory conditions (Williams *et al.*, 2013)) and feed with 1.5 ml 50% sugar water containing approximately 1 µg siRNA per insect every 24 hours.
6. Dependent on the actual experiments bees can be held for several weeks.
7. Once the experiment is finished, bees should be shock-frozen in liquid nitrogen in order to maintain the RNA status.

10.3.2. RNAi in honey bee larvae via feeding

1. Take a comb with second instar larvae out of the colony.
2. Transfer it to the lab.

The whole treatment is conducted at room temperature.

3. Draw a map of the different treatment groups on the very same comb for future identification of the treated individuals.
4. Apply 1 µl of sugar solution containing the respective amount of dsRNA directly into the cells. Deposit it at the bottom of the worker brood cell that contains a drop of food. Avoid touching the larvae. Successful experiments used dsRNA concentrations between 0.5 µg (Nunes and Simões, 2009) and up to 1.26 µg (Aronstein *et al.*, 2006).

In addition to the first dsRNA feeding you may feed another µg of your dsRNA after 12 hours. This feeding cycle can be repeated for several days (Liu *et al.*, 2010) until the life stage of interest is reached.

5. Place the comb back to its host colony two hours after treatment and take samples at the life stage you are interested in.

10.3.3. Gene knock-down by abdominal injection of target-specific dsRNA/siRNA

RNA interfering molecules injected by intra-abdominal injection do not reach every tissue (Jarosch and Moritz, 2011). But especially the fat body can be easily reached by this user friendly method (Amdam *et al.*, 2003; Jarosch and Moritz, 2011).

1. Take age-defined workers (see the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013)).

Note: newly emerged workers are a little bit more difficult to inject as their abdomen is quite flexible.

2. Immobilise bees by cooling down in at 4°C.
3. Fix the bees on wax plates using small fixing pins.
4. Inject 5 µg of freshly diluted dsRNA or alternatively 3 µg of freshly diluted siRNA (treatment and control dsRNA/siRNA) between the 5th and 6th abdominal segment using a 10 µl microsyringe (e.g. Hamilton).
5. Inject negative controls with insect ringer (54 mM NaCl; 24 mM KCl; 7 mM $CaCl_2$ x $2H_2O$).
6. Keep the injected workers on wax plates until they recover and keep bees not showing haemolymph leakage (visible on their substrate or as a droplet on the cuticle) together with about 25 nurse bees (1-10 days) in cages (see the *BEEBOOK* paper on maintaining adult honey bees in vitro under laboratory conditions (Williams *et al.*, 2013)).
7. Sacrifice the bees by shock-freezing in liquid nitrogen.
8. Store them at -80°C until tissue preparation.
9. Prepare the worker tissues on cooled wax plates using an RNA Stabilization Reagent (e.g. RNAlater®) in order to avoid RNA degradation.

10.4. Concluding remarks

Based on the literature (see Huvenne and Smagghe, 2012 for review) five aspects seem to be most important to conduct successful RNAi knockdown experiments in honey bees.

Concentration of dsRNA: For every target gene the most effective concentration of RNAi molecules has to be determined. It does not follow the general rule: The more the better. Nunes and Simões (2009) for example report on the removal of 2nd instar larvae, which were fed with 3 and 5 µg dsRNA. In contrast, larvae fed with just 0.5 µg dsRNA did not show a significant higher removal rate than the control group, and moreover exhibited an mRNA silencing effect of about 90%.

Nucleotide sequence: Sequences of the RNAi effective molecules have to be carefully designed and tested in order to avoid off-target effects.

Length of the dsRNA fragment: When not using siRNAs the length of the dsRNA fragments may be crucial for uptake and efficient silencing. Most experiments used dsRNA ranging from 300 to 520 bp (see Huvenne and Smagghe, 2012 for review). Moreover, a minimal length of 211 bp is suggested in S2 cells (Saleh *et al.*, 2006).

Honey bee life stage: Although adults are easier to handle, literature of other insects suggest a larger silencing effect in younger life stages. E.g. in fall armyworms (*Spodoptera frugiperda*) the silencing effects after RNAi treatment were reported to be less effective than in *S. frugiperda* larvae (Griebler *et al.*, 2006). Thus the usage of larvae rather than adults where feasible may be advisable in honey bees as well.

Application method: The two application methods presented here both have pros and cons. The feeding regimes lead to an individually different consumption of food and therefore to the ingestion of different dosages of dsRNA. But in contrast to injections protocols, feeding is much easier in handling and moreover, it causes less stress in the target animals. Moreover, studies suggest, that the composition of the target tissue may have some influence on the accessibility of dsRNA when choosing injection as application method (Jarosch and Moritz, 2011).

11. DNA methylation in honey bees

11.1. Introduction

Methylation of chromosomal DNA is a flexible epigenetic mechanism that plays a critical role in gene regulation, and patterns of methylation across the genome are often surrogates for interesting sets of proteins that are regulated in concert with each other and with biological traits. In order to detect methylated bases in genomic DNA (essentially only cytosines are methylated), DNA has to be treated with bisulfite to convert non-methylated cytosines to uracil and subsequently to thymine during the PCR amplification step.

11.2. DNA methylation in honey bees

So far, four full methylomes (genome-wide methylation patterns) have been generated for *Apis mellifera* using the following tissues: adult brains of queens and workers and 96 hrs-old queen and worker larval heads (Lyko *et al.*, 2010; Foret *et al.*, 2012). The below protocol describes methylation analyses of DNAs extracted from the dissected brains of 50 age matched active queens and 50 8-day old workers (dissection of clean, gland-free brains is shown at: http://dl.dropbox.com/u/59152790/Brain%20dissection%20Maleszka%20lab.wmv).

A similar protocol was used to generate larval methylomes (Foret *et al.*, 2012) and in principle this procedure should work for any bee tissue and/or life stage from which intact RNA's can be extracted as below (section 11.3.).

11.3. DNA extraction from various tissues for methylation analysis

Methylation analyses do not depend on a particular DNA extraction method. Nevertheless, the below extraction has been validated in a variety of honey bee tissues.

1. Homogenize tissues in a 1.5 ml microcentrifuge tube in a small volume of NTE buffer:
   - 100 mM NaCl,
   - 50 mM Tris pH 8.2,
   - 10 mM EDTA,
   - 1% SDS,
   - Proteinase K (500 µg per ml, freshly dissolved).
2. Add a small amount (0.01%) of a non-ionic detergent such as Triton X100.

The detergent is beneficial (increases the efficiency of proteinase K digestion), but not necessary.

3. Add more buffer (roughly 500 µl per 20-50 mg of tissue).
4. Incubate at 55°C for 1-3 hrs.
5. Extract with 1 volume of phenol: chloroform.
6. Spin for 10 min at 10,000rpm.
7. Collect the upper phase (repeat the extraction if the upper phase looks cloudy).
8. Add 1 µl of RNase A (10 mg/ml).
9. Incubate for 10 min at 37^0C to digest RNA.

This step is not necessary for bisulfite conversion, but the presence of RNA interferes with measuring DNA yield.

10. Precipitate DNA with 1 volume of isopropanol or 2 volumes of EtOH.
11. Spin gently (5,000 rpm for 2 min).
12. Discard the supernatant.
13. Wash the pellet once with 70% EtOH.
14. Remove ethanol, but DO NOT DRY THE PELLET!
15. Dissolve the pellet in TE buffer by heating to 65°C.
16. Store at 4°C (or at -80°C for long term storage).

Clean DNA preps are stable at 4°C for at least 5 years. The majority of DNA strands from the above prep are 200-250 kb in length with the smallest molecules around 70 kb.

Note: DNA preps from larvae might appear milky after this procedure, but such preps are suitable for bisulfite conversions. Alternatively use the MasterPure DNA Purification kit from AMRESCO (Cat. No. MCD85201) yields cleaner larval preps.

11.4. High-throughput sequencing of targeted regions

11.4.1. Fragmentation of DNA

1. Fragment 5µg of high molecular weight DNA using the Covaris S2 AFA System in a total volume of 100µl.
   Fragmentation-run parameters:
   - Duty cycle 10%,
   - Intensity: 5,
   - Cycles/burst: 200,
   - Time: 3min,
   - Number of cycles: 3,
   - This results in a total fragmentation-time of 180s.
2. Confirm fragmentation with a 2100 Bioanalyzer (Agilent Technologies) using a DNA1000 chip, aiming for fragment sizes of 140 bp on average for both queen and worker DNAs.

11.4.2. End-repair of sheared DNA

Having a blunt (neither strand longer than the other) end to each double-stranded DNA section is needed to attach the below adaptors ('handles' that help connect DNA during library formation), and this is achieved as follows:

1. Concentrate fragmented DNA to a final volume of 75 µl using a DNA Speed Vac.
2. End-repair fragmented DNA in a total volume of 100 µl using the Paired End DNA Sample Prep Kit (Illumina, PE-102-1001) following manufacturer's protocol.

11.4.3. Adaptor ligation

Ligate adaptors using the Illumina Early Access Methylation Adaptor Oligo Kit (P/N: 1006132) and the Paired End DNA Sample Prep Kit (Illumina, PE-102-1001), as recommended by the manufacturer.

11.4.4. Size selection of adapter-ligated fragments

For the size selection of the adaptor-ligated fragments use the E-Gel Electrophoresis System (Invitrogen) and a Size Select 2% precast agarose gel (Invitrogen) as below.

1. Load each fragmented DNA on two lanes of an E-gel.
2. Electrophorese using the "Size Select" program for 16 min.
3. Using a size standard (50 bp DNA Ladder, Invitrogen, Cat no. 104 16-014), extract 240 bp fragments from the gel.
4. Pool samples and directly transfer to bisulfite treatment without further purification.

11.4.5. Bisulfite conversion and amplification of the final library

1. Bisulfite treatment can be carried out with the EZ-DNA Methylation Kit (Zymo) as recommended by the manufacturer, with the exception of a modified thermal profile for the bisulfite conversion reaction. Alternatively the QIAGEN EpiTect Bisulfite Kit can be used.

   The conversion is carried out in a thermal cycler using the following thermal profile:

   15 cycles of:
   95° C for 15 sec,
   50° C for 1hr,
   Incubate at 4° C for at least 10 min.
2. Amplify the resulting libraries using the Fast Start High Fidelity PCR System (Roche, 03 553 400 001) with buffer 2, the Illumina PE1.1 and PE2.1 amplification primers, and the below protocol.
   PCR thermal profile:
   95°C- 2min,
   11 cycles of:
   95°C for 30 sec,
   65°C for 20 sec,
   72°C for 30 sec,
   72°C for 7 min,
   20°C hold.
3. Purify products on PCR purification columns (MinElute, Qiagen), eluting in 20 µl elution buffer (Qiagen).

11.4.6. Validation of the libraries

1. Analyse 1 µl of the libraries on a 2100 Bioanalyzer (Agilent Technologies) using a DNA1000 chip.
2. Confirm product size of ca. 240 base pairs and adequate quantity using the DNA1000 size standards.

11.4.7. Sequencing and data analysis

1. Use a Solexa Genoma Analyzer GAIIx with a v2 Paired End Cluster Generation Kit - GA II (Illumina, PE-203-2001) and v3 36 bp Cycle Sequencing Kits (Illumina, FC-104-3002) following manufacturer's protocols, for sequencing.
2. Extract sequences using Illumina Pipeline v1.4 software.
3. Perform image analysis and base calling using Illumina SCS v2.5 software.

8 pM of material is used per sequence lane, generating between 10 and 16M sequence reads.

11.5. Mapping and methylation assessment

1. Trim the above sequence data using the Illumina Data Analysis Pipeline.
2. Align bisulfite-converted sequencing reads to the honey bee genome using the BSSeeker software (http://pellegrini.mcdb.ucla.edu/BS_Seeker/BS_Seeker.html) as described in Foret *et al.* (2012); http://www.pnas.org/content/suppl/2012/03/12/1202392109.DCSupplemental/pnas.201202392SI.pdf#nameddest=STXT.

3. Reads containing consecutive CHN nucleotides are the product of incomplete bisulfite conversion and must first be discarded.
4. To increase the accuracy of methylation calls, only those cytosines fulfilling neighbourhood quality standards are counted (bases of quality 20 or more, flanked by at least three perfectly matching bases with a PHRAP quality score of 15 or more).
5. The methylation status of each cytosine base can be modelled by a binomial distribution with the number of trial equal to the number of mapping reads and the probability equal to the conversion rate.
6. A base is called methylated if the number of reads supporting a methylated status departed from this null model significantly at the 5% level after correcting for multiple testing.
7. Differentially methylated genes are identified using generalized linear models of the binomial family; the response vector *CpGmeth* (number of methylated and non-methylated reads for each CpG in a gene) was modelled as a function of two discrete categorical variables, the caste and the CpG position: CpGmeth = caste * CpGi.
8. P-values are corrected for multiple testing using the Benjamini and Hochberg method. These tests are carried out using the R statistical environment (http://www.r-project.org).
9. Honey bee ESTs and predicted genes are loaded into a Mysql database and visualized with Gbrowse, where CpG methylation levels in queens and workers are added as separate tracks.

11.6. Methylation dynamics and expression of individual genes

Targeted analyses of selected genes can be conducted using 454 sequencing of amplified gene fragments from bisulfite-converted DNAs. For a small-scale testing the amplicons can be cloned into a plasmid, cloned and sequenced using via Sanger dideoxy sequencing (Foret *et al.*, 2009). Both approaches can be used to validate genome -wide methylation data, but 454 sequencing allows for a much higher coverage, as shown in section 8.5.

11.6.1. Amplicon sequence selection

1. Illumina sequencing and BSMAP mapping results can be confirmed by 454 sequencing of a set of bisulfite amplicons.
2. Specific amplicon sequences are selected using raw methylome data and the following arbitrary criteria:
    1. Minimum coverage - 5 mapped reads for each queen and worker sample.
    2. Minimum 2 methylated CpGs within a region of ~400-600 bp of sequence showing at least 50% difference in methylation levels between the two samples. This selection is very stringent, but assures that amplicons with high probability of differential methylation are selected.
    3. In addition a few regions of mtDNA that is not methylated are selected as controls (optional).

11.6.2. Bisulfite DNA conversion

1. The Qiagen EpiTect Bisulfite Kit and the manufacture's protocol is widely accepted as the most efficient and reliable kit for DNA conversion. The amount of starting materials can range from 0.1 to 2 µg.
2. Because DNA conversion with bisulfite is only ~98% efficient it is highly recommendable to repeat this protocol twice.
3. $1/10^{th}$ of the second conversion reaction is sufficient for subsequent amplification.

11.6.3. Bisulfite PCR

Bisulfite amplicons are amplified using a nested PCR protocol (Wang *et al.*, 2006; Foret *et al.*, 2009). Nested primers contained an additional 9 nucleotide-long linkers with EcoRI or HindIII recognition sequences allowing directional cloning of the amplicons.
PCR reactions are performed in 25 µl volume containing:

- 1x PCR buffer,
- mM $MgCl_2$,
- mM dNTP,
- 50 pmol each forward and reverse primer,
- 5 units Taq polymerase.

Reaction efficiencies are optimized via annealing temperature gradients (Mastercycler gradient PCR machine, Eppendorf) and testing multiple Taq polymerases such as GoTaq (Promega) or FastStart Taq (Roche).
Cycling profile is as follows:

- Initial denaturation at 94^0C for 2 min,
- Followed by 40 cycles of:
  15 sec denaturation at 94^0C,
  15 sec annealing at primer-specific optimal temperature,
  60 sec extension at 72^0C,
- A final extension cycle at 72^0C for 5 min.

When using FastStart Taq polymerase, the denaturation temperature was increased to 95^0C, initial denaturation time to 5 min and cycling denaturation and annealing times to 30 sec.

11.7. RNA extraction

1. RNA for analysis can be extracted using the TRIzol®/QIAGEN RNeasy combination method followed by the QIAGEN Mini or Rneasy Minelute Cleanup kit, or by the detailed protocol in the RNA methods section 4.3. above.
2. RNA concentrations are then evaluated via Nanodrop (section 3.2.1) analyses and integrity assessed by gel electrophoresis (see section 4.4.).

11.8. cDNA synthesis and template quantification

1. Typical first-strand reactions consist of a 20µ volume containing:
   - 0.5-2 mg of total RNA, or 50-100 ng of poly(A)RNA,
   - 100 pmol of anchored d(T)20VN primer,
   - 200 units of Superscript III (Invitrogen),
   - 1X concentration of proscribed buffer.
2. The tube is incubated for 1 h at 50°C.
3. Terminate by adding 30 µl of TE buffer and freezing.
4. Resulting cDNAs can be screened for levels of specific genes via quantitative-PCR as described in the RNA methods in section 4.7.

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