CHAPTER 3
GENERAL MATERIALS AND METHODS

In this thesis, two experimental techniques were used. First, ovary dissection was carried out to reveal the degree of ovary activation. This technique was explained in Section 4.1.2. Second, molecular marker technique, microsatellite analysis, was performed to determine paternal and maternal origin of drones and workers (Section 4.1.1 and Section 5.1).

3.1 Genetic Techniques

Microsatellite analysis was used in this thesis to determine the parentage of drones and workers of *A. dorsata*. The procedures using in two investigations were described.

3.1.1 DNA Extraction

Pupae were removed from cells and kept individually in an Eppendorf tube. Using sterile technique, one hind leg or, if missing, one middle leg and one antenna which was equal amount of tissue was removed from each bee and put in a sterilised Eppendorf tube. The tissues were then chopped or squeezed into tiny pieces. The genomic DNA was extracted by adding approximately 1 ml of boiling 5 % Chelex solution (Appendix-1) (Bio Rad 1991) into the tube. Tubes were then placed in a boiling water bath for 15 minutes to allow for extraction (Walsh, Metzger and Higuchi, 1991). Chelexed samples were spun in a microcentrifuge at 13,000 rpm for 7 minutes. Only the upper aqueous part of the solution was used as DNA template. Ten

microlitre of extracted DNA was taken and was diluted with 60 mirolitre of sterilised milliQ-H_2O (1:6 dilution). Diluted DNA templates were stored at 4 ^{o}C for further use in PCR reactions.

3.1.2. Amplification of Microsatellite Loci Using Polymerase Chain Reaction (PCR)

The basic components of PCR reaction are one or more molecules of target DNA, oligonucleotide primers, themostable DNA polymerase, and dNTPs. This reaction mix is repeatedly heated and cooled to the temperature optimums required for template denaturation (95^{o}C), primer annealing (55-65^{o}C), and DNA polymerisation (72^{o}C) (Miesfeld, 1999) (Figure 3.1). The source of DNA template can be either purified DNA or relatively crude nucleic acid. In these experiments DNA from honey bees extracted by Chelexed resin were used.

Four polymorphic microsatellite primers specific to *A.mellifera*, A14, A24, A88 identified by Estoup et al., 1993; 1995 and Ad3 which specific to *A.dorsata* (Parr et al., submitted) at the Bee Laboratory, the School of Biological Sciences, University of Sydney) were used to determine the maternity and paternity of bees in this thesis. The primer sequences shown in Table 3.1. Reversed primers had been end-labeled with the fluorescent dye HEX. These primers were obtained in pelleted form and were redehydrated in 100 ul of milli-Q H_2O. PCRs were conducted by preparing PCR mixtures as listed in Table 3.2. Diluted DNA template of each bee was then added into the PCR mixture. Mixtures were put in a PCR machine (Hybaid Omingene

Thermocycler) and underwent a programme appropriate to each primer (Table 3.3). After cycles completed, PCR products were kept at 4 °C.

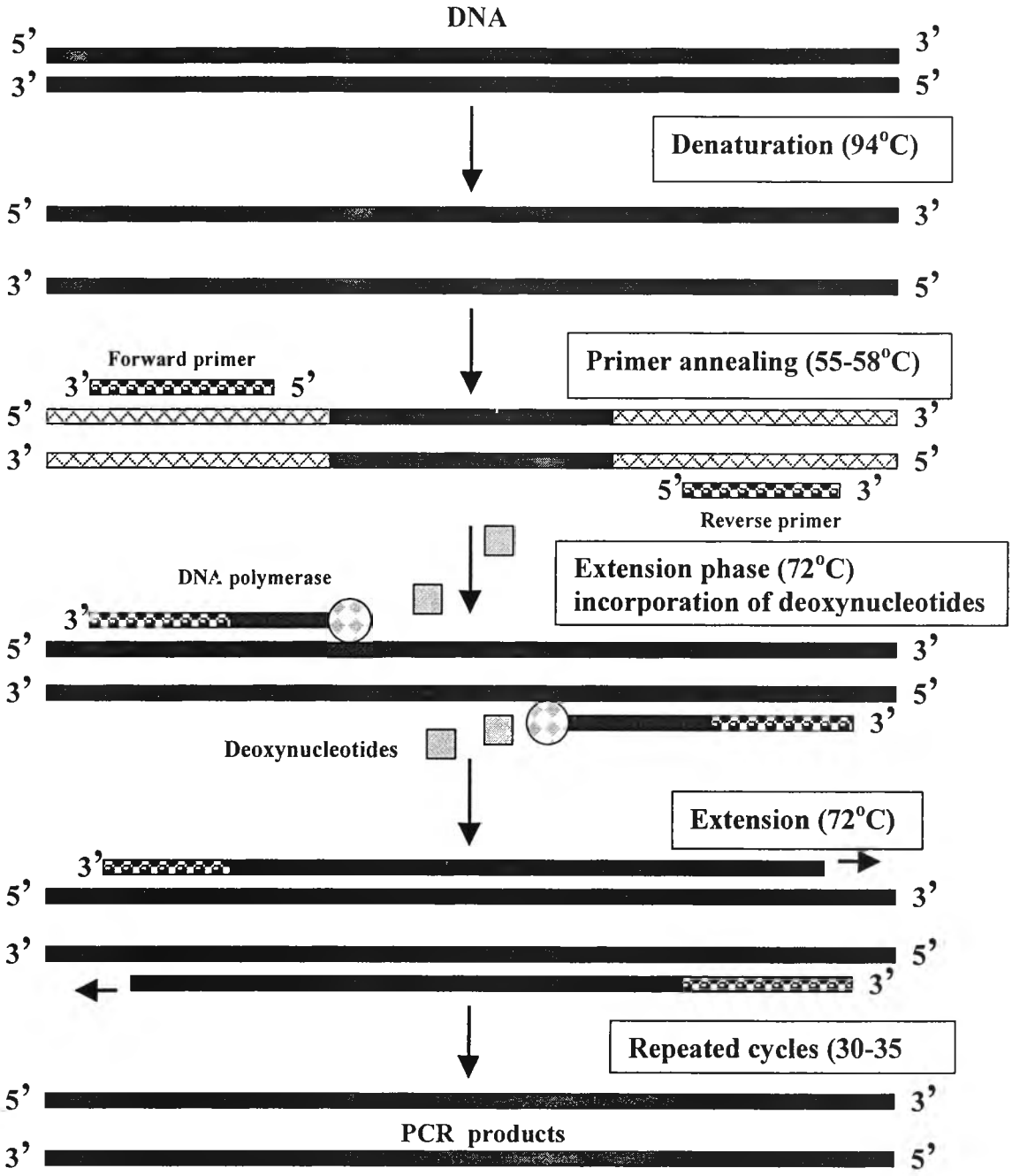

Figure 3.1 Polymerase Chain Reaction for amplifying microsatellite loci (Adapted from Montague, 1996).

Table 3.1. Primer sequences of four microsatellite primers used to detect the paternity of workers of *A. dorsata.*

Locus	Core sequence	Primer sequence	Author
A14	$(CT)_{13}$.....$(GGT)_9$	5'-GTGTCGCAATCGACGTAACC-3' 5'-GTCGATTACCGATCGTGACG-3'	Estoup et al., 1993
A24	$(CT)_{11}$	5'-CACAAGTTCCAACAATGC-3' 5'-CACATTGAGGATGAGCG-3'	Estoup et al., 1995
A88	$(CT)_{10}TC(CCTT)_2$ $(CTTT)_3$....$(GGA)_7$	5'-CGAATTAACCGATTTGTCG-3' 5'-GATCGCAATTATTGAAGGAG-3'	Estoup et al., 1995
Ad3	$(TC)_{13}$	5'-CCGTAACTGGACTTCTTTCCCTCC-3' 5'-GACAATGGCGTACTTTGTGG-3'	Parr et al., submitted

Table 3.2. Optimal PCR mixtures for amplifying microsatellite primers in *A. dorsata*.

Primers	A 14		A 24		A 88		Ad 3	
Reagent	Volume (µl) in 10µl	Final Concentration	Volume (µl) in 10µl	Final Concentration	Volume (µl) in 10µl	Final Concentration	Volume (µl) in 10µl	Final Concentration
Milli-Q H_2O	6.2	-	5.24	-	6.04	-	5.44	-
PCR buffer	1.0	1X	1.0	1X	1.0	1X	1.0	1X
dNTP	0.5	100 µM	0.5	100 µM	0.5	100 µM	0.5	100 µM
$MgCl_2$	0.44	1.1 mM	0.8	2 mM	0.6	1.5 mM	0.6	1.5 mM
Forward primer	0.4	0.8 µM	0.2	0.4 µM	0.4	0.8 µM	0.2	0.4 µM
Reversed primer	0.4	0.8 µM	0.2	0.4 µM	0.4	0.8 µM	0.2	0.4 µM
Taq DNA Polymerase	0.06	0.015 units	0.06	0.015 units	0.06	0.015 units	0.06	0.015 units
DNA template	1.0	-	2.0	-	1.0	-	2.0	-

Table 3.3. Conditions used for all PCR reactions.

Conditions	Pre-denaturation		Denaturation		Annealing		Extension	
Loci	Temp (°C)	Time (sec.)	Temp .(°C)	Time (sec.)	Temp .(°C)	Time (sec.)	Temp. (°C)	Time (sec.)
Ad3	94	60	94	20	58	20	72	20
A14	94	60	94	20	58	20	72	20
A24	94	60	94	20	55	20	72	20
A88	94	60	94	20	58	20	72	20

Each cycle (denaturation + annealing + extension) was repeated 35 times. Final elongation cycle was conducted at 72 °C for 5 minutes.

3.1.3 Electrophoresis

Polyacrylamide gels are polymers of acrylamide monomers link into linear chain by N,N'-methylene bis acrylamide. This gel can be used to purify synthetic oligonucleotide, isolate or analyse DNA less than 1 Kbp in size (Hoy, 1994).

In this thesis, Polyacrylamide Gel Electrophoresis (PAGE) was used to distinguish microsatellite alleles of the bees. PCR product of each bee was denatured by boiling in a water bath for 3 minutes. Gels used in this experiment was denaturing gels in which urea was incoperated into the gel in order to keep the stands of microsatellite DNA separate. They were then

electrophoresed at 1400 volts and 38°C on an automated DNA fragment analyser (Corbett Research, Sydney). Along with the samples, size standards (Gene Scan ®-350 (TAMRA)) which is known allele length (in base pairs; bp) were run as the reference in standard lanes. Each lane was manually checked for its allele size in base pair based on the size standard.

3.2 Genotype Identification

In honey bees, both queens and workers develop from fertilized eggs and are diploid. Because of the kin-structure of honey bee colonies, worker genotypes must occur in one of these 4 categories: 1) Queens carry two alleles and all workers (her daughters) must carry at least one of those two alleles. 2) If any worker carries only 1 allele (i.e. she is homozygous at that locus), her mother (the queens) must carry that allele. 3) If all workers examined carry only one allele, the queen is likely to be homozygous for that allele at that locus. 4) If all workers examined carry one of two alleles, the queen is likely to be heterozygous for those two alleles at that locus (Oldroyd et al., 1996).