Identification of DNA markers from Ant's Stomach in Zoological Findings

ABSTRACT

DNA can be extracted from ants and utilised in forensic analyses. Ants can be potential carriers of ambient DNA important to forensic research, as they are common in a variety of locations. In order to assure the validity of results, the study emphasises the significance of positive and negative controls as it examines the difficulties and techniques involved in extracting DNA from ant guts. Overall, the work broadens the scope of ecological and forensic research by highlighting the potential of ant gut DNA as a useful resource in forensic science.

Keywords: Ant gut, Forensic DNA, Next-generation sequencing, Automated DNA extraction, Emerging technologies etc.

INTRODUCTION

Ants play a role in decomposition ecology and can directly affect decomposition rates by feeding on carcasses and lacerating them. They can also indirectly affect decomposition by predating on other carrion-feeding invertebrates and altering the microhabitat of carrion through nest and mound construction. Studying ants can provide valuable information, such as determining evidence's location, timing, or movement.

There are about 154 species of ants that have been observed on carrion. The most prevalent subfamily of ants found on carrion is *Myrmicinae*, with species such as fire ants (*Solenopsis*), big-headed ants (*Pheidole*), and acrobat ants (*Crematogaster*) being commonly found. Another common subfamily associated with carrion is*Formicinae*, which includes carpenter ants (*Camponotus*), wood ants (*Formica*), and winter ants (*Prenolepis*). (Eubanks et al., 2019). The presence or absence of ants should be considered in forensic cases involving postmortem interval (PMI) estimates because ants can significantly affect the decomposition rate and act as scavengers, feeding on the flesh or exudates from the corpse. They also prey on the eggs, larvae, and adults of other insects, especially flies. (Bonacci et al., 2019). The impact of ants on carrion reduction varies depending on species, abundance, and geographic location. By identifying the species of ants present at a crime scene or analysing the DNA of prey consumed by ants using various techniques, investigators can gain insights into the movement of evidence or the ecological interactions in a given environment. When ants

consume food or prey, DNA from these sources can be found in their digestive system. Therefore, by isolating and extracting DNA from an ant's gut content, it is possible to obtain DNA samples relevant to a forensic investigation.

Table 1: Comparative Analysis of Ant Species in Forensic Entomology: Insights into Putrefaction Time Periods, Life Span, and DNA Analysis Techniques

Ant Species	Putrefaction Time Period	Life Span	DNA Analysis Technique	References
Odontoponera transversa	2-3 weeks	1-2 years	PCR and Sequencing	("Insect Succession on Remains of Human and Animals in Shenzhen, China," 2017)
Solenopsisinvicta	-	2-6 months	next-generation sequencer (NGS)	(Kim et al., 2019)
Pheidole nodus	2 weeks	1-2 years	complete mitochondrial genome sequence	(Sang et al., 2022)

DNA Isolation and Extraction From Ant Stomach

The main prerequisite of molecular biology is to get good quality DNA from any plant or biological samples for further analysis. The examination of genome structure and gene expression, as well as the study of particular DNA sequences, all begin with DNA isolation (Ballinger-Crabtree et al., 1992). Insect molecular systematics and evolutionary ecology have grown quickly in recent years, employing molecular biology methods to investigate systematic issues and identify species. For examinations of forensic samples, when a large portion of the native DNA may be degraded, the ability to isolate the majority of the DNA present in a sample is very crucial(Heikrujam et al., 2020). The nucleic acid isolation technique is determined by the type of sample being submitted for examination. Three main procedures are often involved in nucleic acid extraction, regardless of the source: (i) lysis, (ii) denaturation/degradation of other biomolecules, and (iii) concentration of DNA or RNA or separation of the nucleic acids (Hill, 2011).

Extraction of nucleic acid is a crucial process because it is the starting point of any molecular biology studies(Dairawan& Shetty, 2020). DNA can be extracted from a wide range of sources, including soil, plant and animal tissue, insects, bacteria, yeast, and protozoa. Forensic samples include dried blood spots, buccal swabs, and fingerprints etc. (Dhaliwal, 2022). It is essential to guarantee the quality and quantity of the isolated DNA to carry out the planned downstream applications when selecting an appropriate DNA extraction technique(Dairawan& Shetty, 2020).

Organic method of DNA extraction

Barker et al. introduced the phenol-chloroform DNA extraction method in 1998(Elkins, 2012). To disintegrate nuclear envelope and cell membranes, cells are first treated with a lysis buffer containing detergents such as sodium dodecyl sulphate (SDS). SDS denatures the

proteins efficiently, and isoamyl alcohol prevents emulsification and hence facilitates precipitation of DNA.

Salting-out methods

Miller, Dykes, and Polesky (1988) described a non-toxic DNA extraction technique called the salting-out method. Overnight, the mixture is incubated at 55–65°C. After adding around 6M of saturated NaCl, the mixture is agitated for 15 seconds and centrifuged for 15 minutes at 2500 rpm. Protein precipitation occurs when the solubility of the protein is reduced by the high concentration of salt. The supernatant containing DNA is pipetted into a new tube so that ethanol can be used to precipitate it(Peterson & Sober, 1956). It has been stated that the salting-out approach produces high-quality DNA that is on par with the one that is produced by the phenol-chloroform method. However, the method is preferable since it requires less time and money, and most significantly, the reagents are safe(Doyle, 1991).

Chromatography-based DNA extraction methods

DNA can be extracted from any type of biological material using chromatography-based techniques (Carpi et al., n.d.). Size exclusion chromatography (SEC), which was created by Lathe and Ruthven in 1955, ion-exchange chromatography (IEC), which was created by Peterson and Sober in 1956, and affinity chromatography (AC), which was documented by Cuatrecasas& Wilcheck, are examples of this technique("PROCEEDINGS OF THE BIOCHEMICAL SOCIETY," 1955)(Cuatrecasas et al., 1968). In size exclusion chromatography, molecules are separated based on their molecular size and shape. In contrast to gel permeation chromatography, which uses an organic solvent, when the DNA-containing sample is moved through the chromatography column using an aqueous solution. Porous beads made of agarose, dextran, or polyacrylamide are present in the column. Smaller molecules like mRNA and proteins pass through the tiny pores and channels of the beads when the sample is placed on top and passes through the column, however DNA is prevented from doing so and escapes from the matrix with its higher hydrodynamic volume. As a result, DNA elutes from the column more quickly than smaller molecules(Tan &Yiap, 2009)("The Use of Size-Exclusion Chromatography in the Isolation of Supercoiled Minicircle DNA from Escherichia Coli Lysate," 2020).

Ion-exchange chromatography (IEC) is another chromatography-based technique for extracting DNA. A solution containing DNA anion-exchange resin is utilized to first equilibrate the column. This resin is used to bind DNA with its positively charged diethylaminoethyl cellulose (DEAE) group in a selective manner. Cellular constituents like proteins, lipids, carbohydrates, metabolites, and RNA are eluted with medium-salt buffers, while DNA is held in the column. DNA can be retrieved later too by utilizing high-salt buffers or lowering the pH(Budelier & Schorr, 2001).Affinity chromatography (AC) is another method for purifying nucleic acids; it follows a similar procedure to IEC but uses oligo(dT) or other materials that interact with nucleic acid in a very specific way to remove it from the cell lysate(Carpi et al., n.d.).

Magnetic beads

A patent application for "DNA purification and isolation using magnetic particles" was made by Trevor Hawkins in 1998 (Elkins, 2012). Magnetic nanoparticles coated with a polymer or antibody that has a particular affinity for binding DNA to its surface can be used. The magnetite or maghemite that makes up the majority of magnetic beads can be employed as a

surface material, and functional groups like sulphate and hydroxyl groups can also be used(Saiyed et al., 2008). It is possible to separate the DNA-bound magnetic beads from the cell lysate by creating a magnetic field at the tube's bottom with an external magnet. Rinsing away the supernatant is possible once the beads have gathered at the tube's bottom. The ethanol precipitation method can be used to elute the magnetic pellet, which is then incubated at 65°C to elute the magnetic particles from the DNA(Peterson & Sober, 1956).

Silica Matrices

Vogelstein and Gillespie were the first to describe the strong affinity silicates have for DNA(Höss &Pääbo, 1993). The underlying idea of this method is the selective binding of negatively charged DNA to a positively charged ion-coated silica surface. Since the DNA is firmly attached to the silica matrix, the remaining biological impurities can be removed with distilled water or a buffer like Tris-EDTA before the extracted DNA is eluted from the silica particles(Woodard et al., 1994). The silica matrix approach yields high-quality DNA quickly and affordably, and it can be used for automation.

DNA profiling

DNA profiling, sometimes referred to as forensic genetics or DNA fingerprinting, is a method used in forensic science to identify people or samples based on their individual DNA profiles. On a global scale, DNA fingerprinting is a vital tool used in forensics and laboratories for research. Over 99.1% of the human population has identical DNA, with the remaining 0.9% varying from person to person. (Giardina, 2013). Individuals can be distinguished and correlated using these variable DNA sequences, also known as polymorphic markers. The typical typing procedure includes DNA quantitation, PCR amplification of STR markers, and STR typing using capillary electrophoresis after DNA evidence is collected and extracted from biological samples. Direct PCR can enhance the recovery of trace amounts of DNA by omitting the steps of DNA extraction and quantitation. (Butler, 2023). Traditional DNA fingerprinting methods are being replaced or used in conjunction with DNA sequencing with increasing frequency. "Next generation sequencing" (NGS) technology is rapidly advancing, lowering the cost of sequencing and making large-scale sequencing projects accessible to individual researchers (Roewer, 2013) . The use of forensic genetics in the legal field aims to identify missing persons and mass disaster victims from human remains, establish identity in criminal cases where biological evidence is found at crime scenes, and resolve legal issues like paternity tests and inheritance matters.

Sequencing Technologies

Massive advancements in molecular biology and genetics have been made possible by DNA sequencing technology since the Sanger sequencing method was introduced in the 1970. However, the use of conventional Sanger sequencing technology in deeper and more complex genome analyses has been limited due to its drawbacks, which include low throughput, high cost, and operational difficulties. These issues have mostly been resolved with the advent of next-generation sequencing (NGS) technology, which has been used in forensics, disease diagnosis, Agri genomics, and ancient DNA analysis. NGS technology is low-cost and has a high throughput capacity (Yang et al., 2014). Nowadays, the most common technique for forensic analysis is DNA profiling using STRs typing, and DNA sequencing has primarily

been used to determine the origin and relationship between low-quantity DNA samples by analysing the hypervariable region of mitochondrial DNA (mtDNA). In order to facilitate the quick generation of STR profiles, major efforts have been made recently. These efforts include integrated microfluidic devices for quick sample to profile processing, direct PCR, microfluidic chip-based amplification and detection, and rapid PCR (Alvarez-Cubero et al., 2017).

Next generation sequencing

High-throughput DNA sequencing for biotechnology discovery has long been accomplished with next-generation sequencing (NGS), also referred to as massively parallel sequencing (MPS) in the forensic community. NGS adds another dimension to the data in contrast to current capillary electrophoresis (CE) techniques, which only measure the length of the entire PCR product. Sequences of targeted PCR amplicons for the STR alleles and their corresponding stutter products are generated using NGS of STR markers (Butler & Willis, 2020). Because of its high throughput capacity, low cost, and ability to analyse ancient DNA, next-generation sequencing (NGS) technology has rapidly expanded in the field of genomics research in recent years, surpassing the limitations of conventional Sanger sequencing technology. NGS is without a doubt one of the most significant and notable technological developments in the biological sciences over the past 20 years (Imam et al., 2018).

Automated DNA extraction method

To find trace amounts of DNA, such as DNA from ant stomachs, DNA analysis needs an effective DNA extraction technique.A crucial stage in the analytical process, DNA extraction from forensic evidence samples is essential to the success of applications that come after. To analyse a wide range of DNA in forensic casework, it requires robust DNA extraction methods. One of the most difficult and time-consuming procedures in the sample analysis procedure is often DNA extraction(“Automated DNA Extraction from Pollen in Honey,” 2014). Many Automated DNA extraction kits have been used for the analysis of DNA samples from various sources. Automated robots that reduce operator input and expedite processing times have been made available in recent years.

Silica membrane-based technology

i. QIAamp DNA Investigator Kit: The silica-based approach is a reliable technique for extracting DNA that keeps a high yield of high-quality DNA while eliminating inhibitors from the sample. QIAamp DNA Investigator Kit is designed for the purification of genomic DNA from forensic and human identity testing samples. It provides a method for isolating DNA from various sample types, including blood, saliva, and tissue(Phillips et al., 2012).The four main steps in the QIAamp DNA Investigator Kit protocol are: binding DNA to the silica-based membrane of the QIAamp spin column; using buffers to wash contaminants through the membrane; and eluting DNA. The first step involves disrupting cellular membranes using a combination of enzymatic activity and mechanical lysis (heating and shaking). This kit includes QIAamp spin columns that an operator can utilize manually or the QIAcube robot can automate the process(QIAGEN, n.d.).
ii. Maxwell system: The Maxwell system includes an instrument (e.g., Maxwell RSC or Maxwell 16) that is designed to automate the purification process. The small benchtop

Maxwell ® 16 system (Promega, Madison, WI, USA) is a nucleic acid extraction tool that uses paramagnetic particles to bind nucleic acids to cellulose. The device is intended to be used with pre-dispensed reagent cartridges specifically for DNA and RNA extraction processes, and it comes with pre-programmed purification procedures. It can process up to 16 samples at once in maximum throughput(Dundas et al., 2008).

Magnetic bead based systems

Many forensic laboratories have been using automated technologies more and more to extract DNA from the growing number of forensic casework samples they handle. This reduces the possibility of human error and ensures excellent repeatability. Utilizing robotic platforms to automate various steps in the DNA analysis process is a cutting-edge strategy to manage growing volumes of samples with scarce human resources and reduce errors that may occur while handling and preparing samples by hand(Witt et al., 2012),(Nagy et al., 2005),(Frégeau et al., 2008),(Stangegaard et al., 2011),(Phillips et al., 2012).

i. ***HID Evolution™ :***This system is called as Three Freedom EVO® liquid handling robots as part of an automation system (Tecan). Tecan and Applied Biosystems collaborated to develop the HID EvolutionTM system, which is a fully automated solution for forensic DNA analysis (Witt et al., 2012),(Fang et al., 2010). It was the first HID EvolutionTM system of its type to be installed globally, albeit a prototype. These three robots are designed and built for distinct stages of forensic DNA analysis; one is used to separate nuclear DNA from various biological trace materials(Witt et al., 2012).

ii. **KingFisher^{R} technologies:** Almost from all forensic samples DNA extraction procedures can be standardized and validated by using KingFisher^{R} technologies. As a result, samples are processed using a single protocol(Willard et al., 1998) , which enables us to: (1) avoid the relatively risky organic extraction method (such as the phenol/chloroform method); (2) greatly reduce the number of steps, reagents, or additional procedures (such as CentronR); (3) lower the cost of experiments; and (4) savetime.

Application of DNA from ant gut in forensics

Forensic science stands to benefit greatly from the ability to extract DNA from insects and their excreta, including human and mammalian DNA. In addition to helping to protect DNA from environmental deterioration, insects may contain evidence that lawbreakers are unaware of and thus unable to destroy (Durdle, 2020)2). An investigation may be aided by the examination of DNA taken from the gut contents or insect artefacts gathered from a possible crime scene where the body has vanished. It's possible that DNA recovered from a body that has experienced significant trauma or is in a highly degraded state won't produce a DNA profile that is accurate enough to identify the person. Nonetheless, entomological evidence might contain enough high-quality DNA preserved to generate a DNA profile that could then be compared to reference samples taken from things the person regularly uses, family members, or DNA databases (Chavez-Brones et al., 2013; Marchetti et al., 2013). Human DNA recovered from insects or artefacts may be able to identify an attacker. As a result, insects may consume the offender's DNA, retain it in their stomachs, or leave it behind in artefacts. When it comes to determining a corpse's PMI, insects can be a very helpful source of information. However, in order to guarantee the validity of the estimated PMI, the

investigator must be certain that the entomological evidence utilised in the calculations is sourced from the body of interest.

CONCLUSION

In conclusion, forensic DNA extraction from ant guts offers a viable path forward, circumventing obstacles caused by digestive enzymes and acidic environments. Ant gut DNA is shown as a dependable resource for profiling through painstaking procedures, including as sample collection and customized extraction techniques using enzyme inhibitors. Accurate results are guaranteed by the controls. This paper emphasizes how ant gut DNA can be used to further forensic research, providing insightful information and adding to the arsenal of tools available to investigators. Standardization and future developments in this developing discipline depend on forensic scientists, entomologists, and molecular biologists continuing to collaborate on research projects. Analysis of ant gut DNA has the potential to improve our comprehension of environmental DNA in forensic contexts, advancing both forensic and ecological research.

REFERENCES

1. Alvarez-Cubero, M. J., Saiz, M., Martínez-García, B., Sayalero, S. M., Entrala, C., Lorente, J. A., & Martinez-Gonzalez, L. J. (2017). Next generation sequencing: an application in forensic sciences? Annals of Human Biology, 44(7), 581–592.
2. Automated DNA extraction from pollen in honey. (2014). Food Chemistry, 149, 302–306.
3. Ballinger-Crabtree, M. E., Black, W. C., & Miller, B. R. (1992). Use of Genetic Polymorphisms Detected by the Random-Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) for Differentiation and Identification of Aedes aegypti Subspecies and Populations. The American Journal of Tropical Medicine and Hygiene, 47(6), 893–901.
4. Bonacci, T., Benecke, M., Scapoli, C., Vercillo, V., & Pezzi, M. (2019). Severe post mortem damages by ants on a human corpse. Romanian Journal of Legal Medicine, 27(3), 269–271.
5. Butler, J. M. (2023). Recent advances in forensic biology and forensic DNA typing: INTERPOL review 2019–2022. Forensic Science International: Synergy, 6, 100311.
6. Butler, J. M., & Willis, S. (2020). Interpol review of forensic biology and forensic DNA typing 2016-2019. Forensic Science International. Synergy, 2, 352–367.
7. Carpi, F. M., Di Pietro, F., Vincenzetti, S., Mignini, F., &Napolioni, V. (n.d.). Human DNA Extraction Methods: Patents and Applications. Recent Patents on DNA & Gene Sequences, 5(1), 1–7.
8. Cuatrecasas, P., Wilchek, M., & Anfinsen, C. B. (1968). Selective enzyme purification by affinity chromatography. Proceedings of the National Academy of Sciences of the United States of America, 61(2), 636–643.
9. Dairawan, M., & Shetty, P. J. (2020). Biomedgrid. American Journal of Biomedical Science & Research, 8(1), 39.

10. Dundas, N., Leos, N. K., Mitui, M., Revell, P., & Rogers, B. B. (2008). Comparison of automated nucleic acid extraction methods with manual extraction. The Journal of Molecular Diagnostics: JMD, 10(4), 311–316.
11. Durdle, A. (2020). Insects as vectors of DNA in a forensic context. WIREs Forensic Science, 2 (2).
12. Elkins, K. M. (2012). Forensic DNA Biology: A Laboratory Manual. Academic Press.
13. Eubanks, M. D., Lin, C., & Tarone, A. M. (2019). The role of ants in vertebrate carrion decomposition. Food Webs, 18, e00109.
14. Frégeau, C. J., Lett, C. M., Elliott, J., Yensen, C., & Fourney, R. M. (2008). Automated processing of forensic casework samples using robotic workstations equipped with non-disposable tips: contamination prevention. Journal of Forensic Sciences, 53(3), 632–651.
15. Giardina, E. (2013). DNA Fingerprinting. In S. Maloy & K. Hughes (Eds.), Brenner's Encyclopaedia of Genetics (Second Edition) (pp. 356–359).
16. Heikrujam, J., Kishor, R., & Mazumder, P. B. (2020). The Chemistry Behind Plant DNA Isolation Protocols. In Biochemical Analysis Tools - Methods for Bio-Molecules Studies. IntechOpen.
17. Hill, C. E. (2011). Nucleic Acid Isolation: Overview of Sample Preparation Methods. In Molecular Microbiology (pp. 117–125).
18. Höss, M., &Pääbo, S. (1993). DNA extraction from Pleistocene bones by a silica-based purification method. Nucleic Acids Research, 21(16), 3913–3914.
19. Imam, J., Shrivastava, P., Dixit, S., & Shrivastava, A. (2018). Future of DNA Fingerprinting: Application of NGS in Forensic Science. In H. R. Dash, P. Shrivastava, B. K. Mohapatra, & S. Das (Eds.), DNA Fingerprinting: Advancements and Future Endeavours (pp. 259–275).
20. Cho, H.-J. (2017),Insect succession on remains of human and animals in Shenzhen, China. Forensic Science International, 271, 75–86.
21. Kim, K.-H., Kim, J.-S., Cho, H.-J., Lee, J.-H., Jun, T.-H., & Kang, Y. J. (2019). Identification of Key Genes for the Precise Classification between *Solenopsisinvicta* and S. geminata Facilitating the Quarantine Process. Genes, 10(10), 812.
22. Nagy, M., Otremba, P., Krüger, C., Bergner-Greiner, S., Anders, P., Henske, B., Prinz, M., & Roewer, L. (2005). Optimization and validation of a fully automated silica-coated magnetic beads purification technology in forensics. Forensic Science International, 152(1), 13–22.
23. Peterson, E. A., & Sober, H. A. (1956). Chromatography of proteins. I. cellulose ion-exchange adsorbents. Journal of the American Chemical Society, 78(4), 751–755.
24. Phillips, K., McCallum, N., & Welch, L. (2012). A comparison of methods for forensic DNA extraction: Chelex-100® and the QIAGEN DNA Investigator Kit (manual and automated). Forensic Science International. Genetics, 6(2), 282–285.
25. Roewer, L. (2013). DNA fingerprinting in forensics: past, present, future. Investigative Genetics, 4(1), 22.
26. Saiyed, Z. M., Ramchand, C. N., & Telang, S. D. (2008). Isolation of genomic DNA using magnetic nanoparticles as a solid-phase support. Journal of Physics. Condensed Matter: An Institute of Physics Journal, 20(20), 204153.

27. Stangegaard, M., Frøslev, T. G., Frank-Hansen, R., Hansen, A. J., & Morling, N. (2011). Automated extraction of DNA from blood and PCR setup using a Tecan Freedom EVO liquid handler for forensic genetic STR typing of reference samples. Journal of Laboratory Automation, 16(2), 134–140.
28. Tan, S. C., &Yiap, B. C. (2009). DNA, RNA, and protein extraction: the past and the present. Journal of Biomedicine & Biotechnology, 2009, 574398.
29. Willard, J. M., Lee, D. A., & Holland, M. M. (1998). Recovery of DNA for PCR Amplification from Blood and Forensic Samples Using a Chelating Resin. Forensic DNA Profiling Protocols, 9–18.
30. Witt, S., Neumann, J., Zierdt, H., Gébel, G., & Röscheisen, C. (2012). Establishing a novel automated magnetic bead-based method for the extraction of DNA from a variety of forensic samples. Forensic Science International. Genetics, 6(5), 539–547.
31. Yang, Y., Xie, B., & Yan, J. (2014). Application of next-generation sequencing technology in forensic science. Genomics, Proteomics & Bioinformatics, 12(5), 190–197.