Biotechnological Perspectives in Chemical Ecology of Insects

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Molecular Techniques in the Study of Organismic Biology—Challenges and Opportunities

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INTRODUCTION

Ever since the discovery of the structure of DNA and the deciphering of the genetic code, molecular biology has made rapid strides (Lewin, 1994). A notable feature of the recent advances in molecular biology is the development of increasingly powerful techniques of molecular analysis of biological systems. Organismic biology, especially ecology, behaviour, and evolution, has also entered a new "modern" phase thanks to the clarification of the levels of natural selection and the development of the concept of inclusive fitness (Wilson, 1976).

We are not about to witness a revolution of sorts with the integration of molecular and organismic biology. Such integration is bound to be of mutual benefit. The application of the concepts of adaptation and natural selection developed largely in the context of organisms biology to molecular biology has already demonstrated its ability to engender completely new ways of thinking about life process. The appreciation of the concept of selfish DNA is an excellent example of this (Orgel and Crick, 1980; Doolitle and Sapienza, 1980). On the other side, the application of the powerful techniques of molecular biology to the resolution of questions in organismic biology has begun to lend a level of sophistication to this enterprise that could not have been imagined a decade ago. For the abundant practitioners of molecular biology and the comparatively fewer practitioners of modern organismic biology in India, this revolution in the making offers a host of challenges and opportunities. The purpose of this paper is to demonstrate that this revolution is actually taking place and to begin to articulate its implications for the research agenda of Indian biologists (see also Gadagkar, 1990a).

THE MOLECULAR-ORGANISMIC DICHOTOMY

There is an easily recognizable hierarchy of levels of organization of life process. Genes, cells, tissues, organs, individual organisms, family or other social units, populations, communities, and ecosystems are some of the broad rungs in this hierarchy. There are interesting questions relating to structure, function, and evolution to be asked at each of these levels of organization. There is a fair amount of integration between the branches of biology that deal with different levels of organization at the suborganismic level. An almost entirely reductionist approach and attempts to explain all phenomena in a biochemical or molecular language effectively bind these branches of biology together. At the organismic and super-organismic level, reductionist approaches are not always successful and there is little confidence that complex biological phenomena will be entirely explicable in the molecular language in the foreseeable future or indeed if they even will be. Just as physicists who deal with simpler physical phenomena consider all of biology as a less exact, largely descriptive science, those who work at the suborganismic levels of biological organization tend to treat, or even dismiss, branches of organismic biology such as population biology, ecology, behaviour, community structures, and ecosystem function as inexact and descriptive. This has resulted in a divide between organismic biology on the one hand and cellular and molecular biology on the other. It is fair to say, for example, that a cellular biologist and behavioural ecologist speak rather different languages and have little understanding of the philosophy of each other's scientific approach and methodology.

THE ELECTROPHORETIC REVOLUTION

The origin of the use of biochemical and molecular techniques to answer questions in organismic biology can be traced to two pioneering studies, one by Lewontin and Hubby in the fruit fly *Drosophila pseudoobscura* and another by Harris in humans, both published in 1966 (Harris, 1966; Hubby and Lewontin, 1966; Lewontin and Hubby, 1966). Both these studies separated proteins based on their net electrical charge, by applying an electric current, and came up with a way of quantitatively assessing genotypes of individual organisms. This was possible because small variations in genes that may or may not manifest as phenotypic changes in morphology, anatomy, or physiology often lead to specific changes in the amino acid sequences of the proteins that the genes code for. Slight changes in amino acid sequences often result in altered net electrical charges on the protein molecules, which in turn alter the mobility of the protein during electrophoresis. To quote Lewontin, ". the nature of genetic diversity among organisms, has always seemed the basic problem of evolutionary genetics. Because of the immense methodological difficulties and ambiguities, a characterization of that

genetic variation seemed always to elude us. Then, with the discovery of molecular biology about the relation between genes and proteins, the possibility of an unambiguous characterization of genetic variation among individuals was opened up. The first experiments revealed an extraordinary wealth of genetic diversity. . ." Thousands of studies have since used electrophoresis to separate isoenzymes of allozymes and have confined that natural biological populations harbour a great deal of genetic diversity.

THE POWER OF DNA TECHNOLOGY

There are, however, many limitations to electrophoresis. Not all stretches of DNA code for proteins and, even when they do, not all changes in the DNA lead to changes in the proteins. Clearly, one is better off looking directly at the DNA. DNA technology is in fact rapidly outstripping protein technology in being quick and inexpensive. Restriction enzymes cut DNA at specific sequences and thus generate a predictable number and size class of fragments which, as in the case of proteins, can be separated by electrophoresis and scored. Very small variations in the nucleotide sequences can be detected by using a cocktail of different restriction enzymes. This has led to the technique of DNA fingerprinting, which is so precise that it is becoming a major forensic tool. Because DNA will specifically hybridize to its complementary sequence, radioactive probes containing the desired sequences can be hybridized to the DNA bands separated by electrophoresis. This technique, called Southern blotting, allows us to pick up any specified sequence of any length among the entire billion or more nucleotides that may be present in the sample.

Perhaps the most significant advance in molecular technology has been the development of the polymerase chain reaction or PCR technique. It allows us to amplify small quantities or even a single DNA molecule into millions of copies so that single copy genes are routinely extracted out of total DNA and identified after amplification as distinct bands on electrophoretic gels. This technology has also begun to make possible the analysis of ancient DNA from archaeological samples and even samples preserved in amber, making at least the first step in *Jurassic Park* sound realistic (Lister, 1994)!

Yet another major advance has been the discovery of microsatellites, which are tandem repeats of very short nucleotide motifs, often no more than two or three nucleotides long. But it is the number of repeats that is important. A specific microsatellite sequence with a specific number of repeats such as AAT_{10}, for example, serves as a unique allele with the number of repeats varying between different alleles. Microsatellites are highly variable and solve the problem of insufficient variability in protein molecules (Innis *et al.*, 1990).

In summary, the power of DNA technology appears so great that, at

least for the time being, what we can learn from appears to be limited by "time and money rather than by biology" (Queller *et al.*, 1993).

GENETIC VARIABILITY IN NATURAL POPULATIONS

Although genetic variability is the raw material for natural selection, there was no accurate method for its measurement until the advent of molecular techniques. Our ignorance regarding the levels of genetic variability in natural populations was so great that many people still maintained that there exist nearly universal wild type alleles at every locus and that mutations are rare variants. The frustration due to this ignorance is reflected in Lewontin's (1974) statement that "what we can measure is by definition uninteresting and what we are interested in is by definition unmeasurable". All this changed, however, with the advent of molecular techniques and the use of variations at allozyme loci and more recently using DNA markers has now given us a fair idea of genetic variability in natural populations. We now know that 25–50 per cent of loci in natural population are variable and that individuals may be heterozygous at as many as 5–13 per cent of their loci (Ayala, 1982). Quite naturally these estimates have made models of evolution through natural selection acting on random genetic variation much more plausible.

THE NEUTRALIST-SELECTIONIST CONTROVERSY

Far from wondering whether there is enough genetic variability for natural selection to act upon, the electrophoretic revolution and the age of DNA have prompted us to wonder why there is so much variability. Die-hard Darwinian selectionists continue to maintain that all of this variability is maintained by the force of natural selection and that all the variation is adaptive. Not surprisingly, however, many people find it hard to believe that there is a fitness difference associated with each variant. The so-called neutral theory of evolution proposes that most alleles are neutral and make no difference to the fitness of the bearer. The selectionist-neutralist controversy continues to rage and only further studies using molecular techniques can hope to resolve it.

GENETIC RELATEDNESS AND SOCIAL EVOLUTION

In social insect colonies only one or a small number of individuals function as fertile reproductives, while the rest function as sterile workers. The evolution by natural selection of such altruism on the part of workers remains a major unsolved problem in evolutionary biology. Perhaps the most attractive theory that attempts to explain this paradox relies on the assumption that the sterile workers are directing their altruism only towards close genetic relatives. Any test of such a theory requires estimates of the levels of intra-colony genetic relatedness. The

use of allozyme markers has permitted estimation of intra-colony genetic relatedness in many social insect colonies, permitting a rigorous test of the theory (Gadagkar, 1991, 1991b). Another interesting prediction of this theory is that even within a colony, the nature of interaction between individuals is influenced by their genetic relatedness. The technology of estimating genetic relatedness using allozyme markers is, however, insufficient to test such detailed predictions. DNA technology, especially the use of microsatellites, appears to be on the threshold of overcoming this difficulty (Queller *et al.*, 1993).

GENE FLOW AND QUEEN SELECTION IN FIRE ANTS

In the course of using allozyme markers for estimating genetic relatedness in the American imported fire ant *Solenopsis invicta*, Ross (1992) made a serendipitous discovery. He found that individuals of the genotype *Pgm-3*^{a}/*-3*^{a} never become queens in polygynous colonies of this species. In contrast, this is the most common genotype among queens of the monogynous colonies of the same species. Subsequent studies (Keller and Ross, 1993) have shown that young queens bearing the genotype *Pgm3*^{a}/3^a are selectively killed by the workers. The underlying reason appears to be that these queens have high fecundity and probably produce a strong pheromone. This pheromone signal is probably perceived by the workers, who kill them perhaps to prevent them from so dominating other queens that the colony becomes virtually monogynous. These studies have for the first time provided a genetic marker that indicates fecundity and fitness of queens in social insect colonies. The experimental system provides an attractive model for sophisticated investigation of ant sociobiology. Since *S. invicta* is an introduced species in North America that is perceived as a pest and since it is rapidly extending its geographic range, genetic studies of the colonization process are extremely valuable. DNA technology using randomly amplified polymorphic DNA (RAPD) markers promises to provide extremely precise knowledge of the process of colonization and spread of fire ants in North America. The RAPD data permit the conclusion, for example, that in the hybrid zone between *S. invicta* and *S. richteri* there has been an introgression of alleles from *S. richteri to* S. *invicta* (Shoemaker *et al.*, 1994).

PATERNITY AND MATERNITY ANALYSIS

The determination of paternity and maternity is often extremely crucial in studies of behavioural ecology. While some progress can be made with traditional techniques such as pedigree analysis (see, for example, Gadagkar *et al.*, 1991, 1993), there is no comparison with what molecular techniques can do for us. Using allozyme variation at a non-specific *esterase* locus we were able to determine the genotypes of queens and their daughters and thus infer the genotypes of the fathers

of these daughters in the social wasp *Ropalidia marginata*. From these studies we showed that queens of *R. marginata* mate with at least 1 to 3 males and simultaneously produce multiple patrilines of daughters. As a consequence, intra-colony genetic relatedness drops from the theoretically expected 0.75 to about 0.52, thus largely breaking down the genetic asymmetry created by hyplodiploidy (Murulidharan *et al.*, 1986; Gadagkar, 1990b). This conclusion was primarily responsible for our pursuit of several alternative theories for the evolution of sociality (Gadagkar 1990d, 1991b, 1991c). Sometimes paternity and maternity are required to be determined even more specifically. For example, we may need to known exactly who the father of a given individual is. Such precise estimates have become possible with use of DNA technology, especially with DNA fingerprinting. An important conclusion to emerge from such estimates of paternity is that in many birds that were hitherto thought to be monogamous, extra-pair copulations are not entirely uncommon (Burke *et al.*, 1989; Westneat, 1990; Graves *et al.*, 1993; Dixon *et al.*, 1994).

SYSTEMATIC PHYLOGENY AND RATES OF EVOLUTION

With the advent of numerical taxonomic methods and cladistic methods of phylogenetic reconstruction, there is an increasing premium on precisely estimated quantitative species characteristics. DNA sequence data are extremely useful in this context. They are quantitative and can be estimated with great precision and variation in DNA sequences can be thought of as being at the heart of genetic divergence. Ever since automated DNA sequencing techniques have become available, a flood of sequence data has started pouring in. Allozymes, restriction fragment length polymorphisms (RFLP), variable numbers of tandem repeats (VNTR), and minisatellites and microsatellites all promise to revolutionize the study of systematic phylogeny and evolutionary rates. Crozier (1993) proves a fascinating review of the currently available molecular methods for insect phylogenetics and Simon *et al.* (1994) provide a critique of the phylogenetic utility of mitochondrial gene sequences and conclude that "As more DNA sequence data accumulate, we will be able to gain an even better understanding of the way in which genes and species evolve." It is widely believed that crocodilians are the closest living relatives of birds but this assumption is often challenged by certain kinds of morphological data. Using DNA sequences from four slowly evolving genes, namely, mitochondrial 12S RNA, 16S RNA, $tRNA^{val}$, and nuclear alpha-enolase, Hedges (1994) has now provided strong statistical support for a bird-crocodilian relationship.

GENETIC BASIS OF BEHAVIOUR AND DIVISION OF LABOUR IN HONEYBEES

A striking feature of highly social insect colonies such as honeybees is the division of labour. At any given time in a honeybee colony, the tasks

of feeding larvae, removing dead bees (undertaking), guarding, and foraging are all being done by different sets of bees. It has been known for a long time that division of labour in a honeybee colony is based on age polytheism, meaning that every bee goes through life performing different tasks as it ages. By using allozyme markers to determine genotypes of bees performing different takes, Robinson and Page (1988) demonstrated a genetic basis for division of labour. For example, bees with a certain genotype were more likely to act as undertaker bees and those with another genotype were more likely to act as guard bees. These studies not only proved evidence for the genetic basis of complex behaviour, but also suggest new ways of understanding the evolution of division of labour (for reviews, see Page and Robinson, 1991; Robinson, 1992).

MITOCHONDRIAL GENOME ORGANIZATION IN HONEYBEES

In 1993, Crozier and Crozier published the complete sequence of honeybee (*Apis mellifera)* mitochondrial DNA, which is 16,343 base pairs long. This information has opened up new possibilities. Not only will it permit detailed studies of differentiation and evolution of honeybees, but it will also permit more careful study of the spread of *A. mellifera* and of its interaction with other species of honeybees across the globe. A particularly useful application of this has been in relation to the grave concern regarding the spread of Africanized honeybees across the Americas. A tricky problem in the study of Africanized bees is the unreliability in distinguishing Africanized bees from European bees by morphological criteria. Mitochondrial DNA sequences have now made such discrimination fairly precise. Even more interestingly, these studies suggest that the migration of Africanized bees is mainly due to African maternal lineages spreading as swarms rather than due to African drones mating with European queens (Hall, 1986; Hall and Muralidharan, 1989; Smith *et al.*, 1989; Oldroyd *et al.*, 1991).

BIODIVERSITY

There is now an unprecedented interest in documenting, inventorying, and monitoring the earth's biodiversity. However, that these efforts seldom include microorganism. The problem is not one of negligence towards microorganisms but the extreme difficulty in identifying species of microorganisms through conventional methods. The use of molecular sequence data has for the first time begun to make possible the objective study of microbial biodiversity. A careful examination of the rates of divergence of ribosomal RNA sequences of well-known, laboratory-cultured microorganisms have predicted the existence of many new microbial groups that remain to be isolated (Embley *et al.*, 1994). It is not unreasonable to expect that with continued use of these methods, or estimates of the magnitude of the earth's biodiversity will further

explode. As pointed out by Embley *el al.* (1994), current estimates give a figure of 10–100 million species for arthropods and only 1–10 million for microbes. "Yet all arthropods harbour microorganisms, and experience suggests that in each case at least some of these will be previously unrecorded" (Embley *et al.*, 1994). What then is the likely number of microbial species?

WHY WE CANNOT IGNORE MOLECULAR TECHNIQUES

It must be clear from the foregoing discussion that molecular techniques have revolutionised the study of organismic biology. Until recently, the study of organismic biology was a relatively inexpensive enterprise. It was therefore a better choice for a developing country compared to molecular biology. With the advent of molecular techniques in the study of organismic biology, it may appear that India has lost the little advantage it once had. Can we afford to ignore the use of molecular techniques in organismic biology and continue to study such aspects of organismic biology as ecology, behaviour, and evolution using only conventional techniques? We still have a whole variety of interesting plants and animals that are not so easily accessible to biologists in many developing countries.

Still, we cannot afford to ignore the use of molecular techniques and the consequences of doing so would be dangerous. The following passage (Oldroyd *et al.*, 1994) illustrates the competitive advantages that accrue from use of molecular techniques: "We analyzed bees taken from two *Apis florea* colonies ... located in the fronds of coconut palms. They had populations of about 10,000 bees, so our sampling did not affect colony structure. Colony A was moved from Samut Songkhram to Chulalongkorn University in Bangkok, Thailand. Bees were taken directly from colony B which remained *in situ* in Chathaburi, Thailand. With forces we grasped bees that were observed undertaking the following activities. (1) *Stingers* ... (2) *Guards* ... (3) *Fanners* ... (4) *Waggers* ... (5) *Liquid foragers* .. (6) *Pollen foragers* ... Bees were collected from 0600 to 1000 hours, stored separately on ice according to behavioural class during the daily collection period, and then transferred to liquid nitrogen for transport to the laboratory where they were stored at –80°C until DNA was extracted."

Thus, a scientist from a developed country on a week's holiday in the tropics can collect samples of animals that are in the act of performing interesting behaviours, take the samples back to his or her country, analyse the DNA, and draw conclusions about the correlation between genotype and behaviour. We cannot hope, therefore, that our interesting species will remain unexplored for us to investigate at our own pace. In order to remain competitive even in organismic biology we must adopt modern molecular techniques and stay up-to-date with the rest of the world.

AN OPTIMUM STRATEGY FOR SETTING OUR RESEARCH PRIORITIES

Even if organismic biology becomes as expensivie as molecular biology, there still remains a significant competitive advantage in pursuing it because of our rich and unique biodiversity. In spite of having to use molecular techniques, it would be easier to compete with Western scientists if we are studying the behavioural ecology of some insect or bird unique to our area than if we are studying the molecular biology of *E. coli*. Given the extensive expertise that we already possess in the field of molecular biology, it would not be very difficult for us to pursue organismic biology with molecular techniques. Traditional organismic biologists need to become comfortable with the jargon of molecular biology and traditional molecular biologists need to become excited about the questions in organismic biology. This will require a continued interaction between the two. If we are up to this challenge, the opportunities are immense.

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