DETECTION OF CRYPTIC SPECIES

A.F. COCKBURN, T. JENSEN, J.A. SEAWRIGHT
United States Department of Agriculture,
Agricultural Research Service,
Medical and Veterinary Entomology Research Laboratory,
Gainesville, Florida, USA

Abstract

Morphologically similar cryptic species are common in insects. In *Anopheles* mosquitoes, most morphologically described species are complexes of cryptic species. Cryptic species are of great practical importance for two reasons: first, one or more species of the complex might not be a pest and control efforts directed at the complex as a whole would therefore be partly wasted; and second, genetic (and perhaps biological) control strategies directed against one species of the complex would not affect other species of the complex. At least one SIT effort has failed because the released sterile insects were of a different species and therefore did not mate with the wild insects being targeted.

We use a multidisciplinary approach for detection of cryptic species complexes, focusing first on identifying variability in wild populations using RFLPs of mitochondrial and ribosomal RNA genes (mtDNA and rDNA); followed by confirmation using a variety of other techniques.

For rapid identification of wild individuals of field collections, we use a DNA dot blot assay. DNA probes can be isolated by differential screening, however we are currently focusing on the sequencing of the rDNA extragenic spacers. These regions are repeated several hundred times per genome in mosquitoes and evolve rapidly. Molecular drive tends to keep the individual genes homogeneous within a species.

1. INTRODUCTION

Surprisingly, the problem of cryptic species has not received adequate attention in pest control. Two recent examples of important pests that are cryptic species are the silverleaf whitefly (originally thought to be the sweet potato whitefly), which has caused enormous economic damage in the US in the last few years, and was not described as a separate species until last year [1]; the fall armyworm, which was recently described as two species [2]. In each of these two cases, an enormous amount of research was conducted on the wrong species in an effort to control the pest.

For the past few years, our research program has focused on cryptic species of anopheline mosquitoes. There are four related problems: 1) detection of a species complex in a single morphologically described species, 2) determination of the phylogenetic relationships among these species, 3) elucidation of the ecological and vector biology of the different species, and 4) development of a rapid screening method for mosquito control personnel. In this paper we discuss detection of cryptic species and the use of DNA probes for ecology, vector biology, and routine surveillance.

2. DETECTION OF CRYPTIC SPECIES

The most critical and difficult problem is the detection of a cryptic species complex. In the simplest case, where two species are sympatric, it is usually sufficient to show that the population is

subdivided into two groups that are out of Hardy-Weinberg equilibrium. However, when populations are allopatric or collections are limited, definite proof is often hard to obtain. Hybridization crosses are probably the most powerful method, however many species can not be bred in the laboratory. Luckily, from a practical point of view, it is largely irrelevant whether widely separated allopatric populations are identical or closely related sibling species. In either case, it is necessary to examine the ecology and pest status of each population separately.

Note that it is impossible to prove that a species is monotypic- all we can do is demonstrate that within the limits of our collections and techniques there are no significant differences. This does not rule out the possibility of a new collection or technique showing that a species complex does exist. In a multidisciplinary approach, the conclusive evidence rests with any technique that shows differentiation among groups. Showing that a collection is genetically uniform with one technique, for example polytene chromosome cytology, does not in any way diminish the impact of discovering that it is separable using another technique, for example allozyme electrophoresis.

Our approach has been multidisciplinary (see figure 1), resting on the assumption that we are most likely to detect differences between closely related species by using several different techniques. We routinely screen collections of wild mosquitoes for mtDNA and rDNA RFLPs, allozymes, and polytene chromosomes. All of these techniques are relatively rapid and detect different types of genetic variation.

2.1. Mitochondrial DNA RFLPs

MtDNA RFLPs are easy to interpret, because the mtDNA is circular and a constant length. The fragment sizes should always add to the same value (about 15.5 kb in mosquitoes). Too large a size is due to contamination or partial digestion; too small a size is due to comigrating bands or fragments migrating off of the gel. Differences between mitochondrial patterns are almost always due to gain or loss of restriction sites, which are simple to use in constructing maps or creating phylogenies.

Since mtDNA is maternally inherited and hemizygous, Hardy-Weinberg equilibrium is not applicable. There is no theoretical reason why different individuals of a single species can not have two or more very different mtDNAs, or that individuals of two different species can not have identical patterns. In practice, usually individuals within a species have similar mtDNAs, and different species have different mtDNAs [3]. However, exceptions such as introgression between species and lineage sorting within species have been observed.

MtDNA restriction site data is of dubious usefulness for constructing phylogenies. Unless a large number of restriction enzymes are used, the number of informative characters will be too small to be significant. [4], working with viral isolates with known relationships, used approximately 60 restriction sites to generate phylogenies with various methods. About 10% of the branches in all of their derived phylogenies were incorrect. We find that we seldom get more than 30 significant characters using eight restriction enzymes, so the branches in phylogenies generated using our data are likely to be incorrect 20% of the time. Part of the problem seems to be that the same sites tend to appear in different lineages, because the sequence of the mtDNA is highly restricted due to coding and base composition constraints.

2.2. Ribosomal DNA RFLPs

In our experience, rDNA is the most useful tool for identifying sibling species. Because it is chromosomally inherited and is present in tandem arrays, it is subjected to molecular drive [5] which tends to homogenize the individual repeats. Generally, the rDNA patterns of individuals within a species are essentially identical- there is usually as much variation within an individual as between individuals [6,7]. However, there are usually striking differences between species, either in restriction sites or length [7,8].

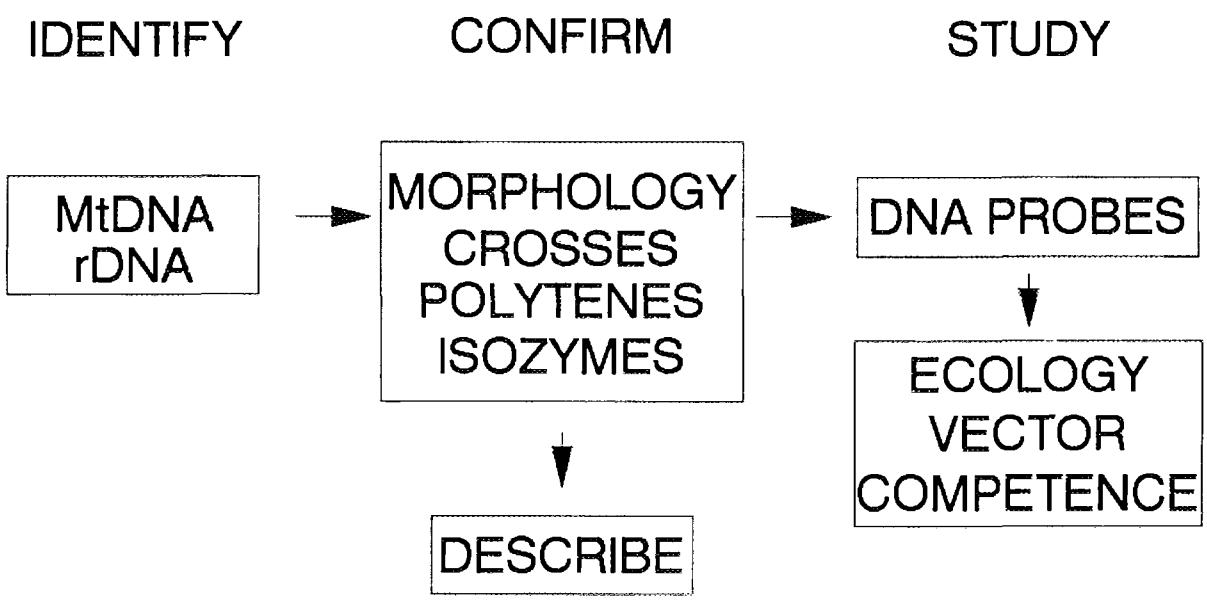

FIG. 1. Characterizing new cryptic species.

The significance of differences in the rDNA between individuals is magnified by the copy number. Mosquitoes and other insects usually have several hundred copies of the rDNA per genome, so any noticeable differences involve dozens of loci. A fixed difference between two individuals represents fixation at hundreds of loci. While these loci are not independent (because of gene conversion or unequal crossing over), neither are they equivalent to a single locus. The probability of getting such fixed differences between individuals in a freely interbreeding population is negligible.

rDNA patterns are much harder to interpret than mtDNA patterns. There are frequently differences in length among the different members of the repeat family even within an individual- this gives rise to bands that are blurred and impossible to measure accurately. It is also possible to gain or lose sites between different repeat units, giving rise to minor bands. Since the nontranscribed spacer diverges rapidly, a generic mosquito rDNA probe will only hybridize to the coding sequences, and fragments that contain only spacer will not be detected. Because much of the variation in rDNA patterns is insertion/deletion length variation and many of the bands are not detected, rDNA is essentially useless for phylogenetics.

3. DNA PROBES

There are three types of data that epidemiologists generally want to know about the individual mosquitoes in a collection: the species, the type of blood meal taken, and whether they are infected with a particular pathogen. Generally these are determined using different techniques- morphology for species identification; antibodies for blood meal identification; and ELISA, cell culture, and others for pathogens. This makes epidemiology expensive and difficult to do, since it requires equipment and expertise for a variety of techniques. We are trying to develop an integrated DNA probe based method for collecting all of this information.

3.1. Mosquito identification

The easiest way to identify a species is to find a single qualitative character that separates it from all other organisms. After determining the existence of a sibling species, we develop DNA probes for the rapid identification of species in samples of natural populations such as would be collected during distribution or epidemiological surveys. We currently have clones of repetitive DNA sequences that are useful for identifying species of the *A. quadrimaculatus* complex [9], species of the *A. crucians* complex,

A. perplexans, *A. punctipennis*, *A. atropos*, and a number of South American species [10]. We have shown that these can be used to identify thousands of individual mosquitoes by means of a convenient squash technique and standard DNA detection methodology [11].

Table 1: Cost estimates for identifying 1,000 individual mosquitoes using three different procedures used in our laboratory. Labor includes not only technical assistance, but also professional time needed for data analysis.

	Equipment	Supplies	Labor
Probes	$500	$50	$75
PCR	$5,000	$2,000	$1,000
Electrophoresis	$1,000	$200	$500

We have used a differential screening strategy to isolate the species-specific clones that we have isolated [9]. However, we have found that many of these clones originate from the non-transcribed spacer of the rDNA locus. Therefore, we are beginning to sequence this region from all of the mosquito species that we have available, in order to create a database of potential species-specific sequences. When a new species is identified, it will be possible to determine whether existing probes will hybridize to it, and it will be simple to predict putative specific probes that will detect it. This should greatly simplify the task of identifying and validating these probes.

3.2. Blood meal identification

We have used a human Alu repeat to detect human blood in field collected mosquitoes. This probe is sensitive enough to detect human blood meals 48 hours after feeding, when the meal is almost totally digested. Using this approach, we have studied collections of blood fed *A. quadrimaculatus* from Manatee Springs, a park in North Florida.

We have previously shown that three species of *A. quadrimaculatus* occur at this location: species A, B, and C1. An excellent probe already exists for species A [9], and we isolated two other probes, one which hybridizes to both species A and B, and one that hybridizes to species C1, C2, and D. Since species C2 and D do not occur at this site, these three probes are sufficient.

Four identical sets of filters were screened with the Alu probe and the three *A. quadrimaculatus* probes. By superimposing the filters, it was easy to determine the species of each individual and which individuals had taken human blood. The higher prevalence of human blood in species A females indicates that this species is much more likely to feed on humans than sympatric species B or C1 (figure 2, table 2). When we pooled the data for the two campgrounds, the human blood feeding rates were significantly higher for species A than for species B or C1. The numbers of mosquitoes collected at the B pond site were too few for statistical analysis, but the low number of human blood meals in species B and C1 is consistent with the two campgrounds.

3.3. Disease detection in individual wild mosquitoes

In collaboration with researchers in Venezuela, we are using our species-specific probes in conjunction with the US Army's ELISA kit for detection of malaria parasites in mosquitoes to study the infection rates of species of the subgenus *Nyssorhynchus* in a hyperendemic region of South America. DNA sequences are available for most important arboviruses, and these can be used to predict sequences for use as probes or PCR primers. Kits for the detection of arboviruses are under development at the Centers for Disease Control and Colorado State University laboratories in Fort Collins. We plan to collaborate with these groups on the development and application of these kits in screening wild populations.

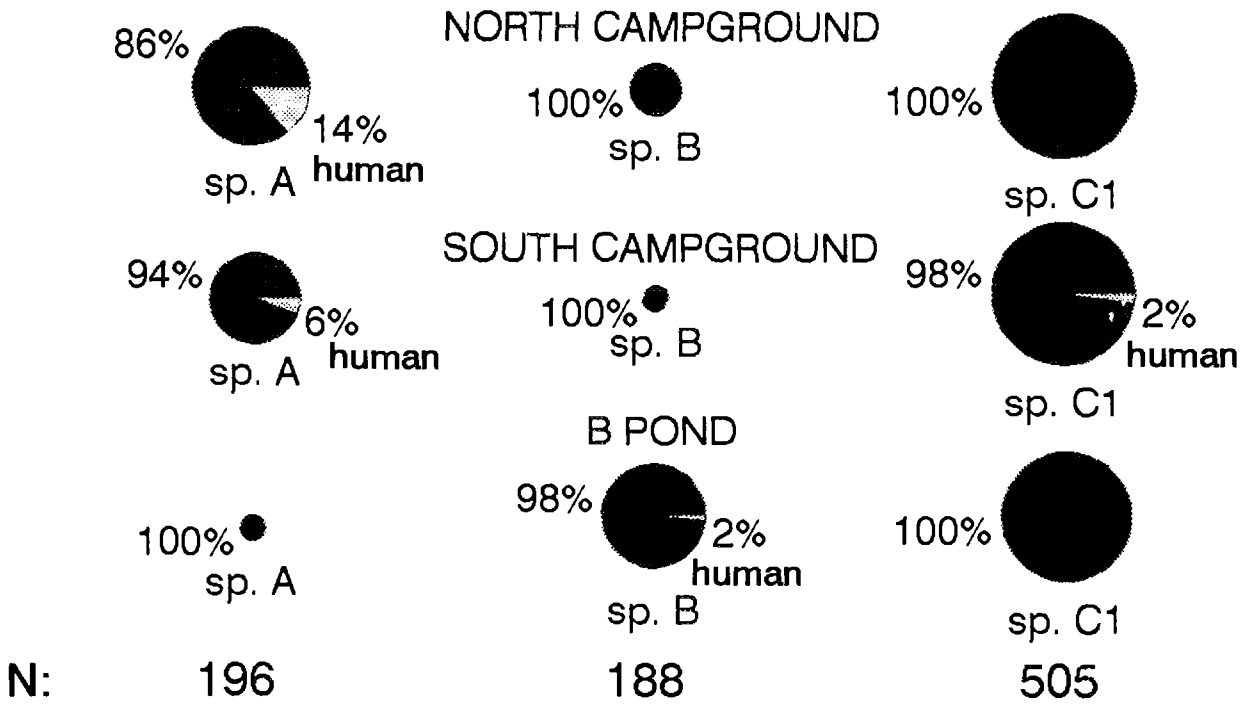

FIG.2. Human and nonhuman blood in An. Quadrimaculatus (from Manatee Springs, Florida).

Table 2: Numbers of blood fed mosquitoes of three species of *A. quadrimaculatus* complex from three locations at Manatee Springs State Park (blooded columns) and numbers that contain human blood (human columns).

	species A		species B		species C1	
	blooded	human	blooded	human	blooded	human
N. campground	106	15	36	0	167	0
S. campground	71	4	23	0	179	4
"B" pond	19	0	129	2	159	0
TOTALS	196	19	188	2	505	4

4. CONCLUSION

A common situation encountered when working with insect pests is the occurrence of several different species that look identical, at least to humans. This can be a problem because control efforts are partly wasted on innocuous species or because specific biocontrol agents (such as diseases or predators) attack the wrong species. Therefore, it is important to know whether a particular pest is a single species or a collection of several indistinguishable species. This paper discusses a multidisciplinary approach to identifying these species complexes, and explains how that approach has been applied to the study of several important mosquitoes.

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