anti-gp39 blocks both the ability of the T_H cell to trigger B cell activation (9) and the binding of CD40 to its ligand (*10*). This effect suggests that anti-gp39 binds at or near the CD40 binding site (*10*). Unlike treatments with other mAbs to T cells or fusion proteins (*19, 20*), it does not appear that treatment of mice with anti-gp39 prevents the priming of antigen-specific T_H cells or causes the cytotoxic or functional deletion of these cells. The results of experiments showing that unaltered T_H function can be adoptively transferred from mice treated with anti-gp39 (*11*) suggest that anti-gp39 administration does not result in T_H tolerance. The blockade of gp39 function has potential therapeutic benefits for affecting the onset of autoimmune disease.

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Synthesis and Degradation of Cyclic ADP–Ribose by NAD Glycohydrolases

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Cyclic adenosine diphosphoribose (cADPR), a recently discovered metabolite of nicotinamide adenine dinucleotide (NAD), is a potent calcium-releasing agent postulated to be a new second messenger. An enzyme that catalyzes the synthesis of cADPR from NAD and the hydrolysis of cADPR to ADP-ribose (ADPR) was purified to homogeneity from canine spleen microsomes. The net conversion of NAD to ADPR categorizes this enzyme as an NAD glycohydrolase. NAD glycohydrolases are ubiquitous membrane-bound enzymes that have been known for many years but whose function has not been identified. The results presented here suggest that these enzymes may function in the regulation of calcium homeostasis by the ability to synthesize and degrade cADPR.

The concentration of cytosolic free Ca^{2+} is a key factor in cellular regulation (*1*). Many extracellular signals stimulate the synthesis of the second messenger inositol 1,4,5-trisphosphate, which activates membrane Ca^{2+} channels resulting in Ca^{2+} release from internal stores (*2*). Reports of Ca^{2+} mobilization that appear to be unrelated to inositol polyphosphate metabolism suggest the presence of other second messengers involved in the regulation of Ca^{2+} homeostasis (*2*). A likely candidate for such a second messenger is cADPR, a metabolite of NAD. Cyclic ADP-ribose is a potent mediator of Ca^{2+} release in sea urchin egg microsomes (*3, 4*) and mammalian cells (*5, 6*). A saturatable site for binding cADPR exists in sea urchin egg microsomes (*7*), and cADPR has been implicated in Ca^{2+}-induced Ca^{2+} release (*8–10*) and insulin secretion (*6*).

Low amounts of cADPR have been found in all mammalian tissues tested (*11*); however, little is known about the enzymes that synthesize and degrade this nucleotide. A molluscan enzyme that catalyzes the conversion of NAD to cADPR has been purified and cloned (*12–14*). In mammalian tissues an activity that generates cADPR has been detected (*7, 15*), but no enzyme has been isolated. Although the presence of enzymes that degrade cADPR has been postulated (9), the occurrence of such an activity has not been demonstrated. We detected cADPR degradative activity in a variety of animal tissue homogenates. We report here the isolation and characterization of an enzyme from canine spleen microsomes that efficiently catalyzes the hydrolysis of cADPR to ADPR. This enzyme also catalyzes the synthesis of cADPR from NAD. The ability of the enzyme to catalyze the net conversion of NAD to ADPR identifies it as an NAD glycohydrolase (NADase). These results suggest the possibility of a new function for these membrane-bound enzymes that have been known for many years, yet whose function has remained an enigma.

We developed a rapid assay in which [^{32}P]cADPR serves as substrate (*16*). The

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Table 1. Purification of cADPR hydrolase from canine spleen.

Fraction	Protein (mg)	Total activity (U)*	Specific activity (U/mg)	Fold purification	Yield (%)	NADase/ cADPRase†	ADPR cyclase/ cADPRase†
Tissue extract	7310	164	0.022	1	100	2.81	0.049
Crude microsome	2300	125	0.054	2.4	76	2.75	0.054
Blue A Sepharose	79.9	33.2	0.416	18.6	20.3	3.59	0.071
Con A–Sepharose	24.3	24.4	3.36	150	14.9	2.72	0.050
Red A Sepharose	0.036	12.8	357	15900	7.8	2.87	0.057

*One unit of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 μmol of cADPR to ADPR per min at 37°C under the assay conditions described in (*16*). †Ratios of activities of NADase or ADPR cyclase to cADPR hydrolase (cADPRase). One unit of activity is defined as the amount of enzyme that results in the net formation of 1 μmol of ADPR (NADase) or cADPR (ADPR cyclase) per min at 37°C under the assay conditions described in (*35, 36*).

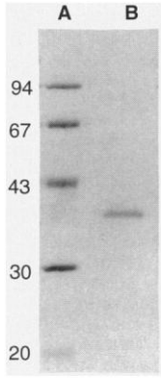


Fig. 1. Coomassie blue–stained SDS-PAGE (10% gel) of purified cADPR hydrolase from canine spleen. Lane A, molecular size standards (in kilodaltons); lane B, purified cADPR hydrolase (0.5 μg). This preparation was used for all experiments described in this report.

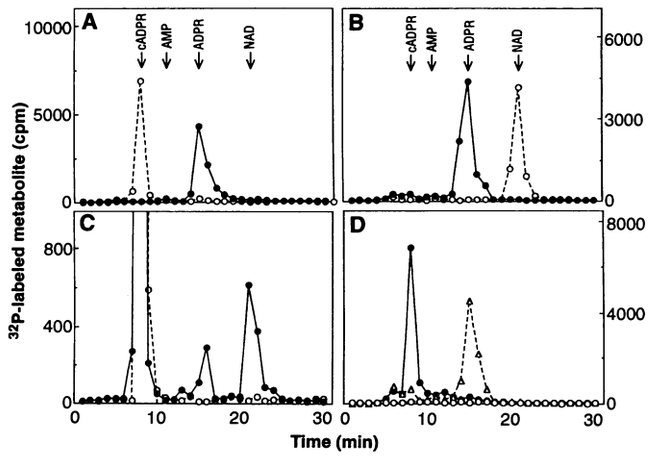


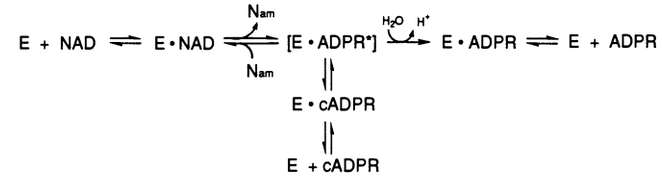
Fig. 2. HPLC analysis of enzymatic products produced by canine spleen cADPR hydrolase. After the incubations described below, samples were prepared for HPLC (*34*). Unlabeled standards were included as carriers during chromatography and their elution positions monitored by absorbance at 260 nm as indicated by the arrows. (**A**) Degradation of cADPR. Each reaction mixture containing 200 μM [^{32}P]cADPR (10^5 cpm), 20 mM Hepes (pH 7.2), and 0.1% Triton X-100 in a total volume of 50 μl was incubated at 37°C for 10 min in the presence (closed circles) or absence (open circles) of 1 milliunit of canine spleen cADPR hydrolase. (**B**) Degradation of NAD. The reaction conditions are as described in (A), except that 200 μM [^{32}P]NAD was used in place of [^{32}P]cADPR. Closed circles represent a complete incubation and open circles an incubation in which enzyme was omitted. (**C**) Formation of NAD from cADPR and nicotinamide. The reaction mixture contained 200 μM [^{32}P]cADPR (10^5 cpm) and 20 mM nicotinamide in a volume of 50 μl with incubation at 37°C for 10 min. The presence and absence of the enzyme during incubation are represented by closed circles and open circles, respectively. (**D**) Formation of cADPR from NAD. Each reaction mixture contained 200 μM [^{32}P]NAD (10^6 cpm) and 0.5 milliunit of enzyme in a total volume of 50 μl containing 20 mM Hepes (pH 7.2) and 0.1% Triton X-100 with incubation at 37°C for 20 min. The sample was incubated with 0.2 U of snake venom phosphodiesterase in 12.5 μl of buffer containing 0.5 M tris (pH 9.5) and 75 mM $MgCl_2$ for 30 min at 37°C. The reaction product was analyzed by HPLC directly (closed circles) or after boiling for 30 min (open triangles). Omission of the enzyme is represented by open circles.

substrate, cADPR, is resistant to hydrolysis by snake venom phosphodiesterase, whereas the product, ADPR, is efficiently converted to adenosine monophosphate (AMP) and ribose 5′-phosphate. Activity detected in an extract of canine spleen was associated with the crude microsomal membrane fraction. The enzyme was purified approximately 16,000-fold by a procedure that included isolation of microsomal membranes, solubilization with Triton X-100, and column chromatography on three different affinity matrices (Table 1) (*17*). The final preparation contained a single protein band of 39 kD when analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions (Fig. 1).

The purified enzyme catalyzes the hydrolysis of cADPR to ADPR (Fig. 2A). Confirmation of the product as ADPR was established by two additional experiments. Treatment with phosphodiesterase yielded material that migrated with AMP, and combined treatment with phosphodiesterase and alkaline phosphatase yielded material that migrated with inorganic phosphate (*18*). The Michaelis constant (K_m) of the enzyme for cADPR was 46 μM. During characterization of the enzyme, NAD was identified as a potent inhibitor with an apparent inhibition constant (K_i) of 8.3 μM. This led us to examine the possibility that NAD was also a substrate for this enzyme. Indeed, the enzyme efficiently catalyzed the conversion of NAD to ADPR (Fig. 2B). The estimated K_m for NAD of 9.9 μM is very similar to the K_i of 8.3 μM for inhibition of the cADPR hydrolase activity by NAD. The ratio of NADase to cADPR hydrolase remained constant throughout each step of the purification (Table 1). From these results, we conclude that this enzyme has both cADPR and NAD hydrolytic activities.

Membrane-associated NADases that catalyze the conversion of NAD to ADPR have been known for many years (*19, 20*). The mechanism of these enzymes has been well characterized (*21, 22*). The cleavage of the nicotinamide-ribose bond has been proposed to generate an enzyme-stabilized ADP-ribosyl oxocarbonium ion intermediate [E·ADPR*] (*23, 24*). This intermediate can react with water to yield ADPR or with nicotinamide to regenerate NAD with retention of the β configuration (*25, 26*) (Scheme 1). To explain the ability of the enzyme characterized in this study to convert both NAD and cADPR to ADPR, we propose a mechanism in which the hydrolysis of cADPR and NAD involves a common intermediate (Scheme 1). If this mechanism is correct, the enzyme should catalyze the synthesis of NAD from cADPR in the presence of nicotinamide. We found that when the enzyme was incubated with [^{32}P]cADPR in the presence of 20 mM nicotinamide and the reaction mixture was examined for the formation of [^{32}P]NAD by high-performance liquid chromatography (HPLC), NAD formation was observed (Fig. 2C). The amount of NAD formed was dependent on the concentration of nicotinamide and was maximal at 20 mM (*18*). In the absence of the enzyme, [^{32}P]NAD was not detected, ruling out the possibility of contamination of [^{32}P]cADPR with [^{32}P]NAD.

In the proposed mechanism, the enzyme can also catalyze the synthesis of cADPR from NAD if the step between E·cADPR and [E·ADPR*] is reversible. Therefore, the enzyme was incubated with [^{32}P]NAD and the reaction mixture was examined for the presence of [^{32}P]cADPR (Fig. 2D). Unreacted [^{32}P]NAD and the [^{32}P]ADPR formed were removed by treatment with phosphodiesterase, and [^{32}P]cADPR was isolated by chromatography on DHB Bio-Rex 70 and by HPLC. The material isolated coeluted with authentic cADPR (Fig. 2D). We have observed that cADPR can be converted quantitatively to ADPR by boiling for 30 min at neutral pH. When the isolated product was boiled, it was quantitatively converted to material that coeluted with ADPR (Fig. 2D). In the absence of the enzyme no detectable cADPR was observed, ruling out the possibility of contamination of [^{32}P]NAD with [^{32}P]cADPR.



Scheme I. Proposed mechanism for the synthesis and hydrolysis of cADPR by NADases.

These data demonstrate that the enzyme has ADPR cyclase activity. Further, the ratio of ADPR cyclase activity to cADPR hydrolase activity remained constant throughout purification (Table 1). Thus, this enzyme catalyzes both the synthesis and degradation of cADPR.

NADases represent one class of enzymes that catalyze ADPR transfer. Enzymes that catalyze ADPR transfer utilize the high group transfer potential of the linkage between nicotinamide and ribose to modify many different cellular nucleophiles with ADPR (*27*). Other classes of ADPR transferases include poly-ADPR polymerase and many different protein:monoADPR transferases (*28*). Although functions for several other ADPR transfer enzymes are emerging (*29*), the function of the NADases has been a long-standing puzzle. The finding that the NADase described here can synthesize and hydrolyze cADPR raises the possibility that NADases function in Ca^{2+} homeostasis. This possibility is consistent with studies showing a close correlation between NAD hydrolysis and Ca^{2+} release in mitochondria (*30*).

To test whether the ability to both synthesize and hydrolyze cADPR represents a general property of NADases, we purified NADase to apparent homogeneity from *Bungarus fasciatus* venom (*31*). This enzyme effectively catalyzed the hydrolysis of cADPR to ADPR, the conversion of cADPR to NAD in the presence of nicotinamide, and the conversion of NAD to cADPR (*18*). Thus, the ability to synthesize and hydrolyze cADPR may be a property of other eukaryotic NADases.

The enzyme described here has properties similar to an enzymatic activity detected in canine brain that synthesized cADPR (*7*). The similarities include (i) its membrane location, (ii) chromatographic properties on Con A–Sepharose, (iii) pH activity profile, and (iv) relative rates of ADPR cyclase and NADase activities. A low ratio of ADPR cyclase to NADase activity (*7*) was attributed to contaminating NADase activity. However, in our proposed mechanism the net accumulation of cADPR would be a function of the relative rates of disassociation of cADPR from the enzyme and of cADPR hydrolysis by the enzyme. Of course, the relative rates of dissociation and hydrolysis (and thus net accumulation) of cADPR that we measure in detergent-solubilized enzyme may not reflect the rates in vivo.

Two possible mechanisms can be envisioned for the involvement of NADases in the metabolism of cADPR and regulation of Ca^{2+} homeostasis. On stimulation, the enzyme may initially function to generate cADPR. This could be achieved, for example, by extrusion of a substrate water molecule from the active site of NADase (*32*), which would prevent the hydrolysis to ADPR and allow the synthesis of cADPR. The cADPR hydrolase activity could function independently in cADPR degradation. An alternative possibility could involve the ADPR cyclase and cADPR hydrolase activities functioning in a concerted manner. On stimulation, the enzyme-cADPR complex may be involved directly in channel activation. Such activation would be transient, because inactivation could occur via the intrinsic cADPR hydrolase activity. Further studies will be required to determine whether NADases function in Ca^{2+} homeostasis. Nevertheless, results described here should stimulate investigations of these membrane enzymes from a new perspective.

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16. [^{32}P]cADPR was prepared with ADPR cyclase isolated from *Aplysia* ovotestis as described (*13*). We measured the activity of cADPR hydrolase by first converting the product, [^{32}P]ADPR, to [^{32}P]AMP by treatment with phosphodiesterase. Subsequently, [^{32}P]AMP was separated from unreacted substrate on the highly selective affinity adsorbent dihydroxyboronyl Bio-Rex 70 (DHB Bio-Rex 70) (*33*). The assay mixture, containing 10 μl of enzyme fraction and 40 μl of 250 μM [^{32}P]cADPR (~50,000 cpm) in buffer A [20 mM Hepes (pH 7.2) and 0.1% Triton X-100], was incubated at 37°C for 10 min. The reaction was terminated by addition of snake venom phosphodiesterase (0.1 U) in 12.5 μl of a buffer containing 0.5 M tris (pH 9.5) and 50 mM $MgCl_2$. After incubation at 37°C for 15 min, samples were diluted to 1.0 ml with 0.25 M ammonium formate, pH 9.0 (buffer B), and applied to 0.4-ml columns of DHB Bio-Rex 70 equilibrated in buffer B. The flow-through and wash fractions (4 ml) were combined, and [^{32}P]AMP was quantitated by liquid scintillation counting.
17. For purification of cADPR hydrolase from canine spleen, tissue (100 g) was homogenized in 350 ml of ice-cold buffer [20 mM Hepes (pH 7.2), 1 mM $MgCl_2$, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and soybean trypsin inhibitor (50 μg/ml)] in a Waring blender for 1 min. Tissue extracts were obtained by centrifugation of the homogenate at 8,000*g* for 10 min. Crude microsomes were prepared by centrifugation of the tissue extract at 50,000*g* for 1 hour. The hydrolase activity was solubilized with homogenization buffer (350 ml) containing 2% Triton X-100, and the insoluble material was removed by centrifugation at 50,000*g* for 1 hour. The supernatant fraction was applied to a 150-ml column of Blue A Sepharose (Pharmacia) equilibrated with 0.2 M NaCl in buffer A (*16*). The column was washed with 300 ml of 0.5 M NaCl in buffer A and eluted with 300 ml of 0.5 M KSCN in buffer A directly onto a 50-ml column of hydroxyapatite (Bio-Rad) connected in series. After washing of the hydroxyapatite column with 100 ml of 0.1 M NaCl in buffer A, the enzyme activity was eluted with 120 ml of 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl. The eluate was then applied to a 20-ml column of concanavalin A (Con A)–Sepharose (Sigma) equilibrated with 0.2 M NaCl in buffer A. The column was washed with 50 ml of 0.1 M NaCl in buffer A and then with 40 ml of buffer A containing 0.2 M glucose and 0.1 M NaCl. The bound enzyme was eluted with 55 ml of 0.5 M methyl-α, D-glucopyranoside, and 0.1 M NaCl in buffer A. The eluate was further fractionated on a 2.0-ml column of Red A Sepharose (Pharmacia) equilibrated with 0.2 M NaCl in buffer A. After washing of the column with 0.2 M NaCl in buffer A, the enzyme was eluted with 15 ml of a buffer containing 50 mM isonicotinic acid hydrazide, 0.2 M NaCl, 0.1% Triton X-100, and 50 mM sodium phosphate (pH 7.2). The enzyme solution was desalted and concentrated by binding to a 1.0-ml column of Con A–Sepharose and elution with 2.0 ml of elution buffer as described above.
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34. After incubation (Fig. 2), samples were diluted to 1.0 ml with 0.25 M ammonium formate buffer (pH 9.0) and applied to 0.3-ml columns of DHB Bio-Rex 70. The columns were washed with the same buffer and eluted with 4 ml of deionized water. Portions containing 10,000 cpm were analyzed by HPLC. Chromatography was done on a 0.46 by 12.5 cm Partisil 5 SAX column and a 0.46 by 12.5 cm μBondapak C_{18} column connected in tandem and eluted with 0.25 M ammonium formate buffer (pH 4.0) at a flow rate of 1 ml/min. This system allows the convenient isocratic separation of

cADPR, AMP, ADPR, and NAD in a single chromatographic step. Fractions were collected and counted for radioactivity.

35. NADase activity was measured by separation of released [carbonyl-^{14}C]nicotinamide from NAD with Bio-Rad AG X4 anion exchange resin. The assay mixture (50 μl) contained 10 μl of protein fraction and 200 μM [carbonyl-^{14}C]NAD (50,000 cpm) in buffer A (*16*) and was incubated at 37°C for 5 min. The reaction was terminated by addition of 1 ml of 0.1% SDS. The sample was applied to a 0.4-ml column of AG X4 resin, which was subsequently washed with 4 ml of deionized water. The flow-through and wash fractions were combined, and [^{14}C]nicotinamide was determined by liquid scintillation counting.
36. ADPR cyclase activity was determined by addition of 10 μl of enzyme fraction to 40 μl of 250 μM [^{32}P]NAD (0.5 × 10^6 cpm) in buffer A (*16*). After incubation at 37°C for 10 min, the reaction mixture was treated with phosphodiesterase (0.1 U), diluted, and applied to a 0.4-ml column of DHB Bio-Rex 70 equilibrated with buffer B (*16*). After washing of the column with 30 ml of buffer B, [^{32}P]cADPR was eluted with 5 ml of deionized water and quantitated by liquid scintillation counting.
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TECHNICAL COMMENTS

Determining Whitefly Species

The statement in the report by T. M. Perring *et al.* (*1*) that the "superbug" is not a strain of the sweetpotato whitefly, *Bemisia tabaci* (Gennadius), but a new species, seems premature. When more than 25 pairs of males and females of both strains were placed together, interstrain mating resulted in the production of viable, hybrid females (*2*). Field collections made in the Imperial Valley of California in 1992 revealed that feral populations of the two strains had interbred. Hybrid whiteflies that had fixed (not induced) esterase loci from both "A" and "B" strain parents were clearly identified (*2–4*).

Perring *et al.* used single primer polymerase chain reaction amplification (RAPD-PCR) and found that genetic differences between the strains were at a "species" level, but RAPD-PCR fragments have revealed only arbitrary differences between the DNAs. "Genetic distances" of a size similar to those between *B. tabaci* strains are likely to be observed if either strain is compared to RAPD-PCR fragments generated from any number of randomly selected taxa (for example, another whitefly strain or species, dogs, or nematodes). The RAPD-PCR results in the report by Perring *et al.* are of potential diagnostic value, but of little phylogenetic utility.

When one of us (B.C.C.) compared more than 2000 nucleotides of genes in the ribosomal RNA (rRNA) transcript from *B. tabaci*, which included three variable expansion regions, the rDNA in those strains was identical (*5*, *6*). Sequences of 28S rDNA D2 expansion regions (550 nucleotides) have been found to be identical in the *B. tabaci* strains, whereas 40 nucleotide substitutions have been found in ash and greenhouse whiteflies (*5*). The D2 expansion region has been used to deduce phylogenies of subgenera and sibling species of *Drosophila* (*7*). Whiteflies also have uncommonly elongated (≈2450 nucleotides) 18S rDNAs (*6*). This extra length stems from two internal, variable expansion regions (*8*). The 18S rDNA of the two *B. tabaci* strains has been found to be identical, whereas 60 to more than 100 nucleotide substitutions have been found in ash, iris, and greenhouse whiteflies (*5*, *6*). Sternorrhynchans (for example, aphids and whiteflies) have maternally heritable, procaryotic endosymbionts (*9*). An earlier study of endosymbiont 16S rDNA found that aphid endosymbiosis resulted from a singular infection of a primordial ancestor during the Triassic. Since that time, aphids and their endosymbionts have cospeciated, resulting in congruent phylogenies (*10*). Whitefly endosymbiosis follows a similar congruency, wherein endosymbiont 16S rDNA distinguishes whitefly species (*6*, *11*). Both strains of *B. tabaci* have two endosymbionts. The nucleotide sequences of 16S rDNAs (≈ 1600 nucleotides each) of the respective endosymbionts have been found to be identical in the *B. tabaci* strains, whereas 70 nucleotide substitutions have been found in greenhouse and ash whiteflies (*11*). In summary, our mating and phylogenetic studies do not support the conclusion of Perring *et al.* that the "superbug" is a new species of whitefly.

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Perring *et al.* (*1*) interpret the results of experiments on two forms of *B. tabaci* to mean that the two forms are distinct species. This conclusion seems premature because the results are not only preliminary, but unconvincing. Their estimation of differences between populations, based on the genetic distance measurements, is not appropriate for taxonomic purposes. The standard error of the Nei statistic depends on both the numbers of loci sampled (eight polymorphic loci were reported) and the sample size (which was not reported). It is not possible to judge the significance of the reported value without some estimate of the standard error of the calculated genetic distance. Perring *et al.* give a range of genetic distances between whitefly species, of 0.24 to 0.83. Which value really separates whitefly species? Perring *et al.* give no indication of whether the populations were in Hardy-Weinberg equilibrium (an assumption for the calculation of the Nei statistic), and the genetic differences they report could be a result of laboratory selection or founder effects.

Perring *et al.* find no evidence of mating between the A and B biotypes (*1*). Other researchers (*2*) have observed A × B hybrids when mating was attempted under conditions different from those used by Perring *et al.*

Products of RAPD-PCR are useful as genetic markers and for the construction of molecular genetic maps (*3*). However, we are not aware of any agreement in the literature about the interpretation of RAPD data for insect taxonomy. Our own unpublished data indicate that whiteflies (morphologically typical of *B. tabaci*) collected from several areas of the world show RAPD-PCR differences as great as those found between the A and B forms [as defined