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DNA BARCODING FOR TARGETING INVASIVE SPECIES AND NATURAL CONTROL AGENTS IN THE CARIBBEAN

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ABSTRACT: The development of DNA barcoding for species identification is a broad tool that has been emphasized in the recent decade. Since the 1980's, the increase of trade and exchange of goods has raised the number of invasive species reported. The new invaders are threatening the development of a sustainable agriculture, the preservation of natural habitats and raising the costs for urban landscaping. Molecular barcoding is part of an innovative approach to accurately identify and fingerprint life species. Barcoding methods are a valuable application for both pest ecology and biocontrol agents. Several universal mitochondrial and nuclear molecular markers of conservative regions such as Cytochrome Oxidase I (COI) and Internal transcribed spacer (ITS) have been used in barcoding for fingerprinting both pests and their natural enemies and have demonstrated high replication of results. Moreover, the development of new molecular markers targeting important arthropod and microorganism species has elucidated their taxonomic status. The objectives at the *Center of Excellence for Quarantine and Invasive Species* (CEQIS) laboratory are to generate and apply DNA barcoding tools and to build an integrative database of pests and beneficial organisms within the Caribbean and track their pathways. Current work at the laboratory addresses the identification of parasitoid communities associated to whiteflies in Solanaceae, Leguminosae and alternate host plants. Plant host species are also identified by both classic morphology and DNA barcoding. Barcoding clarifies discrepancies between morphological and molecular identification. Molecular approaches used clarify the taxonomy of the parasitoid complex associated to the Harrisia cactus mealybug, *Hypogeococcus pungens*. So far, other biological control agents have been targeted for barcoding at the *CEQIS* and include mites, coleopteran and coccinellid.

Keywords: DNA barcoding, molecular marker, biological control, invasive arthropods

RESUMEN: El desarrollo de códigos de barras de ADN para la identificación de especies es una herramienta amplia que se ha utilizado en las últimas dos décadas. El aumento del comercio e intercambio de bienes en las últimas décadas aumentó el número de especies invasoras reportado en todas partes. Los nuevos invasores amenazan el desarrollo de una agricultura sostenible y la conservación de los hábitats naturales. El uso de estas técnicas moleculares es parte de un enfoque innovador para identificar especies nocivas y beneficiosas con precisión. Los métodos de código de barras genético son una valiosa aplicación de la ecología de las plagas y de agentes de control biológico. En estos códigos de barras se han utilizado varios marcadores moleculares tanto mitocondriales (citocromo oxidasa I; COI), como nucleares (ITS) de

uso universal, de regiones conservadas que muestra alta reproducibilidad de los resultados, para la identificación de plagas y sus enemigos naturales. Por otra parte, el desarrollo de nuevos marcadores moleculares dirigidos a artrópodos importantes y especies de microorganismos podría esclarecer su estado taxonómico. Los objetivos de nuestro laboratorio son generar y aplicar las herramientas de "barcoding" y crear una base de datos integral de plagas y organismos benéficos en el Caribe. El trabajo actual se ocupa de la identificación de las comunidades de parasitoides asociados a las moscas blancas en las solanáceas, leguminosas y plantas hospederas alternativas. Las especies de plantas huéspedes también son identificadas por la morfología clásica y por códigos de barras de ADN. El "barcoding" aclara discrepancias entre la identificación morfológica y molecular. Enfoques moleculares ayudaron a esclarecer la taxonomía del complejo de parasitoides asociados a la chinche del cactus, *Hypogeococcus pungens*. Otros agentes de control biológico han sido identificados en el Centro de Excelencia para las Especies Invasoras y Cuarentena, como ácaros, coleópteros y coccinélidos.

Palabras claves: código de barras del ADN, marcadores moleculares, control biológico.

Introduction

One of the most difficult problems in working with new plant pests and diseases is the accurate identification of their main and associated species. This problem is largely due to the small size of arthropods, bacteria, fungi and viruses' and lack of easily distinguishable morphological characteristics. Molecular tools, including DNA barcoding, have provided clear advances toward the early and rapid identification of invading organisms. The deficiencies in the diagnosis of plant diseases or in identifying their vectors cause extreme quarantine limitations (Childers and Rodrigues 2005). It is important to combine new technologies such as DNA molecular markers for barcoding identification with morphological identification. A molecular marker is a particular sequence of DNA that is identifiable within the context of an entire genome. There are different types of DNA molecular markers that are useful for studies of genetic diversity and phylogenetic relationships. DNA barcoding is a technique useful for identifying species of organisms using a short DNA sequence from a standard position in the genome. DNA barcodes are short sequences of genome and they can be developed quickly and cheaply. The cytochrome C oxidase subunit 1 mitochondrial region (COI) is the standard barcode region for higher animals (Ratnasingham and Hebert 2007)

Due to the rapid spread of organisms associated with trade and quick transportation in the world, it is important to develop strategies for rapid identification and design of management plans, especially when related to new species invasions.

The Caribbean region is regarded as one of the world's biodiversity "hotspots" with approximately 7000 species of endemic plants and 780 species of endemic vertebrates (Mittermeier et al. 2005, BirdLife International 2010). The invertebrate endemism is extensive, but poorly documented. Advances in world transportation have enabled invasive organisms to move easily between geographic zones, leading to an increase in

biologic invasions in the past few years (Seebens et al. 2013). This is a major problem related to the dispersion of pests, vectors and diseases.

Current Work and Protocol

Puerto Rico is a key location to to survey for invasive organisms. There is a strong topographic effect on the environmental and ecological conditions, with the wet regions predominant in the northern region, high altitudes in the central mountains and semi arid regions in the south (Gould et al. 2008). In addition, six different life zones can be distinguished across the island (Holdridge 1967, Ewel & Whitmore 1973). In work related to projects on mitigating invasive species in Puerto Rico we collected samples from the all over the island which were studied at the Río Piedras Experimental Research Station, University of Puerto Rico (18°23'27" N, 66°03'23" W) (see Figure 1). DNA was extracted from a minimum of 10 single specimens of each population using the CTAB protocol (Rodrigues et al. 2004). To characterize the specimens of each population, DNA was amplified from a mitochondrial cytochrome oxidase I (COI) gene fragment, which is generally conserved in its composition and gene order and has shown reliable sequence divergence at the species and higher level for many insect taxa (Simon et al. 1994, ScheVer & Grissell 2003). To amplify the mitochondrial (COI), ribosomal (18S, 28S) and nuclear (ITS) regions from different organisms the primers used are detailed in Table 1. The PCR cycling conditions depend on the markers used, and the fragment size (bp, base pair) that is expected to be amplified. Our best results come while amplifying DNA fragments for fingerprinting is between 400 bp and 900 bp. Amplified fragments are visualized in a 1% agarose gel stained with etidium bromide under UV light. The predicted amplicon were cleaned up using the Exo-Sap protocol (PCR product: 5 ul; Exo: 0.30 ul; SAP: 0.45 ul; ddH2O: 2.25 ul), mixed to homogenize, incubated at 37°C by 30 min and heated to inactivate ExoSAP 85°C during 15 min. The products were sequenced at the UPR-Sequencing and Fingerprint Facility (SGF) core laboratory using BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3130 xl DNA sequencer.

Different organisms have been studied at the CEQIS laboratory including invasive species and natural control agents. Invasive species studied include plant feeding mites, Harrisia cactus mealybugs and whitefly, and their natural enemies such as the parasitoids *Leptomastidea* spp. and *Encarsia formosa*.

Results and Discussion

Mealybugs

Because of the unclear taxonomic and molecular identification of Harrisia Cactus Mealybugs (HCM), field samples of this species were collected in places where HCM has been observed and samples of other mealybugs species were present in the zone in association with cacti. Field sampling was conducted in the south of Puerto Rico, in sites with large areas with cacti in the dry forest. At the laboratory, the species of mealybug that is attacking the cactus in Puerto Rico was identified by means of molecular techniques. The specimens of mealybugs were collected from infested

columnar cacti and other alternate hosts in Puerto Rico. Some specimens were mounted on slides and deposited in the Museum of Entomology at the Agricultural Experiment Station of the UPR. For characterization of the DNA samples of each population, nine different regions were amplified with primers specific to them, using both mitochondrial and nuclear regions (see Table 1). Table 2 summarizes the identification of specimens by DNA analysis and their correspondent target species names for recent projects. For the HCM, the only species found in PR was *H. pungens.*

Whiteflies

Whiteflies are a complex species group of cosmopolitan distribution and have largely impacted production and food economy. About 1,200 species have been described, most have different host plants, but some are specific. Among the most important crop pests in the world are found *Bemisia tabaci, Trialeurodes vaporariorum, Bemisia argentifolii* and *Aleurothrixous flocossus*. These species attack a wide variety of ornamental, wild and cultivated plants. The aim of the present work is to determine the occurrence and distribution of whitefly populations, their natural hosts as well as main invasive plant viruses associated with their hosts, on the island of Puerto Rico. Collection routes were established to survey different geographical and ecological areas around the island. Each sampling site was photo documented and georeferenced by GPS. Eighty-three sites were visited to collect 291 insect organisms and 1200 plant samples, an average of three populations per site. Plant samples of the collection part of the laboratory, had their DNA extracted for identification and generation of barcode using a chloroplast gene (rbcLa) and subsequent analysis of associated viral hosts. The whiteflies were prepared for morphological identification (slide-mounted) and DNA was extracted for diversity analyses. Emerging parasitoids were similarly identified (see Table 1). The most frequent hosts plant families for whiteflies in Puerto Rico found during the survey were Convolvulaceae, Euphorbiaceae and Moraceae. Three whitefly species were identified: *Aleirodicus dispersus*, *Aleurocanthus spiniferus* and *Bemisia tabaci* (see Table 2). So far, the incidence of whitefly in inspected plants in Puerto Rico was 86% in plants and 88 % at the study sites. Frequency appears to decrease with increased humidity.

Natural enemies

Leptomastidea spp. parasitoids (Encyrtidae, Hymenoptera) are important in the biological control of mealybugs. Three populations of *Leptomastidea* sp. (Barbados, Puerto Rico and Florida) were analyzed and resolved using molecular techniques to detect differentiation in their genetic profile using specific primers to amplify COI mitochondrial and ITS regions (see Table 1).

In relation to *Leptomastidea* sp., one unique haplotype was found among the three populations of Puerto Rico (n=3) Barbados (n=1) and Florida (n=2). Using both genomic regions the haplotypes did not present geographical clustering (see Table 2). *Encarsia formosa* is another parasitoid that was found associated to whiteflies in Puerto Rico.

Mites

A complex of Tetranychoidea mites have been identified at the molecular level as well as the association of *Brevipalpus* mites with viruses monitored by use of molecular tools. In addition, Rivera-Rivera et al. (2012) reported the development of molecular tools to identify and testing prey DNA fingerprinting on *Amblyseius largoensis* (Acari: Phytoseiidae) predation of *Raoiella indica.*

Conclusion

The molecular tools used and the morphological examinations of specimens is crucial to accurately identify a growing number of target species reaching new agricultural and natural landscapes. The increase of training programs and development of integrated efforts among Caribbean nations to track pathways and prevent the introduction of invasive species is imperative to preserve local agriculture and natural resources. The use of molecular tools enhanced the CEQIS laboratory capability in detecting and clarifying taxonomic status for several target arthropod groups of quarantine relevance.

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Table 1. List of target arthropod genomic regions and molecular probes routinely performed at CEQIS laboratory, at University of Puerto Rico, Agricultural Experimental Station, Río Piedras, Puerto Rico.

Organism	Locus	Primer names	Primer sequence	PCR product (bp)	References
Whitefly	COI	C1-J-2183	CAACATTTATTTTGATTTTTTGG	385	Gullan et al. 2003
		C1-N-2568	GCWACWACRTAATAKGTATCATG		
	ITS1	TW81	GTTTCCGTAGGTGAACCTGC	950	Brust et al. 1998
		5.8R	ATCCGCGAGCCGAGTGATCC		De Barro et al. 2000
	16S	operon F2	GAGGATCCCT	600-900	De Barro and Driver 1997
		F12	ACGGTACCAG		
		H9	TGTAGCTGGG		
		H16	TCTCAGCTGG		
		4119	CGCCTGTTTAACAAAAACAT	500-600	Xiong and Kocher 1991
		4118	CCGGTCTGAACTCAGATCACGT		
	28s	None(D2)	AGAGAGAGTTCAAGAGTACGTG	320	Belshaw and Quicke 1997
		None(D2)	TTGGTCCGTGTTTCAAGACGGG		
Mealybugs					
	COI	LCO-M-2d-F	ATAACTATACCTATYATTATTGGAAG	491	Malausa et al. 2011
		LCO-M-2d-R	AATAAATGTTGATATAAAATTGG		
	ITS2	ITS2-M-F	CTCGTGACCAAAGAGTCCTG	800	Malausa et al. 2011
		ITS2-M-R	TGCTTAAGTTCAGCGGGTAG		
	rpS15-16ST	leuA	GTATCTAGAGGNATHCAYCARGAY GGNG	1050	Baumann et al. 2002
		U16S	GCCGTMCGACTWGCATGTG		
	18S	18S-2880	CTGGTTGATCCTGCCAGTAG	589-671	von Dohlen and Moran

					1995
		18S-B	CCGCGGCTGCTGGCACCAGA		
	28S	none	GTAGCCAAATGCCTCGTCA	738-767	Dietrich et al. 2001
			CACAATGATAGGAAGAGCC		
	EF-1a	M3	CACATYAACATTGTCGTSATYGG	306-384	Cho et al. 1995
		rcM44.9	CTTGATGAAATCYCTGTGTCC		
		M51.9	CARGACGTATACAAAATCGG	372-506	Cho et al. 1995
		rcM53-2	GCAATGTGRGCIGTGTGGCA		
Leptomastidea					
	COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	680	Folmer et al. 1994
			TAAACTTCAGGGTGACCAAAAAATC		
		HCO2198	A		
	18SrRNA	ITS5	GGAAGTAAAAGTCGTAACAAGG	550-600	White et al. 1990
			CACGAGCCGAGTGATCCACCGCTA		
	5.8S RNA	rna2	AGAGT		
	CAS18Fs1		TACACACCGCCCGTCGCTACTA		Ji, Y et al. 2003
Acari					
	COI	COI F	TGATTTTTTGGTCACCCAGAAG	410	Navajas et al. 1996
Tetranychoidaea		COI R	TACAGCTCCTATAGATAAAAC		Rivera-Rivera et al. 2012

Table 2. Example for identification of specimens of mealybugs, whitefly and parasitoids using different primer combination and sequences. Sequences producing significant alignments (Max ident) at GeneBank database (BLAST, NCBI)

Gene	*Species – closest match*	Max ident.	Locality	Hosts or origin
18S	*Hypogeococcus pungens*	100%	Hawaii	*Nototrichium divaricatum*
(589–671 bp)	*Hypogeococcus pungens*	98%	Guanica-PR	Galls-*Pilosocereus royeni*
	Hypogeococcus pungens	95%	Guanica-PR	Galls-*Pilosocereus royeni*
	Hypogeococcus pungens	99%	San Juan-PR	Portulaca
	Hypogeococcus pungens	84%	Cabo Rojo-PR	Galls-*Pilosocereus royeni*
	Planococcus citri	100%	San Juan-PR	Portulaca
	Hypogeococcus pungens	77%	Cabo Rojo-PR	Galls-*Pilosocereus royeni*
28S	*Hypogeococcus pungens*	99%	Hawaii	*Nototrichium divaricatum*
(~320 bp)	*Hypogeococcus pungens*	97%	San Juan-PR	Portulaca
	Planococcus citri	98%	San Juan-PR	Portulaca
	Hypogeococcus pungens	97%	Cabo Rojo-PR	Galls-*Pilosocereus royeni*
M51.9	*Hypogeococcus pungens*	99%	Hawaii	*Nototrichium divaricatum*
(372–506 bp)	*Hypogeococcus pungens*	96%	San Juan-PR	Portulaca

	Planococcus citri	99%	San Juan-PR	Portulaca
	Hypogeococcus pungens	98%	Cabo Rojo-PR	Galls-*Pilosocereus royeni*
LCO-COI	*Atrococccus paludinus*	94%	San Juan-PR	Portulaca
(~491 bp)	*Planococcus citri*	99%	San Juan-PR	Portulaca
ITS2	*Planococcus citri*	94%	San Juan-PR	Portulaca
(~800 bp)	*Phenacoccus solani*	100%	San Juan-PR	Portulaca
	Planococcus citri	99%	San Juan-PR	Portulaca
C1-J-2183	*Aleirodicus dispersus*	99%	Canovanas-PR	-
C1-N-2568	*Aleurocanthus spiniferus*	90%	Carolina-PR	-
	Bemisia tabaci	98%	Carolina-PR	-
HCO-LCO	*Metaphycus flavus*	88%	Puerto Rico	*Hypogeococcus pungens*
(680 bp)				
	Metaphycus flavus	88%	Barbados	*Hypogeococcus pungens*
	Metaphycus flavus	88%	Florida	*Hypogeococcus pungens*
C1-J-2183	*Encarsia formosa*	98%	Carolina-PR	-

BLAST - Basic Local Alignment Search Tool; NCBI - The National Center for Biotechnology Information

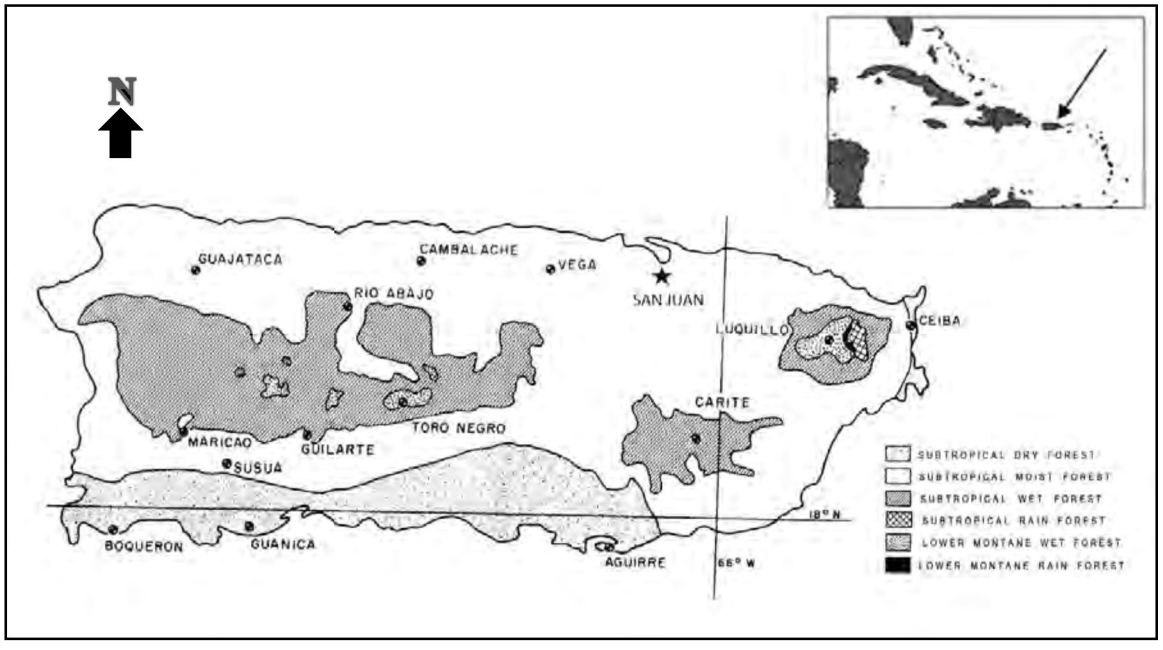

Figure 1. Life zones of mainland Puerto Rico took from Ewel and Whitmore (1973).

Black star indicate the laboratory location.