USDA-ARS National Clonal Germplasm Repository for Citrus & Dates
Riverside, California

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Introduction:

The National Clonal Germplasm Repository for Citrus & Dates (NCGRCD) in Riverside, California, is a unit of the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA). The mission of the Repository is to acquire, preserve, distribute, and evaluate germplasm of *Citrus,* 32 related Aurantioideae genera, and date palms *(Phoenix dactylifera)* and related species, and to conduct research that enables the mission to be better accomplished. The NCGRCD is located on the campus of the University of California, Riverside (UCR).

The Repository was established in 1987 on the campus of UCR, and UCR provides a number of services and support to NCGRCD through a Research Support Agreement (RSA) with the Dept of Agricultural Operations, UCR. Specific Cooperative Agreements are in place with Drs. Mikeal L Roose, Tracy L Kahn, Dept of Botany & Plant Science, UCR. More information on these SCAs is presented below. One direct funded SCA is administered by the Repository, with Dr Thomas Bellows, Dept of Entomology (Biological Control of the Giant Whitefly) and this SCA is not reported on here. Additional information on cooperation between the NCGRCD and UCR is detailed in the appropriate sections. Additional UC facilities utilized include the Coachella Valley Agricultural Research Station (CVARS), located in Thermal, and the South Coast Research and Extension Center (SCREC), located in Irvine.

The Repository is served administratively by the ARS Riverside Location housed at the USSL, Riverside and by the ARS Pacific West Area (PWA), Albany. The Repository is a part of the USDA National Plant Germplasm System (NPGS), under National Program 301: Plant, Microbial, and Insect Genetic Resources, Genomics, and Genetic improvement.

Germplasm Collections

The NCGRCD may be thought of as a 'collection of collections'. These collections include the Protected Collection; the Citrus Variety Collection (CVC); the Citrus Relatives Collection; and the Date Palm Collection. All these collections consist of living trees due to the limitations associated with preservation and distribution of these clonally propagated crops as seed.

There are over 1,000 accessions in the CVC and Citrus Relatives Collection and about 400 accessions in the Protected Collection which are available for distribution as pathogen-tested budwood. Currently, 28 of the 33 genera in the subfamily *Aurantioideae* are represented in the various collections. However, some of these genera are represented by only one species. A complete listing of Repository holdings may be found at the GRIN website: http://www.ars-grin.gov/cgi-bin/npgs/html/site.pl?RIV.

Protected Collection

The Protected Collection consists of small potted trees that are propagated from pathogen-tested budwood. These trees are the source of budwood for distributions. Except under unusual circumstances, budwood is not distributed from other sources. The pathogen-

tested collection is maintained under screen to prevent infection via insect vectors. Stringent precautions are also taken as far as sanitation and to prevent entry of insect vectors. There are currently over 400 accessions under screen, represented by over 850 individual trees. Most of the accessions maintained in the screenhouse are available for distribution. The rest are under non-propagation agreements, have not been released, or are being evaluated prior to official accessioning.

Accessions maintained in the Protected Collection are re-tested annually for CTV by ELISA. Trees to be used for distributions are often re-tested for CTV by ELISA prior to budwood cuts. In addition, we have begun re-testing every 5 years for exocortis, other viroids, and psorosis. This will put the Protected Collection in accordance with California state regulations for registered nursery trees. In 2007, the trees in the Protected Collection were tested for *Citrus leprosis virus* by ELISA, and they have previously been tested for *Citrus leaf blotch virus* (CLBV) using RT-PCR.

Accessions are added to the Protected Collection after being pathogen tested following quarantine at either the Repository or the UC Citrus Clonal Protection Program (CCPP). If an accession originates from outside the US, it must be quarantined and released from quarantine by USDA-APHIS and CDFA officials before it can be added to the virus-tested collection (or planted outside the quarantine facilities). Accessions originating domestically from outside of California (and from some areas within California) must be released from quarantine by CDFA but not USDA-APHIS. Pathogen testing has been ongoing at NCGRCD and a number of accessions are being held under quarantine. Material, especially those received as seed, which has not flowered or fruited are not normally distributed except by special request.

Citrus Variety Collection (CVC)

The UCR Citrus Variety Collection (Dr Tracy L Kahn, Principal Museum Scientist, UCR Dept of Botany & Plant Science) is used cooperatively by the NCGRCD for characterization/evaluation, as a seed source, and occasionally as a source of tissue or materials for distribution which are not vegetatively propagated (leaves, flowers, pollen, or DNA extracts). Trees in the CVC which are used for seed collection are indexed for CLBV using RT-PCR. The Repository maintains a Specific Cooperative Agreement (SCA) with Dr Kahn to support the evaluation of trees in the CVC. Cooperation with Dr Kahn is ongoing in the area of evaluation.

There are currently over 1000 accessions maintained in the CVC. This includes accessions only planted in the CVC, not those maintained only in greenhouses or at SCREC (see below). In 2007, 27 new accessions were planted in the CVC including 10 citrus relatives, and a total of 139 trees were replanted. Some of these replanted trees include trees which had to be relocated because of construction of the freeway. About 150 monoembryonic seedlings, which are part of the collection, are maintained in the field at CVARS.

Because of concern of continuing support from UCR, Dr. Kahn has started an endowment campaign for the CVC. The long range goal is to generate sufficient annual funds to cover the costs associated with the maintenance and preservation of this important source of citrus genetic information.

Citrus Relatives Collection

There are approximately 80 accessions of species of Aurantioideae genera other than *Citrus* that are maintained in greenhouses and in field locations in the CVC or at SCREC and/or CVARS. Eighteen citrus relative trees were planted at SCREC in 2007. Field 45 is being removed and accessions are being reestablished into Field 25. Some accessions that are commonly used for seed collection are remaining in Field 45. This activity will result in a more consolidated planting and has removed several non-thrifty trees.

The SCREC is located adjacent to the El Toro Marine Air Base (ETMAB) which was closed several years ago. The future of SCREC is uncertain. It is likely the ETMAB will be utilized as a recreational/cultural/residential area, thus increasing the value of the land, and the SCREC may be sold in the future.

The citrus relatives often are more sensitive than citrus to factors such as cold, heat, pesticides, fertilizers, etc. Because of these factors, citrus relatives are maintained under the more moderate coastal temperatures of the SCREC. For these reasons, several plants of each accession are maintained in NCGRCD in greenhouses as a backup.

The planting at CVARS was established in order to determine if the higher heat load in this low desert environment would produce more flowering and fruiting than at the more moderate locations, even if the environmental conditions are more subtropical. Many of the citrus relatives were lost at CVARS in the January 2007 freeze. Forty trees have been replanted, and a total of 22 accessions are now established. Additionally at CVARS, there are plantings of wild or semi-wild citrons, some have no flesh similar to Yemeni type and as such are of interest to Rabbis, there are 20 selections of Poncirus trifoliata, 36 accessions with a total of 177 trees of seedling populations (monoembryonic and zygotic), and the Persian lime SPB-7 which is a budline recently released from quarantine of a wood pocket-free clone selected in Florida. The wood pocket disorder is a genetic problem and is expressed more rapidly in warmer growing areas.

Date palm Collection

The date palm collection currently consists of 128 accession total. This includes about 500 trees at CVARS and about 100 trees at the older collection located in Brawley. Repropagation of the main collection at CVARS has been completed. A CDFA permit was obtained in 2007 to enable receiving 10 new varieties from Arizona, and six seed populations were acquired from a exploration made to Baja California. The disease-free status of the date plams at CVARS is maintained by a California state quarantine for the desert areas. Due to the difficulty of obtaining and quarantining new date palms, it is unlikely that this collection will grow very quickly in the future from overseas locations.

These date palms were originally a collection developed by the USDA Date and Citrus Station in lndio. When that station was closed in 1979, the dates were moved to the USDA

irrigated Desert Research Station (IDRS) in Brawley, and came under NCGRCD responsibility shortly after the establishment of NCGRCD in 1987. In the early 1990's, it was apparent that the Brawley station would be closed; the date palms were then repropagated beginning in 1993 and planted in CVARS. The CVARS site in Thermal is better suited for the growth of date palms due to soil type and weather conditions.

Currently the date palms exist in both Thermal and Brawley. The status of the Brawley station is unclear due to the complicated arrangement between the Federal government, the Imperial County government, and a private committee that was made when the site was originally established. It is currently maintained by the Imperial County Farmer's Committee (and is now called Imperial Valley Agricultural Research Station). The collection at Brawley needs major renovation/pruning at an estimated cost of about $20,000, cultural care is less than adequate, and this collection has limited value as older date palms do not produce the off shoots which we need for distributions.

Germplasm Distributions

Distributions of germplasm from NCGRCD are summarized in Table 1 for the past five years. Distributions for the year 2007 totaled 911 accessions, most of which were citrus. Of the citrus distributions, about 30 percent were distributed to domestic requestors; the remainder of the distributions was to foreign requestors. While the distributions were down from the record number of CY2006, the general trend has been for a greater number of distributions.

The above figures are for all distributions, and include budwood, seed, pollen, leaves, DNA extracts, etc. Although NCGRCD is a clonal repository, it continues to distribute a fairly large amount of material as seed. There are several reasons for this: many of the seeds distributed are used for virus indicators or in rootstock trials when requestors do not want to wait the years necessary for trees to start producing seeds when propagated from budwood; requestors wish to avoid quarantine hassles associated with vegetative tissue; and most distributions of citrus relatives are in the form of seeds since quarantine requirements are not well defined and the relatives generally come true-to-type form seed. All trees used for seed collection must now be check for CLBV before seed can be distributed. Budwood distributions mostly fall in a few categories: production of seed sources of indicator plants for virus testing or production of rootstocks; establishment of a clean stock program, commercial trials; or breeding research.

Databases

The NCGRCD uses several local databases as well as the national Germplasm Resources Information Network (GRIN) database maintained by the Database Management Unit (DBMU) of the National Germplasm Resources Laboratory (NGRL) in Beltsville. Review of the local databases is ongoing, but the inventory in the GRIN database is now up to date. In CY2007, the local databases have been consolidated into a unified Access format. This makes it easier to find specific plants and in updating the information in the GRIN database. Progress is being made on intergrating the pathogen testing information into the access database.

Citrus Germplasm Activities

While the NCGRCD in the past has been primarily a service unit in the past with its primary focus on providing others with the materials necessary to do research, research also is now part of the mission since R. Lee was hired as a Research Plant Pathologist Category 1 scientist. Research and other activities supporting and enhancing the Repository's mission are described in this section.

One of the real strengths of the Riverside Repository, as compared to some of the other repositories, is the breadth of its program. Due to our status as a quarantine facility, we are able to directly introduce new accessions, and we also enjoy excellent cooperation with the CCPP. This is in contrast with other clonal repositories, which must introduce new materials through the National Plant Germplasm Quarantine Office in Beltsville. Our pathogen testing program and maintenance of materials under screen also allows us to offer pathogen-tested 'clean stock' material, which other repositories are unable to do. In the area of evaluation and research, the Riverside repository is active in several areas. During CY 2007, extramural funds were accessed for the development of quantitative real time PCR method for the detection of huanglongbing (HLB, commonly called citrus greening) in the psyllid vectors. In addition, we are active in the areas of horticulture and plant pathology in both the applied and basic research areas. The Repository also is closely involved in state and national phytosanitary issues through participation in several committees.

The Repository has been working with the Florida Department of Agriculture and Consumer Service, Division of Plant Industry, the California Department of Agriculture, the California Citrus Clonal Protection Program to develop a "citrus passport" system. Once implemented, the three participating facilities would recognize the therapy and pathogen testing performed at one location, and germplasm being shipped under the "citrus passport" system would need only to be tested for presence of Citrus tristeza virus, Citrus psorosis virus, and citrus viroids upon receipt. Once biological tests confirm freedom of these pathogens, the germplasm would be released from quarantine and ready for release in 6-8 months after receipt rather than the 3-4 years as per current protocol.

Date Palm Activities

The Repository is continuing to work with the Arizona State University (ASU) Arboretum in Tempe (Mr Richard Harris, Coordinator) to access date palms are not in the NCGRCD collection. In the past there have been difficulties in obtaining these varieties due to California state quarantines. Date palms from outside the desert areas are restricted due to Palm Lethal Yellows mycoplasm, *Fusarium,* and *Ozonium.* The first two are not present in Arizona and would not prevent bringing date palms to the Cochella Valley. *Ozonium* is not known to occur in the Tempe area; however, it is difficult to certify this due to the complexity of the *Ozonium* assay. In CY2006, an import permit was obtained which enabled the Repository to access the varieties of date palm in Arizona which were not present in California in CY2007, and we anticipate additional accession in CY2008.

RESEARCH

Molecular Characterization of the Citrus Variety Collection (CVC)

This project was cooperative with Dr Mikeal Roose, UCR Dept of Botany & Plant Science, and is supported by a SCA. Previously simple-sequence-repeat (SSR) markers were developed and used to evaluate the genetic diversity in 380 sexually-derived accessions in the CVC and resulted in designating a core collection containing the majority of the genetic diversity. The core collection has been flagged in GRIN. Information regarding the primers developed is posted at **http://www.plantbiology.ucr.edu/people/faculty/rooselink2.html** .

This research was followed by identifying 33 representative cultivars and related accessions and comparing the sequence of five randomly selected nuclear genes (~1 Kb). The phylogenetic relationships between the different cultivars of citrus are not clearly understood because of hybridization, nucellar embryony and somatic mutations. Earlier studies on citrus taxonomy and phylogeny were based on isozymes analyses, microsatellite and RAPD data. The study included 4 citrons, 4 limes, 1 lemon, 7 mandarins, 2 sour oranges, 1 sweet orange, 1 tangor, 3 pummelos, 2 papedas, 2 trifoliates, 1 kumquat, 1 *Microcitrus*, 4 citrus relatives belonging to the sub-tribes Citrinae and Balsamocitrinae. The citrus accessions selected for the study were representatives from a core collection selected based on SSR marker analysis and included several known hybrids. Several of the cultivars had a high level of heterozygosity. The heterozygous accessions were resolved using the software PHASE and/or by cloning and sequencing. Haplotype information was generated and used for phylogenetic studies using PAUP and Mr. Bayes analysis. Two of the six phylogenetic trees had polytomies reflecting uncertainty about relationships. Coalescence simulations using Mesquite were done to test whether hybridization or the sorting of ancestral polymorphisms best explains incongruence seen between several loci for alleles from some accessions. The possible ancestry of the accessions in the study was inferred based on the different kinds of analyses done. Relationships among haplotypes from various accessions provide new depth to knowledge about relationships among citrus taxa.

Huanglongbing (HLB)

Huanglongbing (HLB), or citrus greening disease, is probably the most destructive disease of citrus. The disease has been present in Asia and Africa for several decades and was first reported in the Western hemisphere from Brazil in 2004 and Florida in 2005. The disease is spreading at an alarming rate in both areas, and several groves have already been severely affected resulting in either abandonment, or replacement with other crops. In 2006 surveys in Florida indicated the disease was widespread in at least 10-12 southern counties of the state. *Diaphorina citri*, one of the psyllid vectors of the disease, is already present in Texas, Mexico, and Hawaii and poses an immediate threat of introduction into California.

HLB is caused by a bacterium of the genus, *Candidatus* Liberibacter. At present, three species, *Ca*. L. asiaticus, *Ca*. L. africanus and *Ca*. L. americanus are known. The bacteria are phloem-limited in the plant, and are not well characterized since the bacterium has not been cultured yet. While HLB bacteria can be detected from infected plant tissue by polymerase chain reaction (PCR), it is difficult to detect the same from non-symptomatic tissue, or prior to appearance of symptoms. PCR detection of HLB from plants is generally done as a confirmatory test, rather than as a diagnostic test. Early detection of the disease is critical for successful

management of the disease.

Diaphorina citri, the psyllid vector of HLB, has been present in Brazil for over sixty years. The psyllid was found in Florida in 1998, and is now present in Texas, Mexico (as close as Sonora State) and Hawaii. The psyllid has not yet been found either in Arizona or California.

We reported the application of a Taqman based real-time quantitative PCR assay for the detection of HLB in psyllids and the development of a Taqman probe to monitor the quality of DNA extractions from the psyllids. The summary of results of the analyses of over 1200 samples of psyllid adults and nymphs collected from Florida are reported. The results show that HLB may be detected in psyllids in an area 1-2 years before the development of HLB symptoms and confirmation from plants, also the study suggests that garden stores and retail nurseries may have played a important role in the distribution of infected psyllids and plants.

Cytoplasmic Citrus leprosis virus (CCLV)

Citrus leprosis disease, vectored by *Brevipalpus* species mites, is an emerging disease in Central America. Without control of the mite vector and reduction of inoculum in the field by pruning, the disease will kill citrus trees in three years. In cooperation with Dr. R. H. Brlansky and A. Guerra at the Citrus Research and Education Center, University of Florida, we have further characterized the genome of the CCLV including the subgenomic RNAs, and reported the presence of defective RNAs in infected plants. We developed a sensitive RT-PCR assay based on an highly expressed open reading frame present in infected tissue, and reported raising polyclonal antibodies specific for CCLV and their application for detection of the virus using ELISA protocols. The serological protocols are undergoing patent application.

Citrus tristeza virus (CTV)

A serological protocol has been developed which utilized the expressed coat protein gene of CTV to raise antibodies which are efficient at trapping virus particles in double antibody sandwich ELISA assays. This technology is being transferred to other parties through a CRADA that is being developed.

Dweet mottle virus (DMV)/Citrus leaf blotch virus (CLBV)

In cooperation with UCR Department of Plant Pathology, the coat protein gene of DMV has been identified and inserted in an expression vector for the expression of a fusion protein which has been used to raise an antibody specific for DMV. The antibodies appear to be effective in recognizing and diagnosing CLBV using ELISA format procedures.

Citrus measles

The graft transmissible nature of citrus measles was confirmed by biological indexing, and the citrus measles disease was found in California (near Farmersville) for the first time.

Facilities and Resources

We struggle to find space for the activities at the Repository. With the growth chamber purchased in CY2005 and the old growth chamber, we have the capacity to thermotherapy 18-21 accessions per year. At the end of CY2007, Repository facilities consisted of 538 ft^2 of laboratory space, 400 ft^2 of office space, 1375 ft^2 of headhouse space, 5948 ft^2 of greenhouse space, 16,600 ft^2 of screenhouse space (including the new quarantine screenhouse), and 280 ft^2 storage space. Additional greenhouse space belonging to the University (three greenhouses and shared space in two additional greenhouses) is also used by the Repository. The laboratory is used for pathogen testing and elimination, research, and as a general work area for order processing, etc. Pathogen-tested potted trees belonging to our Protected Collection are maintained in the screenhouse. Greenhouse space is used for propagation, virus indexing, and maintenance of cold-sensitive germplasm (mostly citrus relatives); one greenhouse on loan from UCR is used as a quarantine greenhouse. A 480 ft^2 office trailer provides office space and laboratory space for incubators, freezers, and the transfer hood utilized mainly for shoot tip grafting. The greenhouses were upgraded in 2001-2002 and the screenhouse which holds the Protected Collection was enlarged in 2002-2003, but we are at capacity with these facilities.

Personnel

During CY2007, Repository (permanent, full-time) staffing was 2.0 SY: Research Leader/Research Plant Pathologist and Horticulturist/Curator, and 3 FTE Biological Technicians. These positions were supplemented with a 0.75 FTE Office Assistant 'term' (temporary) position, a .4 FTE mechanic, two post-doctoral researchers (only one as of May 2007), and approximately 2.0 FTE student and casual positions hired through the UCR via the RSA.

The Research Leader, R. Lee, is the only category 1 scientist in the unit, and his research has been directed at development of new or improving diagnostic techniques for citrus pathogens to strengthen the ability of the Repository to provide the highest quality pathogen-tested germplasm and at applying the diagnostic techniques to determine sources of tolerance/resistance to selected pathogens.

The Location support for the Repository is provided by the Location personnel housed in the US Salinity Laboratory. Administrative support from the PWA Area Office is under the direction of the Area Director, Dr Andrew Hammond, with able assistance from the Associate Area Director, and Assistant Area Director.

Major Issues

The most critical issue facing the Repository at the end of CY2007 is the threat of the Asian citrus psyllid and huanglongbing (HLB). At the moment, neither the vector nor the disease are known to be present in Californai. With the finding of HLB and the presence of citrus canker in Florida, there is an urgent need to protect germplasm resources such as the core citrus mapping population, in Florida, being utilized by the international citrus genome group to sequence the citrus genome. If the psyllid and HLB should be found in California, the repository would have additional pressure to recover germplasm that presently is only in field plantings. It is estimated that about 325 varieties are present only in the CVC in the field and are not backed

up in greenhouses or screenhouses anywhere in the USA. If there was an urgent need to protect these germplasm resources because of an immediate threat by HLB, the present facilities at the Repository would not be adequate. The laboratory space is very crowded, and will become more crowded in the foreseeable future. One room in the trailer is now dedicated to facilities for STG and real time PCR, separation of the real time PCR instrumentation and assay area from the area used for extraction of samples reduced the risk of contamination. Office space is crowded and visitors/graduate students have to use bench space in the laboratory for office tasks, further crowding the laboratory.

While the screenhouse which houses our Protected Collection was completed in CY2002, we are nearing capacity in this expanded space. The shortage of greenhouse space has been temporarily helped by being able to borrow the use of greenhouse space from UCR, these have the disadvantage of not being able to make improvements to the structures because they do not belong to the USDA, and they are not adjacent to the core collection of buildings which are serviced by the back-up generator in case of prolonged power failure. In July we lost power in one of the greenhouses, resulting in the loss of all plants being maintained in this facility. The power failure was due to a mechanical breakdown, and although it was discovered within 24 hours, the extreme heat and low humidity in July resulted in a total loss of the plant material. This puts our collection of citrus relatives at risk of loss because they are almost totally housed in borrowed greenhouses.

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Table 1. Germplasm distributions from the National Clonal Germplasm Repository for Citrus and Dates for CY2003, CY2004, CY2005, CY2006 and CY2007.

	CY2003	***CY2004***	***CY2005***	***CY2006***	***CY2007***
Citrus	394	230	799	1,209	899
Dates	99	5	16	34	12
Total	493	235	815	1,243	911