The effects of symbiont induced haploid thelytoky on the evolution of brevipalpus mites
Groot, T.V.M.

Citation for published version (APA):
Amsterdam: IBED
THE EFFECTS OF SYMBIONT INDUCED HAPLOID THELYTOKY ON THE EVOLUTION OF *BREVIPALPUS* MITES
On the cover

The front cover contains four pictures of mites representing the four asexual *Brevipalpus* species studied in this thesis. In clockwise order, starting with the upper picture on the left, the species are: *B. californicus*, *B. phoenicis*, uninfected *B. obovatus* and *Cardinium* infected *B. obovatus*. When maintained on common bean (*Phaseolus vulgaris*), cultures of the different species can be distinguished based on the shape and color of the adult females. Female mites of *B. californicus* and *B. phoenicis* are more elongated whereas females of the two *B. obovatus* species are broader. The elongation is most pronounced in *B. californicus* females which have V-shaped abdomens, whereas the abdomens of *B. phoenicis* females are more U-shaped. The two *B. obovatus* species differ mainly in color. The *Cardinium* infected mites are reddish brown and the uninfected ones are more greenish. The differences between the four species are most clearly visible from older females that have started oviposition already.

The back cover shows mites of various life stages in a culture of *B. phoenicis*.

Photos and design: J. van Arkel.

The work presented in this thesis was funded by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), grant number W89-141, and was carried out at the University of Amsterdam, Institute for Biodiversity and Ecosystem Dynamics.

THE EFFECTS OF SYMBIONT INDUCED HAPLOID THELYTOKY ON THE EVOLUTION OF *BREVIPALPUS* MITES

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus

prof. mr. P.F. van der Heijden

ten overstaan van een door het College van Promoties ingestelde
commissie, in het openbaar te verdedigen in de Aula der Universiteit

op donderdag 26 oktober 2006, te 12:00 uur

door Thomas Volkert Marie Groot
geboren te Alkmaar
Promotiecommissie

Promotores
Prof. dr. S.B.J. Menken
Prof. dr. M.W. Sabelis

Co-promotor
Dr. J.A.J. Breeuwer

Overige leden
Prof. dr. L.W. Beukeboom
Dr. M. Maraun
Prof. dr. R. Stouthamer
Prof. dr. P.H. van Tienderen
Prof. dr. M.K.H. Veith

Faculteit der Natuurwetenschappen, Wiskunde en Informatica
Contents

Samenvatting pp.  1

Summary pp.  9

General Introduction pp. 17

Chapter 1 Cardinium Symbionts Induce Haploid Thelytoky in Most Clones of Three Closely Related Brevipalpus Species pp. 31

Chapter 2 Adaptation in the Asexual False Spider Mite Brevipalpus phoenicis: Evidence for Frozen Niche Variation pp. 49

Chapter 3 Clonal Diversity and Host Plant Specialization in Asexual Brevipalpus Mites pp. 61

Chapter 4 Horizontal Transmission of Cardinium Symbionts Within and Between Closely Related Parthenogenetic Mite Species pp. 75

Chapter 5 Male Function in the Asexual False Spider Mite Brevipalpus phoenicis pp. 91

Chapter 6 Recombination and Intragenomic Variation in Asexual Brevipalpus Mites pp.103

General Discussion pp.125

References pp.133

Dankwoord pp.151

Curriculum vitae pp.154
Samenvatting

Theoretische achtergrond

Het merendeel van de diersoorten plant zich seksueel voort; om een nieuw individu te produceren moeten een mannelijke en een vrouwelijke gameet fusioneren. Het tegengestelde, aseksuele reproductie waarbij vrouwtjes dochters produceren zonder de bijdrage van een mannelijke gameet, is zeldzaam. Deze observaties suggereren dat seksuele voortplanting de beste manier van voortplanting is. Aan de andere kant, seksuele voortplanting zou ook minder voordelig kunnen zijn omdat het inefficiënt is en specifieke kosten heeft. Bijvoorbeeld, in seksueel voortplantende soorten produceren de mannetjes niet direct nakomelingen, en verder wordt veel energie besteed aan het zoeken naar een geschikte partner. Deze schijnbare tegenstelling is bekend geworden als de ‘paradox of sex’ en wordt gezien als een van de belangrijkste vraagstukken in de evolutionaire biologie.


De theorieën van beide groepen worden ondersteund door wiskundige modellen. Vooral wanneer de verwachte voordelen van verschillende theorieën gecombineerd worden, kunnen de nadelen van seksuele voortplanting volledig gecompenseerd worden. In de laatste jaren zijn er al meer studies gekomen die bewijs voor de theorieën aantonen in levende organismes. Met dit bewijs kan de paradox of sex als verklaard worden beschouwd; seksuele voortplanting is inderdaad voordeliger dan aseksuele voortplanting. Als dat zo is, dan kan men zich terecht afvragen waarom er toch ook nog aseksuele soorten bestaan. Het doel van mijn thesis is het bestaan van een bepaalde groep aseksuele soorten te verklaren: namelijk de aseksuele soorten van het mijten geslacht Brevipalpus.
Er zijn drie mogelijke verklaringen waarom aseksuele soorten, tenminste tijdelijk, kunnen voortbestaan. Ten eerste is het mogelijk dat aseksuele soorten speciale aanpassingen hebben die de nadelen van het gebrek aan seksuele reproductie compenseren. Ten tweede, aseksuele soorten zouden kunnen bestaan omdat ze continu worden geproduceerd vanuit seksuele soorten. De nieuwe aseksuele soorten zullen het een relatief korte periode goed doen, maar op langere termijn zullen ze toch extinct gaan. Ten derde, aseksuele soorten zouden kunnen bestaan omdat ze niet volledig aseksueel zijn, maar zich af en toe seksueel voortplanten.

Niet alleen het bestaan van aseksuele soorten op zich dient verklaard te worden. Een verklaring is ook nodig voor het feit dat veel aseksuele soorten vaak zijn aangepast aan een opmerkelijk brede ecologische niche, een die vaak nog breder is dan de niche van de seksuele voorouder soort. Er zijn twee theoretische modellen die beschrijven hoe een aseksuele soort een brede niche kunnen bezitten. Het ‘Generalist Purpose Genotype’ model voorspelt dat een aseksuele soort zal bestaan uit een klein aantal klonen met zeer brede niches die tolerant ten opzichte van veel verschillende omstandigheden. Dit model gaat er van uit dat specialistische types uitsterven vanwege veranderingen in hun omgeving. Maar, het ‘Frozen Niche Variation’ model voorspelt dat een aseksuele soort zal bestaan uit veel klonen met verschillende smalle niches. Dit model gaat er juist van uit dat generalistische klonen niet kunnen overleven door concurrentie met meer gespecialiseerde klonen.

Het model organisme

Het genus *Brevipalpus* is het grootste genus van de familie Tenuipalpidae en telt ongeveer 300 beschreven soorten. Echter, vanwege het kleine formaat van de mijten zijn de taxonomische relaties tussen al die soorten weinig begrepen. Het genus bevat zowel seksueel als aseksueel voortplantende soorten. Deze thesis gaat over de aseksuele soorten. Er is een groot aantal aseksuele *Brevipalpus* soorten beschreven, maar de drie belangrijkste zijn *B. phoenicis*, *B. obovatus* en *B. californicus*. Het morfologische onderscheid tussen deze soorten is beperkt en intraspecifieke variatie wordt vaak gemeld. Ieder van de drie soorten komt wereldwijd voor in (sub)tropische gebieden en wordt gevonden op honderden verschillende waardplantsoorten. De meeste waardplantsoorten wordt gedeeld door twee of alledrie de mijten soorten. Deze mijten worden gezien als een belangrijke plaga op groente, fruit en siergewassen. Ze veroorzaken schade zowel direct door te eten van de planten, als indirect door het overdragen van plantvirussen. Bestrijding
Samenvatting

van de mijten gebeurt doorgaans met chemische middelen. Echter, de mijten ontwikkelen opmerkelijk snel resistenties tegen deze acariciden. Gezien hun klonale voortplanting, is deze snelle aanpassing onverwacht.

Het is aangetoond dat in *B. phoenicis* de aseksualiteit wordt veroorzaakt door een endocellulaire bacteriële symbiont van het genus *Cardinium*. De seksuele soorten van de Tenuipalpidae zijn echter arrhenotook (haplo-diploïd). De *Cardinium* geïnfecteerde *B. phoenicis* vrouwtjes produceren geïnfecteerde, maar onbevruchte eieren. Omdat de eieren ongeïnfecteerde zijn, en dus haploïd, zouden ze normaal gesproken tot mannetjes ontwikkelen. Echter, de *Cardinium* in het ei feminiseren deze zodat het zich ontwikkelt tot een haploïd vrouwelijk dier. In *B. californicus* zijn het ook *Cardinium* bacteriën die de aseksualiteit veroorzaken. Echter, dit ook voor alle populaties en ook voor *B. obovatus* geldt nog niet bekend.

Na de recentelijk ontdekking van *Cardinium* in 2001, is dezelfde bacterie aangetroffen in aan aantal andere soorten insecten en mijten. Naast feminisatie, is *Cardinium* instaat tot nog twee vormen van manipulatie van de voortplanting van de gastheer: het induceren van parthenogenese en cytoplasmatische incompatibiliteit. Een andere endosymbiont die ook in insecten en mijten wordt gevonden en dezelfde effecten heeft op zijn gastheren is *Wolbachia*. Echter, *Wolbachia* is veel eerder ontdekt en daarom is er ook al veel meer bekend over zijn biologie. In mijn thesis zal ik de twee bacteriën regelmatig vergelijken om te zien of ook de zelfde processen plaats vinden, wat men wel zou verwachten gezien de overeenkomsten in levenswijzen.

Om voorspellingen te kunnen doen over de relatie tussen gastheer en symbiont, is het essentieel te weten hoe de symbiont wordt doorgegeven. Indien een symbiont alleen verticaal doorgegeven wordt is een mutualistische relatie te verwachten. Echter, wanneer de overdracht (soms) verticaal is kan de symbiont zich ook ontwikkelen tot een parasiet.

Om het evolutionaire lot van de aseksuele *Brevipalpus* soorten te kunnen begrijpen moeten we weten hoe nieuwe genetische variatie verkregen wordt. Daarom heb ik een groot deel van mijn thesis gewijd aan deze vraag. In theorie zijn er vier manieren waarop klonale variatie kan ontstaan. Ten eerste, nieuwe klonale types kunnen ontstaan als bestaande types veranderen door mutaties. Ten tweede, nieuwe klonale types kunnen ontstaan wanneer de symbiont horizontaal wordt doorgegeven naar een seksuele mijt, die daardoor het begin van een nieuwe aseksuele lijn wordt. Ten derde, nieuwe klonale types kunnen ontstaan wanneer aseksuele vrouwtjes paren met de mannetjes die ongeveer 1% van de aseksuele populatie opmaken. Ten vierde, nieuwe aseksuele types kunnen ontstaan
wanneer aseksuele vrouwtjes paren met mannetjes van verwante seksuele soorten.

**Deze thesis**

Voor hoofdstuk 1 heb ik de manier van aseksuele voortplanting onderzocht voor een aantal verschillende genotypen representatief voor de drie soorten. Eerst zijn er isofemale lijnen gemaakt om te gebruiken in de verschillende experimenten. Er is aangetoond dat onder de standaard laboratoriumcondities alle *B. phoenicis* en *B. californicus* lijnen mannetjes produceren met een frequentie variërend van 0.15 tot 6.72 procent. De *B. obovatus* lijnen produceerden geen mannetjes onder deze condities. Wanneer de dieren met antibiotica behandeld werden produceerden alle *B. phoenicis*, *B. californicus* en een aantal *B. obovatus* lijnen een hoger aantal mannetjes wat aangeeft dat de *Cardinium* symbiont verantwoordelijk is voor de aseksualiteit in deze lijnen. Andere *B. obovatus* lijnen produceerden helemaal geen mannen na behandeling met antibiotica. Deze lijnen bleken dan ook niet geïnfecteerd te zijn met *Cardinium* of andere endosymbionten. De conclusie was dat in deze lijnen de aseksualiteit waarschijnlijk een genetische eigenschap van de mijt zelf is. Tot slot, een vergelijking tussen de taxonomieën gebaseerd op morfologische eigenschappen enerzijds en op genetische eigenschappen anderzijds gaven verschillende resultaten. De lijnen die geen *Cardinium* bevatten groepeerden met *B. obovatus* op basis van genetische eigenschappen, maar werden geïdentificeerd als *B. phoenicis* gebaseerd op morfologie.

In hoofdstukken 2 en 3 beschrijf ik experimenten om te testen tussen de General Purpose Genotype (GPG) en Frozen Niche Variation (FNV) modellen als verklaring voor de brede ecologische niche van deze mijten. In hoofdstuk 2 werd een ecologische benadering genomen. Mijten van drie natuurlijke populaties op drie verschillende waardplantsoorten zijn overgeplaatst naar ieder van de drie waardplantsoorten. Wanneer mijten naar een andere waardplantsoort werden overgeplaatst hadden ze vaak lage reproductieve waardes en/of stierven. Dit suggereerde dat mijten gespecialiseerd zijn voor de waardplantsoort waar zijn op voorkomen, en is dus bewijs voor het FNV model. In hoofdstuk 3 werd een genetische benadering genomen. Mijten werden gegenotypeerd voor hun mitochondriale *COI* gen en de haplotype diversiteit werd gerelateerd aan de waardplantsoorten. Deze analyse leverde bewijs op voor beide modellen. Bewijs voor FNV werd gevonden in een cluster van mijten die allemaal van acerola verzameld waren. Bewijs voor GPG was gevonden in een aantal *B.
De conclusie was dan ook dat *Brevipalpus* bewijs levert voor beide modellen. Daarnaast liet dit hoofdstuk ook zien dat op de meeste planten verschillende genotypen tegelijkertijd voorkomen. Op zes van de 22 bemonsterde planten zijn genotypen gevonden die tot verschillende genetische clusters behoorden. Dit laat dus zien dat op een enkele waardplant verschillende mijtensoorten tegelijkertijd kunnen voorkomen.

In hoofdstuk 4 beschrijf ik de resultaten van een experiment om de transmissie van *Cardinium* symbionten te bestuderen. Van een groot aantal mijten, van alle drie de soorten, werd zowel een gen van de mijt als een gen van de symbiont gesequenced. Hiermee werden twee aparte fylogenieën geproduceerd die vervolgens werden vergeleken. De fylogenie van de symbiont bevatte drie clusters die elk geassocieerd waren met een enkele mijtensoort, wat aangeeft dat er in grote lijnen overeenstemming is tussen de fylogenieën van gastheer en symbiont. Deze overeenstemming was verwacht omdat de symbionten verticaal doorgegeven worden. Echter, de symbiont die voornamelijk geassocieerd was met *B. phoenicis* werd ook gevonden in een aantal *B. obovatus* en *B. californicus* mijten. Deze ongelijksoortigheden tussen de fylogenieën van mijt en symbiont zijn aantonend voor horizontale transmissie van *Cardinium* symbionten tussen de verschillende *Brevipalpus* soorten. Er waren ook kleine ongelijksoortigheden tussen de gastheer en symbiont fylogenieën binnen *B. phoenicis*, aantonend dat horizontale transmissie waarschijnlijk ook op dit niveau plaats heeft gevonden.

Voor de laatste twee hoofdstukken heb ik de mogelijkheid dat de aseksuele soorten zich af en toe seksueel voortplanten onderzocht. In hoofdstuk 5 beschrijf ik een experiment dat testte of *B. phoenicis* vrouwtjes hun eieren bevruchten nadat ze gepaard hebben met mannetjes van dezelfde soort. Hiervoor werden mannetjes en vrouwtjes van twee nauw verwante isofemale lijnen reciprook gekruist. Vervolgens werd de aanwezigheid van het vaderlijke genotype in de nakomeling getest door middel van twee microsatelliet markers. Het effect van de symbiont op de mate van bevruchtingssucces werd getest door vier verschillende kruisingen uit te voeren. In alle mogelijke combinaties werden natuurlijk voorkomende mannetjes en mannetjes die resulteren van antibiotica behandelde vrouwtjes gekruist met onbehandelde en antibiotica behandelde vrouwtjes. Het resultaat van al deze kruisingen was dat geen van de nakomelingen het vaderlijke genotype had, en dus dat *B. phoenicis* vrouwtjes hun eieren niet bevruchten, ongeacht of ze geïnfecteerd zijn of niet. Er wordt beargumenteerd dat het falen van de kruisingen veroorzaakt zou kunnen worden door een ‘functioneel maagdelijkheids mutatie’; een mutatie die de
Samenvatting

bevruchting van de eieren voorkomt. Een dergelijk mutatie zal zich snel door een aseksuele populatie verspreiden omdat individuen die het uiten bespaard blijven van de kosten van genoom verdunning die gepaard gaan met seksuele voortplanting.

Tenslotte, in hoofdstuk 6 beschrijf ik een experiment dat testte of er in de verschillende Brevipalpus soorten bewijs bestaat dat seksuele reproductie al een lange periode niet meer heeft plaats gevonden. Hiertoe werd de fylogenie van de mijten gebaseerd op een mitochondriale marker (COI sequenties) vergeleken met de fylogenieën gebaseerd op twee nucleaire markers (AFLP en ITS1 sequenties). Indien de voortplanting inderdaad strikt klonaal is geweest sinds het begin van de divergentie van een groep, dan is te verwachten dat mitochondriale en nucleaire markers dezelfde fylogenieën opleveren. Anderzijds, ongelijkheid tussen beide groepen markers werden geïnterpreteerd als bewijs voor seksuele voortplanting. Op hoger taxonomisch niveau waren mitochondriale en nucleaire fylogenieën niet in overeenstemming, wat aantoont dat seksuele reproductie heeft plaats gevonden nadat deze groep soorten is begonnen te divergeren. De analyse op hoger taxonomisch niveau liet ook zien dat er vier duidelijk aparte groepen van mijten te onderscheiden zijn, namelijk B. phoenicis, B. californicus en de Cardinium geïnfecteerde en niet geïnfecteerde B. obovatus als aparte groepen. Op een lager taxonomisch niveau was het bewijs voor strikte aseksualiteit variabel. In B. phoenicis en de geïnfecteerde B. obovatus was er geen bewijs voor strikte aseksualiteit. Deze soorten zijn of pas recentelijk aseksueel geworden, of planten zich nog steeds af en toe seksueel voort. Er was wel bewijs voor strikte aseksualiteit in B. californicus en de ongeïnfecteerde B. obovatus. In deze soorten is de voortplanting waarschijnlijk voor een aanzienlijke periode strikt aseksueel geweest. Verder laat hoofdstuk 6 zien dat er zeer veel intragenomsche variatie in de ITS1 sequenties is. Deze variatie is waarschijnlijk een consequentie van het feit dat deze mijten haploïd zijn. Hierdoor wordt een aantal processen die leiden tot gene conversion onmogelijk.

Algemene discussie

Dit zijn de belangrijkste conclusies met betrekking tot het verklaren van het bestaan van aseksuele Brevipalpus soorten. Op basis van de resultaten van mijn thesis is er geen grond om aan te nemen dat Brevipalpus mijten speciale aanpassingen hebben die de nadelen van aseksualiteit compenseren. Hiermee kan de eerste verklaring voor het bestaan van aseksuele soorten worden verworpen. De tweede verklaring, dat nieuwe aseksuele lijnen
continu worden geproduceerd vanuit een seksuele soort, is aannemelijk. Dit zou vereisen dat de symbiont horizontaal over gedragen kan worden heegen inderdaad mogelijk is gebleken. Het zou ook vereisen dat er sympathische seksuele en aseksuele populaties zijn. Echter, of dergelijke populaties daadwerkelijk bestaan is niet zeker. De derde verklaring, dat aseksuele soorten zich af en toe nog seksueel voortplanten zou op kunnen gaan voor *B. phoenicis* en de geïnfecteerde *B. obovatus*, maar niet voor de andere twee soorten. Als *B. phoenicis* en de geïnfecteerde *B. obovatus* zich inderdaad af en toe seksueel voortplanten, dan moet dat zijn met mannetjes van hun eigen soort. Indien zij met andere soorten zouden kruisen zou dat hebben geleid tot vreemde introgressies in het nucleaire genoom die opgemerkt zouden zijn in deze analyses. Ik concludeer dat een recent ontstaan van de aseksualiteit en/of af en toe seksuele voortplanting het bestaan van de aseksuele *B. phoenicis* en geïnfecteerde *B. obovatus* kan verklaren. Het is onduidelijk of aseksualiteit in *B. californicus* en de ongeïnfecteerde *B. obovatus* op een zelfde manier te verklaren is. Deze soorten zijn al een tijdje strikt aseksueel maar het is onbekend of deze periode te lang heeft geduurd om de aseksualiteit nog te kunnen verklaren als recentelijk ontstaan. Het is namelijk onbekend hoe lang de periode van strikte aseksualiteit precies geduurd heeft. Bovendien is het onbekend na hoeveel tijd de nadelen van aseksualiteit echt problematisch worden.

De resultaten die ik heb gepresenteerd in mijn thesis geven aan dat de *Cardinium* symbionten parasitair zijn ten opzichte van hun *Brevipalpus* gastheren. Wanneer een seksuele mijt geïnfecteerd raakt en aseksueel wordt, dan zorgt de symbiont ervoor dat zijn gastheer de (meeste) kosten van seks bespaard blijft, en dus wordt de fitness van de gastheer aanzienlijk verhoogd. Wanneer bevruchting dan niet meer nodig is voor de voortplanting, is er geen selectie druk meer om de eigenschappen die nodig zijn voor seksuele voortplanting in stand te houden. Deze zullen dan mutaties oplopen en uiteindelijk gaat de mogelijkheid om zich seksueel voort te planten verloren. Wanneer dit het geval is, is de mijt volledig aseksueel en zal op lange termijn de nadelen hiervan ondervinden met als gevolg dat hij uiteindelijk extinct zal gaan. Dus, hoewel een infectie met *Cardinium* op de korte termijn een fitness voordeel op levert, uiteindelijk gaat de gastheer extinct door de infectie, en daarom beschouw ik de symbiont een parasiet. Voordat zijn gastheer extinct gaat moet de symbiont een nieuwe gastheer bereiken door middel van horizontale transmissie. De resultaten van hoofdstuk 4 laten zien dat de symbiont hier inderdaad toe in staat geacht kan worden.
Summary

Theoretical background

The majority of animal species reproduces sexually; the fusion of a male and a female gamete is required to produce a new individual. In contrast, asexual reproduction, where a female produces a daughter without the contribution of a male gamete, is rare. This observation suggests that sexual reproduction is the best way to reproduce. On the other hand, sexual reproduction may not be the best way because it is inefficient and costly. For example, in sexual species males do not directly produce offspring, and furthermore, much time and energy is spent finding a suitable mate. Known as the paradox of sex, this apparent conflict has become one of the major issues in evolutionary biology.

A large number of theories have been proposed to solve the paradox of sex. These theories ascribe various advantages to sexual reproduction that compensate its inefficiency and costs. Based on the kind of advantage, these theories are divided in two major groups. The theories of the first group state that sexual reproduction speeds up adaptation and evolution. This enables sexual species to respond to changes in their environment. The theories of the second group state that sexual reproduction enables the removal of deleterious mutations. Asexual species lack this possibility and deleterious mutations are expected to accumulate in their genomes.

The theories of both groups are supported by mathematical model studies. Especially when the advantages expected from various theories are combined, they can indeed compensate the disadvantages of sexual reproduction. In recent years an increasing number of studies have also revealed evidence for these theories in living organisms. With this evidence the paradox of sex becomes resolved; despite its inefficiency, sexual reproduction is indeed more advantageous than asexual reproduction. If so, one may wonder why there still are asexual species at all. This thesis aims to explain the existence of one particular group of asexual species; the asexual species of the mite genus *Brevipalpus*.

There are three explanations to why asexual species, at least temporarily, may persist. Firstly, the asexual species may have special adaptations that counter the disadvantages of not reproducing sexually. Secondly, asexual species may persist because they are continuously produced from within a sexual species. These new asexual species will do well for a short period of evolutionary time, but will not persist in the long
run. Thirdly, asexual species may persist because they are not entirely asexual, but occasionally reproduce sexually.

Besides that the existence of asexual species in itself requires an explanation, many asexual species also have adapted a remarkably broad ecological niche, often extending the niche of their sexual ancestors. Two theoretical models can explain how an asexual species may acquire such a broad niche. The General Purpose Genotype model predicts that asexual species will consist of a few clones with very broad niches tolerant to many different environmental conditions. The model assumes that specialist clones can not survive over longer periods of evolutionary time because their niches will be lost due to environmental changes. Contrastingly, the Frozen Niche Variation model predicts that asexual species will contain many different clones with different, and narrow, niches. The model assumes competition among clones allows only the niche specialists to survive whereas more generalist clones are out competed.

The model organism

With about 300 described species, the genus *Brevipalpus* is the largest genus of the family Tenuipalpidae. However, due to the small size of the mites, the taxonomic relations between the species are poorly understood. The genus contains both sexual and asexual species. This thesis is concerned about the asexual species. Although many asexual species have bee described, the three main species are *B. phoenicis*, *B. obovatus*, and *B. californicus*. The morphological characters that discriminate these species are limited and intraspecific variation has frequently been reported. Each of the species is found worldwide in (sub) tropical areas and occurs on hundreds of different host plant species, which shows their remarkably broad niche. Most of these host plant species are shared between two or all three mite species. These species are considered a pest on various crop and ornamental plant species, either because of the direct damage they inflict by feeding, or because of their capacity to vector plant viruses. Generally mites are controlled by applying chemical acaricides. However, mites seem to develop resistance against acaricides rapidly, which is unexpected given their clonal mode of reproduction.

In *B. phoenicis* the asexuality has been shown the effect of a vertically endocellular symbiont of the genus *Cardinium*. The mode of reproduction in sexual species of Tenuipalpidae is arrhenotoky. *Cardinium* infected *B. phoenicis* females produce infected but unfertilized eggs.
Because they are unfertilized and therefore haploid, the eggs would normally develop in males. However, the symbiont feminizes the offspring resulting in haploid females. The same symbiont has been shown causing asexuality in *B. californicus* too. However, whether it has the same effect in all lineages of these species and also in *B. obovatus* is yet unknown.

Since its recent discovery in 2001, the *Cardinium* symbiont has been found in various insect and mite species. Besides feminization, it has been shown capable of two other manipulations of host reproduction: induction of parthenogenesis and cytoplasmic incompatibility. Another endosymbiont that also infects insects and mites and has similar effects on the reproduction of its host is *Wolbachia*. However, *Wolbachia* has been known much longer and consequently much more is understood about its biology. Therefore, in this thesis I will regularly compare both bacteria to see whether the same processes apply, as one might expect from their similar biologies.

To make predictions on the relation between the mite and its symbiont, understanding of the mode of transmission of the symbiont is critical. If symbionts are strictly transmitted vertically a mutualistic relationship is expected. However, if transmission is at least occasionally horizontal, the symbiont might become a parasite to its host.

To understand the evolutionary fate of the asexual *Brevipalpus*, it is necessary to know how clonal genetic variation might arise. Therefore, a large part of this thesis is devoted to this matter. Theoretically there are four ways in which clonal variation might arise. Firstly, new clonal varieties may arise when mutations alter the existing genotypes. Secondly, new clonal varieties may arise if the symbiont is transferred horizontally to a sexual individual that then becomes the start of a new asexual lineage. Thirdly, new clonal varieties may arise when asexual females mate with the males that make up about 1% of the asexual populations. Fourthly, new clonal varieties may arise when asexual females mate with males from related sexual species.

**This thesis**

For chapter 1 I have studied the mode of asexual reproduction across a range of different genotypes covering the three common asexual species. First isofemale lines were produced to be used in the various experiments. It was shown that under standard laboratory rearing conditions, all *B. phoenicis* and *B. californicus* isofemale lines produce males in percentages ranging from 0.15 to 6.72 percent. In contrast, males were absent from all *B. obovatus* lines. When treated with antibiotics, all *B. phoenicis*, all *B.
Summary

californicus and half the B. obovatus lines produced increased numbers of males indicating that their Cardinium symbiont is responsible for their asexuality. Other B. obovatus isofemale lines did not produce males after antibiotic treatment, nor were these lines infected with Cardinium. It was concluded that in those isofemale lines asexuality probably was a genetic property of the mite itself. Finally, a comparison of taxonomies based on morphological and genetic characters revealed an incongruity. The isofemale lines not infected with Cardinium were identified as B. obovatus based on mitochondrial haplotype, but as B. phoenicis based on morphology.

In chapters 2 and 3 I have described experiments that test between the General Purpose Genotype (GPG) and Frozen Niche Variation (FNV) models to explain the broad ecological niche of these mites. In chapter 2 an ecological approach was taken. Mites from three natural populations on different host plant species were transplanted to each of the other host plants. When transplanted to a different host plant species, mites often had low reproductive values and/or died. This showed that mites are specialized to the host plant species they feed on, and thus was evidence for FNV. However, the level of host plant specialization varied among the three populations. In chapter 3 a phylogenetic approach was taken. Mites were genotyped at the mitochondrial COI gene and haplotype diversity was related to host plant species. This analysis revealed evidence for both models. Evidence for FNV was observed in one clade of mites collected exclusively from acerola host plants. Evidence for GPG was found in several B. phoenicis haplotypes that occurred on four, or more, different host plant species. The conclusion was that Brevipalpus contains evidence for both models. In addition, this chapter showed that on most host plants several genotypes of mites occurred simultaneously. On six out of the 22 sampled plants, different genotypes were found that belonged to different genetic clades. This showed that on a single host plant several mite species may occur simultaneously.

In chapter 4 I have described an experiment to test the mode of transmission of the Cardinium symbionts. From a large number of field-collected mites representing all three species, both one gene of the mite and one gene of the symbiont were sequenced. Based on these, the two phylogenies were constructed separately and compared. The symbiont phylogeny contained three clades, each associated with one of the mite species, showing that there is general congruence between host and symbiont phylogeny. This congruence was expected because the symbionts are generally transmitted vertically. However, the symbiont that was mainly associated with B. phoenicis also occurred in some B. obovatus and B.
Summary

californicus samples. These incongruities between mite and symbiont phylogenies are indicative of horizontal transmission of Cardinium among Brevipalpus species. There was some minor incongruity between host and symbiont within B. phoenicis too, indicating that horizontal transmission might take place at this level as well.

For the final two chapters I have investigated the possibility that the asexual Brevipalpus species occasionally reproduce sexually. In chapter 5 I have described an experiment that tested whether B. phoenicis females fertilize their eggs after having mated with males from the same species. Males and females of two closely related isofemale lines were crossed reciprocally, and the presence of the paternal genotype in the offspring was tested using two microsatellite markers. The effect of the symbiont on the fertilization rate was tested by making four separate crosses. In all four combinations naturally occurring males and males that result from antibiotic treated females were mated with untreated and antibiotic treated females. The results of all four crosses were that all tested offspring contained the maternal genotype only, which showed that these B. phoenicis females do not fertilize their eggs, regardless of their infection status. It is argued that this failure may be caused by a ‘functional virginity mutation’; a mutation that prevents the fertilization of eggs. Such a mutation will spread through an asexual population because individuals expressing the mutation will save the cost of genome dilution that is associated with sexual reproduction.

Finally, in chapter 6 I have described an experiment that tested if there is evidence for long-term absence of sexual reproduction in the various Brevipalpus species. The phylogeny of the mites based on a mitochondrial marker (COI sequences) was compared with the phylogenies based on two nuclear markers (AFLP and ITS1 sequences). If reproduction has been strictly clonal since the divergence of a clade, both mitochondrial and nuclear markers were expected to reveal similar phylogenies. On the other hand, incongruities were interpreted as evidence for sexual reproduction. At a higher taxonomic level mitochondrial and nuclear phylogenies were incongruous showing that sexual reproduction has occurred after this group of species started to diverge. Analysis at this level also showed that four distinct groups of mites could be identified; these were B. phoenicis, B. californicus, and the Cardinium infected and uninfected B. obovatus as separate groups. At a lower taxonomic level the evidence for strict clonality was variable. Within B. phoenicis and the infected B. obovatus there was no evidence for strict clonality. These species have either become asexually only recently, or still occasionally reproduce sexually. There was evidence for strict clonality in B. californicus and the uninfected B. obovatus. These two species probably have reproduced strictly asexual for a considerable
period of time. In addition, chapter 6 shows that there is a high amount of intragenomic variation in ITS1 sequences. This is most likely the result of a decrease in the efficiency of gene conversion caused by the fact that these mites are haploid.

**General discussion**

These are the main conclusions in respect to explaining the existence of the asexual *Brevipalpus* species. Based on the results from this thesis there is no evidence for special adaptations in *Brevipalpus* that may counter the disadvantages of asexuality. Therefore, the first explanation for the existence of asexuality in *Brevipalpus* can be discarded. The second explanation, that new asexual lineages are produced continuously from a sexual population, is conceivable for *Brevipalpus*. This would require that the symbiont can be transmitted horizontally which is indeed possible. It would also require that there are sympatric sexual and asexual populations. Whether such populations exist is still uncertain. The third explanation, that asexual species still have occasional sex, might apply to *B. phoenicis* and the infected *B. obovatus*, but occasional sex is absent in the other two species. If occasional sex does occur in *B. phoenicis* or the infected *B. obovatus*, it must be with male of the same species. If they would cross with males of other species it would have resulted in the introgression of strange genotypes in the nuclear genome that would have been revealed in these analyses. I concluded that a recent origin of asexuality and/or occasional sex can explain the existence of the asexual *B. phoenicis* and infected *B. obovatus*. It has remained unsure whether asexuality in *B. californicus* and uninfected *B. obovatus* can be explained in the same way. The latter species have reproduced strictly asexual for some time but it is uncertain whether this period has lasted too long that a recent origin of asexuality may explain their persistence. It is yet unknown how long they have been strictly asexual. Additionally, it is unknown after what period of time the disadvantages of asexuality become really problematic.

The results I presented in my this thesis indicate that the *Cardinium* symbiont is parasitic to its *Brevipalpus* hosts. When a sexual mite is first infected and becomes asexual, the symbiont relieves the mite from paying the cost of sex, and thus increasing the fitness of the host. When fertilization is no longer required for reproduction, traits involved in sexual reproduction are not maintained by natural selection and will accumulate deleterious mutations. Because of these mutations, the possibility to reproduce sexually
will eventually be lost. From this moment on, the mite will experience the long term disadvantages of asexuality, in the end driving it extinct. Hence, although the *Cardinium* infection renders a short term benefit, eventually it drives the mite extinct and therefore I considered the symbiont a parasite. Before its host goes extinct, the symbiont needs to find a new host through horizontal transmission. This thesis has revealed that *Cardinium* indeed is capable of horizontal transmission.
General Introduction

Thomas V.M. Groot

The paradox of sex

Nearly all animal species have both males and females that together produce the next generation. This common pattern of two sexes together producing offspring prompts the idea that sexual reproduction is the best way to reproduce. On second thought, this view may appear wrong because sexual reproducing is highly inefficient. This problem was first pointed out by (Weismann 1889) and, now known as the paradox of sex, has become one of the major issues in evolutionary biology. I will start the general introduction of my thesis by explaining the paradox of sex, and how it has been resolved. Next, I will show that because sexual reproduction is indeed advantageous, it is remarkable that not all species reproduce sexually. The past four years I have conducted research to explain how one particular group of asexual mite species persists without the advantages that sexual reproduction provides: species of the mite genus *Brevipalpus*, known as important pests on various economically important crops.

Sexual reproduction as a paradox

To explain the paradox of sex a definition of sexual reproduction is required. Sexual reproduction can be defined as the process where two cells (gametes) fuse to form a zygote that in turn will develop into a new individual. Alternatively, asexual reproduction can be defined as reproduction that does not require the fusion of two cells. Applying this definition, two types of asexual reproduction can be distinguished. Firstly, there is female parthenogenesis (thelytoky) where females produce female offspring from unfertilized eggs, and secondly there are females of arrhenotokous species that produce sons from unfertilized eggs. Although males are produced asexually in the latter case, arrhenotokous species generally are considered sexual because sexual reproduction is required to produce females. I will therefore always imply thelytoky when I refer to asexual reproduction.

The paradox of sexual reproduction stems from the observation that sexual reproduction in higher organisms is rather inefficient compared to asexual reproduction, yet sexual reproduction is much more common than asexual reproduction. The inefficiency of sexual reproduction arises from
the costs of sex. Various costs have been described, the most important ones being the cost of males (Maynard Smith 1978) and the cost of genome dilution (Williams 1975). The cost of males is based on the observation that males do not directly produce offspring. In a sexual population with an equal sex-ratio only half the individuals will produce offspring, whereas in an asexual population all individuals will produce offspring. Hence, the asexual population will have a twofold reproductive advantage over the sexual population, and, all else being equal, will out-compete the sexual one. The cost of genome dilution is based on the notion that sexual individuals only transmit half their genes to each offspring. They reduce their genome by 50% during meiosis and then dilute it by fusion with 50% of their partner’s genome. Asexual species do not reduce their genome but pass it on entirely to their offspring. Thus, asexual species again have a two-fold advantage over sexual species. Although being two different things (Joshi and Moody 1998), both costs are referred to as the twofold cost of sex. These costs are not necessarily exactly twofold, but depend on the mating system (Charlesworth 1980; Joshi and Moody 1998). Besides these two costs, there are various other costs associated with sexual reproduction (Crow 1999; Lewis 1987). To mention just some, there are the cost of sexual selection, the cost of finding a mate, and the costs of sexually transmitted diseases and transposons.

The ubiquity of sexual reproduction is in strong contrast to the notion that there are various costs to sex: 99% of all animal species reproduce sexually and only 1% asexually (White 1978). The paradox of sex has received a lot of attention from evolutionary biologists and is known as “the queen of problems in evolutionary biology” (Bell 1982).

Theories explaining the paradox

The ubiquity of sexual reproduction implies that, in spite of its various costs, there should also be some advantages to sexual reproduction. Many potential benefits of sexual reproduction have been proposed that may explain the persistence of sex (Barton and Charlesworth 1998; Hurst and Peck 1996; West et al. 1999). The theories explaining the advantages of sexual reproduction can be divided in two classes, one class states that sex enables the production/spread of advantageous traits, the other class states that sex enables the efficient removal of deleterious mutations.

The two main hypotheses of the first class are the “Red Queen” (Hamilton 1980; Jaenike 1978) and the “Tangled Bank” (Bell 1982; Ghiselin 1974). The Red Queen hypothesis states that sex is advantageous when selection pressures fluctuate over time. A certain genotype might be adapted well to the current environment and consequently have a high
fitness. However, when the environment changes it might be less adapted to the new environment and therefore experience a decreased fitness. In such a situation sex is favored because it constantly creates new genotypes that might be better adapted to the changed environment. Typically, this process is used to describe the interaction between host and parasites; a change in the behavior of one of the two players, results in a changed selection pressure on the other player. Besides its typical description, the principle is easily extended to any other factor in the organisms’ environment that may cause a change in the selection pressures. The Tangled Bank hypothesis states that sex is advantageous when resource competition is intense. In asexual species all members of a clone have the same niche and compete for the same resources, but leave other resources underexploited. Sex is advantageous because it creates new genotypes that can escape the strong competition by using these underexploited resources.

The two main models supporting the idea that sex enables the efficient removal of deleterious mutations are “Muller’s Ratchet” (Muller 1964) and the “deterministic mutation hypothesis” (Kondrashov 1988). Both theories state that asexual populations will suffer from an increasing mutational load. Sexual species do not suffer from this because recombination allows for effective purging of deleterious mutations. According to Muller’s Ratchet, the mutational load of an asexual population will increase because of stochastic processes. The individuals in population can be divided in classes according to the number of deleterious mutations each carries in its genome. By chance alone, the class of individuals with the lowest number of deleterious mutations in a population will get lost. The best class then exists of individuals with one additional deleterious mutation. This class too will eventually go extinct because of stochastic processes. This successive loss of the best class is an irreversible process and will eventually lead to a situation where the best class has a fitness that is so low that the population will go extinct. The ratchet does not exist in sexual species; in sexual species classes with fewer mutations can be re-established through recombination. Because Muller’s ratchet is a stochastic process, the rate at which the ratchet works depends much on population size: the larger the population, the smaller the chance that all individual in the best class are lost, and thus the slower the ratchet. Not dependent on population size is the deterministic mutation hypothesis. This model has two main assumptions: there is a high input of new deleterious mutations in every generation, and epistatic interactions among the deleterious mutations make that the fitness reduction of all mutations combined is larger than the sum of the fitness reduction of each mutation alone. The model predicts that the mutational load of an asexual species will inescapably increase until it drives the
species extinct. In sexual species recombination produces more variation in the number of deleterious genomes, enabling selection to select the better genotype, and thus preventing the accumulation of deleterious mutations. Although Muller’s Ratchet and the deterministic mutation hypothesis describe two different processes, the differences between them are small. In practice both processes work simultaneously and are hard to distinguish.

The various theories of the advantages to sexual reproduction have been tested using mathematical models (e.g. Doncaster et al. 2000; Green and Noakes 1995; Hadany and Feldman 2005; Otto and Lenormand 2002; Otto and Nuismer 2004; Pound et al. 2004; Wilke 2004). These models have shown that the advantage of each theory alone is generally insufficient to compensate for all the costs of sex. However, West et al. (1999) have advocated a pluralistic approach in which different theories are combined to come to an advantage that is large enough to compensate all the costs.

Not only theoretical models have been used to validate the theories on the advantages of sex, but recently an increasing number of empirical studies have confirmed the same theories. These studies either used organisms with short generation times (such as microorganisms or *Drosophila*) to study their evolution in the lab (Colegrave 2002; de Visser and Rozen 2005; Fischer and Schmid-Hempel 2005; Goddard et al. 2005; Grimberg and Zeyl 2005; Rice 2002; Rice and Chippindale 2001; Xu 2004), or studied evolution in natural populations of various asexual species, mainly by using molecular genetic data (Butlin 2002; Cutter and Payseur 2003; Jokela et al. 2003; Mark Welch and Meselson 2001; Paland and Lynch 2006; Schon et al. 2003; Xu 2004).

**Persistence of asexual species**

Because various advantages of sexual reproduction have been postulated and confirmed, the ubiquity of sexual reproduction does not seem very paradoxical anymore. In fact, considering all the advantages to sexual reproduction, one might wonder why asexual species still exist at all. If a certain mechanism describes an advantage of sexual reproduction, one may also consider the same mechanism a disadvantage of asexual reproduction. In this sense, the main disadvantages of asexual reproduction are a lowered adaptive ability and the inability to purge deleterious mutations. An explanation for the persistence of asexual species will have to account for these disadvantages. There are a few reasons why asexual organisms exist, at least temporarily.

First, asexual species might still exist because they have special characteristics that allow them to survive without sex. Examples of such characteristics to overcome the disadvantages of asexuality could be very
low mutation rates or very efficient DNA repair mechanisms. These special characteristics may explain the persistence of ancient asexuals such as the bdelloid rotifers (Mark Welch and Meselson 2000), darwinulid ostracods (Van Doninck et al. 2002), and oribatid mites (Maraun et al. 2003).

Second, asexual species might exist because new asexual lineages arise constantly. Although the various processes that give benefits to sexual reproduction act at different time scales, each theory states that sex is mainly advantageous in the long run. Consequently, asexual species might be quite successful in the short run. Because a new asexual species will arise from within a sexual population, it will start off with a genome that is well adapted to the current environment, has a low mutational load, and most importantly, does not pay the costs of sex. Therefore, provided that there is a mechanism that regularly allows sexual individuals to somehow become asexual, the persistence of asexual species might be explained by the initial benefits of not having to pay for the costs of sex. This however implies that asexual species suffer the disadvantages of asexuality in the long run and therefore will be short-lived. Indeed, asexual species are generally considered to be short lived (Bell 1982; Maynard Smith 1978; White 1973).

Third, asexual species might occasionally reproduce sexually by crossing with spanandric males (males that are occasionally produced by asexual females) or with males from related sexual populations. Mathematical model studies have shown that only a very limited amount of recombination is required to make up for nearly all the disadvantages of asexual reproduction (Green and Noakes 1995; Hadany and Feldman 2005; Hurst and Peck 1996) [see also Otto and Jarne (2001) for a discussion specifically on Brevipalpus mites]. By only occasionally reproducing sexually, such species can combine the best of two worlds; not paying the costs of sex, but also not suffering from the disadvantages of asexuality. Species that have a similar reproductive strategy are the cyclical parthenogens. For example some aphid and daphnia species in the temperate regions are asexual in spring and summer but have one sexual generation in autumn (Innes and Singleton 2000; Vorburger et al. 2003). However, occasional sex still has a cost and therefore might not be an evolutionary stable strategy in many systems without strong seasonality.

Adaptation in asexuals

Despite the expected difficulties of asexual species to adapt to different environments (see above), many asexual species have a broad niche, often extending that of their sexual ancestor (Bell 1982; Hughes 1989; Lynch 1984; Vrijenhoek 1998). Apparently, these asexual species have succeeded to adapt to many different environments. Two theoretical models exist that
explain how an asexual species can realize a broad niche; the General Purpose Genotype (GPG) model, and the Frozen Niche Variation (FNV) model.

The GPG model (Baker 1965; Lynch 1984) predicts that only clones that can tolerate many different environmental conditions will survive over longer evolutionary times. The model assumes that the clonal genotypes in a population are fixed; the genotypes do not change and no new genotypes are produced. At the start, the asexual species may contain many different clones that have variable niche widths. Because the environment changes over time, clones that have narrow niches will go extinct because at certain times their niches cease to exist. Generalist clones, however, are tolerant to such environmental changes and therefore face a better chance to survive. Once enough time has passed and sufficient environmental changes have occurred, all specialist clones will have gone extinct. The surviving clones are said to have a General Purpose Genotype.

The FNV model predicts that clones will be selected that are highly specialized and have very narrow niches (Vrijenhoek 1979). This model works on a relative short evolutionary time scale and allows for continuous production of new clones. The model assumes that many different clones originate from a sexual ancestor; either these clones all originated at the onset of asexual reproduction, or new clonal lineages arise from within the sexual ancestor population continuously. Each of these clones will inherit a different suite of characters from the sexual ancestor and therefore will freeze a different part of the ancestor’s niche. Competition with the sexual ancestor and among the various asexual clones will result in selection for clones that are highly specialized and have non-overlapping niches. The surviving genotypes will be a set of genotypes that each has frozen a different part of the niche of the sexual ancestor.

Although GPG and FNV appear to be alternatives in theory (Parker and Niklasson 2000), in practice the separation between them is not always straightforward. The GPG and FNV models operate on different time scales and make different assumptions on the production of new genotypes. The GPG model focuses on what happens over long periods of evolutionary time in a situation where no new clonal types are produced, whereas the FNV model considers short term clonal interaction in situations where new clonal genotypes have recently been produced or are still being produced regularly. Because of these differences, both models are not mutually exclusive (Vrijenhoek and Pfeiler 1997). Therefore, it might not always be easy to assign one of these models to a certain asexual species. Other complicating factors are that a specialist and a generalist are two alternative extremes
from what in reality is a continuum, and that a clone that is a specialist for one trait might be a generalist for another (Vrijenhoek 1998).

**Biology of the mite**

Now I will introduce the model organisms of this study: asexual *Brevipalpus* mites. Most of the basic information can be found in reviews by Kennedy (1995) and Childers et al. (2003a).

**Taxonomy and Species diversity**

With about 300 described species, *Brevipalpus* is the largest genus in the family Tenuipalpidae (Welbourn et al. 2003). Species identification, however, is difficult, mainly because of the small sizes of the mites (approximately 200 - 410µm) and the limited amount of distinguishing morphological characters. The genus as a whole is more easily recognized. The three asexual species most commonly referred to are *B. phoenicis* (Geijskes), *B. obovatus* Donadieu, and *B. californicus* (Banks) and I focus on these species in this thesis. Childers et al. (2003b) list a total of 928 host plant species of these three species, representing 513 plant genera from 139 families. Although only one mite species has been reported from some plant species, most plant species are host to two or all three *Brevipalpus* species. Each species occurs worldwide in tropical and subtropical areas, but is also found in temperate regions in greenhouses. Morphological differentiation between these three species is limited and intraspecific variation in the key characteristics has been reported frequently (reviewed by Welbourn et al. 2003). Due to this intraspecific morphological variation, the extensive diet breadth, the extensive geographic distributions, and the large number of plant-specific viruses that are vectored by the mites, Childers et al. (2003b) expressed the concern that cryptic species complexes might be hidden in these species.

**Development**

The complete life cycle of *Brevipalpus* consists of eight developmental stages. After the egg stage, the mite goes through three active and three inactive stages before adulthood is reached. In between the active stages (larva, protonymph, deutonymph, and adult) are three inactive chrysalis stages during which the mites mold. Depending on temperature, humidity and host plant species or variety, the egg to adult development time of *B. phoenicis* varies from 16 to 48 days (reviewed by Kennedy 1995).
Pests
All three species are frequently reported as pests on many host plant species. The mites inflict damage on the plants either directly through feeding or indirectly by transmitting plant pathogens. Feeding damage results in various symptoms such as defoliation, gall formation, scarring, and browning of the foliage. Examples of crops that can be severely affected by direct feeding damage are papaya, passion fruit (Haramoto 1969), and tea (Kennedy 1995; Oomen 1982). Indirect damage is mainly caused by viruses that are transmitted by the mites. An increasing number of economically important host plant species are found to be infected with rhabdoviruses that are transmitted by *Brevipalpus* mites (Kitajima et al. 2003). Of special interest is Citrus Leprosis Virus. The Brazilian citrus industry spends 80 million Euro annually to control the mites that vector this virus (Rodrigues et al. 2003). Although this virus mainly occurs in South America, recent reports from Central America have raised the concern that the virus might spread (Childers and Rodrigues 2005). Unfortunately, very little is known about the specificity between the three components of the mite, virus and host plant species interactions (Rodrigues et al. 2005; Kitajima pers. comm.). Knowledge on how efficient different mite species transfer different types of viruses to different plant species is urgently needed for proper control of these pests. Currently, the control of *Brevipalpus* is mainly through the application of chemical acaricides (Campos and Omoto 2002) although biological alternatives are being developed (Magalhaes et al. 2005). A serious problem with chemical control of the mites is the relatively rapid development of resistance (Campos and Omoto 2002; Omoto 1998). This rapid development is surprising given that the mites are asexual. Apparently, they are able to adapt to changing selection pressures despite their asexual mode of reproduction.

The fact that *Brevipalpus* mites are important pests gives socioeconomic relevance to this research. Considering its economic importance, it is amazing how little is known about them. For example, a robust phylogeny is lacking, hardly anything is known about clonal variation in natural population and levels of host specificity. It appears that the mites are quite capable to adapt to different environments; they occur on many different host plant species and geographic areas, and also develop resistance to acaricides quickly (Campos and Omoto 2002). This capacity to adapt is unexpected based on the asexual mode of reproduction. Insights in how the mites succeed to adapt and evolve will give valuable insights in how the mite can be managed. For example, still little is known about the capacity of mites to feed on alternative host plant species when the crop is sprayed with acaricides. If the mites can easily switch between different host
General Introduction

plant species, this will affect the efficiency of chemical control. Similarly, although all species are asexual, nothing is known about the strictness of the clonal reproduction. The asexual reproduction is induced by an intracellular bacterium (see below). If the applied acaricides have anti-bacterial effects, the mites can lose this bacterium and then may revert to sexual reproduction. This sexual reproduction would be a most unwelcome side-effect of the acaricide because it could enable the production of resistant genotypes.

Reproduction
The genus *Brevipalpus* contains both sexual and thelytokous species (Helle et al. 1980). The sexual species reproduce by arrhenotoky, where fertilized eggs develop into diploid females and unfertilized eggs develop into haploid males. The most common species, however, are thelytokous. In the early 1980’s Pijnacker et al. (1980) suggested that females of thelytokous *Brevipalpus* mites are haploid because thelytokous females had the same number of chromosomes as males of arrhenotokous species. In addition, G banding patterns suggested that the two chromosomes were not homologous. Weeks et al. (2001) settled the issue and showed that the two chromosomes are not homologous in *B. phoenicis* using fluorescent in situ hybridization techniques and microsatellites. Quantification of the amount of DNA in the cells during egg development revealed that prior to meiosis the amount of DNA in the cells is doubled (Pijnacker et al. 1981). This premeiotic doubling enables haploid females to produce haploid gametes through normal meiosis. Weeks et al. (2001) showed that *B. phoenicis* was infected with a vertically transmitted intracellular symbiont and that curing this infection with antibiotics resulted in the production of males. The authors concluded that this endosymbiotic bacterium was responsible for the thelytoky by feminizing the individuals that hatch from the unfertilized eggs, and thus become haploid females. Whether the other asexual *Brevipalpus* species are also feminized by the same symbiont still remains to be seen.

Biology of the symbiont

Occurrence and effects
The bacterial endosymbiont that induces thelytoky in *B. phoenicis* was new to science when it was discovered in this species (Weeks et al. 2001). It was shown to be a new strain within the Cytophaga-Flavobacterium-Bacteroides phylum (CFB). This phylum contains species that show an extremely diverse range of physiological and morphological characters (Reichenbach 1991). Species from this phylum have been found in most environments,
including the human gut, soil, fresh water and sea water and activated sludge (Horn et al. 2001). Endosymbiotic CFB bacteria are known from cockroaches (Bandi et al. 1994), termites (Bandi et al. 1995), and ladybirds (Hurst et al. 1999; Hurst et al. 1997). The CFB symbionts of the ladybirds manipulate the reproduction of their hosts by male killing, but they belong to a different lineage than the *B. phoenicis* symbiont.Shortly after its discovery in *B. phoenicis*, the same bacterium was shown responsible for inducing parthenogenesis in *Encarsia* wasps (Zchori-Fein et al. 2001). The symbiont of *Encarsia* has been formally described as *Candidatus Cardinium* hertigii and therefore *Cardinium* now is the general name for this group of endosymbionts. The presence of *Cardinium* has been checked using specific PCR in 320 insect and mite species and 6 - 7% of them are indeed infected (Weeks et al. 2003; Zchori-Fein and Perlman 2004). Studies that have systematically looked for *Cardinium* in other organisms than insects and mites are currently lacking. Since its discovery, *Cardinium* has been shown capable of various reproductive manipulations in various host species, such as feminization in *Brevipalpus* mites (Chigira and Miura 2005; Weeks et al. 2001), induction of parthenogenesis in *Encarsia* wasps (Zchori-Fein et al. 2001; Zchori-Fein et al. 2004) and scale insects (Provencher et al. 2005), and inducing cytoplasmic incompatibility in again *Encarsia* wasps (Hunter et al. 2003) and in the mite *Metaseiulus occidentalis* (Weeks and Stouthamer 2004).

**Comparison to Wolbachia**

As described above, *Cardinium* has only recently been discovered. Another endosymbiont that can manipulate host reproduction and is known for a much longer period is *Wolbachia*, a member of the Proteobacteria. Although taxonomically unrelated, both bacteria do share much of their biology (Weeks and Breeuwer 2003). Like *Cardinium*, *Wolbachia* is a vertically transmitted endocellular symbiont that infects a wide range of arthropod host species (reviewed by Stouthamer et al. 1999). *Wolbachia* is also capable of inducing parthenogenesis, feminization, and cytoplasmic incompatibility. In addition, *Wolbachia* can cause male killing which is the only reproductive manipulation that has (not yet) been shown for *Cardinium*. The sharing of these manipulations of host reproduction by the unrelated bacteria is a striking example of convergent evolution. This observation begs for further comparisons to see if both bacteria also use the same mechanisms to manipulate their hosts. I will therefore regularly compare the results of this thesis with what is known for *Wolbachia*.
Relation to the host
This thesis concerns the evolution of asexual *Brevipalpus* mites. The evolutionary fate of these mites is very much connected with, and influenced by, the symbiont. Therefore, it is necessary to investigate the relationship between the two. Bacterial symbionts that manipulate the reproduction of their hosts are often referred to as reproductive parasites (Werren et al. 1995b), implying a host-parasite interaction. Such a description implies that the symbiont imposes a fitness cost onto its host. When the symbionts induce cytoplasmic incompatibility, male killing, or feminization, this cost is easily seen; cytoplasmic incompatibility will result in a reduced fitness of infected males because they cannot fertilize uninfected females, male killing reduces the fitness of infected females because part of their offspring is killed, and feminization reduces fitness of infected females because there is a shortage of males to fertilize their eggs. However, the costs are not so clear when the symbiont induces parthenogenesis. In fact, one might argue that the symbiont enhances the fitness of its host because it relieves the host from paying the costs of sex. On the other hand, asexual reproduction has several long term disadvantages. These disadvantages can be regarded as the fitness costs imposed on the host by the symbiont. If this is the case for the thelytokous *Brevipalpus*, the symbiont is a parasite and infection with *Cardinium* causes the infected lineage to be evolutionarily doomed. Under this scenario, the question whether the symbiont is capable of horizontal transmission is of particular importance. For the symbiont to survive, it needs to reach a new host before it drives its current host extinct. Therefore, a certain level of horizontal transmission is required for the symbiont to guarantee its continuation as a reproductive parasite. Apart from the reproductive manipulations, the symbiont can have other costs or benefits that further complicate the relation to the host. Evidence for the physiological costs and benefits of *Wolbachia* infections have been reviewed by Bordestein and Werren (2000) and Vala et al. (2003). A benefit of *Cardinium* infection is known for *Metaseiulus occidentalis* where infection results in higher fecundity (Weeks and Stouthamer 2004).

**Origin of clonal variation in *Brevipalpus***

Crucial to the understanding of the persistence of an asexual species is to know how variation in clonal genotypes is generated. For the asexual *Brevipalpus*, clonal variation might be generated in four different ways.
1. Mutations
New clonal types might originate from mutations that alter existing types. The establishment of divergent genotypes through mutations will be a slow process. Individual mutation will have only limited effect and most mutations will be deleterious and therefore selected against. However, given enough time, the effect of the accumulated mutations might become substantial. For example, the bdelloid rotifers are believed to have been strictly asexual for the last 35 - 40 million years, leaving accumulated mutations as the exclusive explanation for their diversification, and still 360 different species have been described (Mark Welch and Meselson 2000; Mark Welch et al. 2004).

2. Horizontal transmission of the symbiont
New clones may arise when sexually reproducing individuals become infected through horizontal transmission of the symbiont. Currently no sexual populations are known of the species studied here, and therefore it is unlikely that new clones are still being generated in this way. However, there must have been a period when sexual and asexual mites occurred in sympathy before the infection had reached fixation. Thus, if the symbiont is capable of regular horizontal transmission, there might be a polyclonal origin of the thelytokous *Brevipalpus*.

3. Occasional sex with spanandric males
New clones may result from eggs fertilized by spanandric males. Like in many asexual organisms (Lynch 1984), spanandric males have frequently been reported in thelytokous *Brevipalpus* species (Chigira and Miura 2005; Haramoto 1969; Kennedy 1995; Nagesha Chandra and Channabasavanna 1974; Oomen 1982; Pijnacker et al. 1980; Razoux Schultz 1961). These males also mate with thelytokous females (Haramoto 1969; pers. obs.). It is possible that thelytokous *Brevipalpus* females receive sperm and fertilize their eggs as do thelytokous *Trichogramma* wasps (Stouthamer and Kazmer 1994; Stouthamer and Luck 1993; Stouthamer et al. 1990).

4. Occasional sex with males of related sexual species
In the same way that occasional sex with spanandric males could generate new clones, new clones could also be generated if thelytokous females occasionally hybridize with males of related sexual species. These sexual species would have to be closely related and occur sympatrically with the thelytokous species. During my field work, I have never come across such a candidate sexual species; all sexual species I have encountered were rather unrelated to the asexual species with respect to morphology and molecular
phylogeny. However, sympatric sexual and asexual species might exist elsewhere. For example, Gonzalez (1975) described both males and females of the species *B. phoenicoides*, which is highly similar to and supposedly sympatric with *B. phoenicis*.

This thesis

This thesis contains six chapters describing the experiments I performed to study various aspects of the evolution of thelytokous *Brevipalpus* mites. Chapter 1 reports on several experiments to assess the mode of reproduction in the three common asexual *Brevipalpus* species. Each of the species was represented by a number of isofemale lines. First, the genetic relationships between the species were studied using mitochondrial *COI* sequences and these relationships were compared to the morphological species descriptions. Then, each isofemale line was checked for infection with *Cardinium*, and whether this bacterium was the inducer of the observed thelytoky.

The following two chapters describe experiments that tested the GPG and FNV models by studying how natural populations of *Brevipalpus* are adapted to their host plant species. In chapter 2 an ecological approach was taken. Mites were reciprocally transplanted between three host plant species and the performance on the original and novel hosts was compared. When mites were adapted according to the GPG model, it was expected that they are able to switch between the host plant species. Alternatively, if mites are adapted according to the FNV model, it was expected that they are unable to successfully switch between host plant species. In chapter 3 a genetic approach was taken. Mites collected from different host plant species from various locations in Southern Brazil were genotyped, and the relation between mite genotype and host plant species was studied. When mites were adapted according to the GPG model, it was expected that the same mite genotype occurs on various host plant species. Alternatively, when mites are adapted according to the FNV model, each mite genotype is expected to occur on only one host plant species.

In chapter 4, I took a closer look at the mode of transmission of the *Cardinium* symbiont. For a large number of individual mites both a mitochondrial gene (*COI*) of the host and a gene of the symbiont (*gyrB*) were sequenced. Gene trees were constructed for both genes and the trees were compared. If transmission would be strictly vertical, both genes would be inherited maternally. Therefore, it was expected that both gene-trees will show similar phylogenies if transmission was strictly vertical. In contrast,
incongruities between both gene-trees were taken as evidence for horizontal transmission of the symbiont.

In the final two chapters, I tested the possibility that new clonal variation is created by occasional sex. Chapter 5 describes an experiment where attempts were made to create hybrid offspring from two closely related *B. phoenicis* isofemale lines. Males and females were mated in reciprocal crosses and the presence of the paternal genotype in the offspring was checked using microsatellite markers. Chapter 6 describes an experiment that tested for the occurrence of recombination in the relatively recent evolutionary past of the different asexual *Brevipalpus* species. The phylogenetic signal from a mitochondrial marker was compared with the phylogenetic signal from two nuclear markers. If no recombination had taken place for a long evolutionary period, both types of markers were expected to reveal the same phylogeny. Incongruities between mitochondrial and nuclear markers, on the contrary, can be taken as evidence for recent recombination events. The presence of these recombination events may indicate that either occasional sex takes place, or that the mites have only recently become asexual.

After these six chapters, the results of the various experiments are combined in the general discussion. In addition, I discuss a number of other subjects that could not be treated in any of the research chapters.

**Acknowledgements**

I am thankful to the people who provided suggestions to improve this introduction. These people were Vera Ros, Steph Menken, Hans Breeuwer, Arne Janssen, and Maus Sabelis.
Chapter 1

*Cardinium* Symbionts Induce Haploid Thelytoky in Most Clones of Three Closely Related *Brevipalpus* Species

Thomas V. M. Groot and Johannes A.J. Breeuwer

Abstract

Bacterial symbionts that manipulate the reproduction of their host to increase their own transmission are widespread. Most of these bacteria are *Wolbachia*, but recently a new bacterium, named *Cardinium*, was discovered that is capable of the same manipulations. In the host species *Brevipalpus phoenicis* (Acari: Tenuipalpidae) this bacterium induces thelytoky by feminizing unfertilized haploid eggs. The related species *B. obovatus* and *B. californicus* are thelytokous too, suggesting that they reproduce in the same remarkable way as *B. phoenicis*. Here we investigated the mode of thelytokous reproduction in these three species. Isofemale lines were created of all three species and 19 lines were selected based on variation in mitochondrial *COI* sequences. All *B. phoenicis* and *B. californicus* lines (10 and 4 lines, respectively) produced males under laboratory conditions up to 6.7%. In contrast, males were absent from all *B. obovatus* lines (5 lines). Additional experiments with two *B. phoenicis* isofemale lines showed that males can be produced by very young females only, while older females produce daughters exclusively. For most lines it was shown that they are indeed feminized by a bacterium as treatment with antibiotics resulted in increased numbers of males up to 13.5%. Amplification and identification of specific *gyrB* sequences confirmed that those lines were infected with *Cardinium*. Three out of the five *B. obovatus* lines did not produce males after treatments with antibiotics, nor did they contain *Cardinium* or any other bacterium that might induce thelytoky. In these lines thelytoky is probably a genetic property of the mite itself. Despite the different causes of thelytoky, flow cytometry revealed that all 19 lines were haploid. Finally, the taxonomic inferences based on the mitochondrial *COI* sequences were incongruous with the classical taxonomy based on morphology, suggesting that a taxonomic revision of this group is necessary.
Introduction

Bacterial symbionts that manipulate host reproductive behavior are widespread (Werren and O'Neill 1997). They increase their own fitness by manipulating the reproduction of their hosts in different ways, which include: the induction of parthenogenesis, feminization, cytoplasmic incompatibility, and male-killing. Most of these bacteria are *Wolbachia*, but male-killing bacteria comprise a variety of unrelated bacteria and microsporidia (Hurst et al. 2003). Recently, a new bacterial genus named *Cardinium* has been described which consists of species that are also capable of manipulating host reproduction (Hunter et al. 2003; Weeks et al. 2001; Zchori-Fein et al. 2004). It appears to be less widespread than *Wolbachia* (Weeks et al. 2003; Zchori-Fein and Perlman 2004). In one of its hosts, the phytophagous false spider mite, *Brevipalpus phoenicis* (Geijskes) (Acari: Tenuipalpidae), *Cardinium* induces thelytokous reproduction (Weeks et al. 2001). Moreover, because females in this mite are haploid, *Cardinium* seems to feminize unfertilized eggs. Without *Cardinium* unfertilized eggs of *B. phoenicis* develop into haploid males. The existence of haploid females in itself is an intriguing exception to the rule that females of metazoan species are diploid or polyploid (Weeks et al. 2001).

The genus *Brevipalpus* contains about 300 arhenotokous and thelytokous species (Helle et al. 1980; Welborn et al. 2003). Because species descriptions are based upon a limited set of morphological characters that are often variable within species, it remains to be seen to what extent species delineations are supported by molecular data. Nevertheless, a large proportion of these species is thelytokous suggesting that the haploid females of *B. phoenicis* and *Cardinium* infections are not unique. Pijnacker et al. (1980; 1981) have shown that females of the thelytokous species *Brevipalpus obovatus* Donnadieu have equal numbers of chromosomes as males and therefore females might be haploid. Weeks et al. (2003) have shown that this species too is infected with *Cardinium*. In addition, the symbiont has also been reported to be the cause of thelytokous reproduction in *Brevipalpus californicus* (Banks), and it has also been found in *Brevipalpus lewisi* McGregor (Chigira and Miura 2005).

Thelytokous reproduction in *B. phoenicis* and *B. obovatus* is not complete since males frequently occur at low frequencies (Haramoto 1969; Kennedy 1995; Nagesha Chandra and Channabasavanna 1974; Oomen 1982; Pijnacker et al. 1981; Razoux Schultz 1961). From parthenogenesis inducing *Wolbachia* it is known that the production of sons is generally caused by the failure to transmit (sufficient numbers of) the symbiont (Stouthamer et al. 1999) and the occurrence of males in *Brevipalpus*
probably has the same reason. The number of males in natural *Brevipalpus* populations varies (results presented in this paper). Whereas some populations do contain males up to 3.3 % of the population, males are entirely absent from others. This variation in the number of males between populations can have four causes that are not mutually exclusive; 1) transmission efficiency of the symbiont might be influenced by variable environmental conditions in the field, 2) population age structure differences between populations might influence the total number of males if males are produced at a specific age, 3) different populations might contain different types of mites and / or symbionts that differ in their transmission efficiency, and 4) there might be different causes for thelytoky in different populations of mites.

For a better understanding of the evolution of these species with their unique reproduction by haploid females, more information about the occurrence in and the effect of *Cardinium* on the different mite species is necessary. Here we present the results of a study into the cause of thelytoky in *Brevipalpus* mites. We have looked at the presence of *Cardinium* and studied the transmission efficiency and the effects on thelytoky of the symbiont.

**Material and Methods**

**Sampling and maintenance of isofemale lines**

*Brevipalpus* mites were collected from various host plants in Brazil, in the states São Paulo and Minas Gerais, and in the Netherlands, in the Burger’s Bush tropical greenhouse (Table 1). The collections from São Paulo were made in 1999, the collections from Minas Gerais and the Netherlands were made in 2004. *Brevipalpus* collections made from a single host plant frequently contain different genotypes (Groot and Breeuwer unpub.). Therefore, isofemale lines were produced to study the characteristics of the different genotypes separately. Isofemale lines were initiated by placing individual adult female on detached leaves of common bean (*Phaseolus vulgaris*) placed on soaked cotton wool. Later, isofemale lines were maintained on seedlings of common bean placed in florist’s foam because this resulted in higher population densities and required less labor. Isofemale lines were reared in a climate room with constant temperature, relative humidity, and photoperiod (24°C, 70%, and 16L:8D). All experiments with living mites described below were conducted in the same room.
Table 1. Details of the *Brevipalpus* isofemale lines and the number of males they produced. Indicated in the first half are the sampling location, host plant species, the percentage of males that occur in each isofemale line under laboratory conditions, and the number of adult individuals (N) that was counted to determine this percentage. The percentage of males was not counted in line 20 because this line was lost shortly after the experiment on the timing of male production was performed. Indicated in the second half are the percentage of males and the total number of adult individuals in 13 isofemale lines resulting from the first and second replicate treatments with antibiotics. Abbreviations for sampling locations are: SP = São Paulo state and MG = Minas Gerais state.

<table>
<thead>
<tr>
<th>Line</th>
<th>Sampling location</th>
<th>Host plant species</th>
<th>% males</th>
<th>N</th>
<th>First replicate</th>
<th>Second replicate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% males</td>
<td>N</td>
</tr>
<tr>
<td>1</td>
<td>Brazil, SP</td>
<td>Citrus sp.</td>
<td>3.6</td>
<td>500</td>
<td>14.4</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>Brazil, SP</td>
<td>Citrus sp.</td>
<td>1.9</td>
<td>523</td>
<td>7.4</td>
<td>81</td>
</tr>
<tr>
<td>3</td>
<td>The Netherlands</td>
<td>Monodora crispata</td>
<td>2.1</td>
<td>579</td>
<td>2.9</td>
<td>204</td>
</tr>
<tr>
<td>4</td>
<td>The Netherlands</td>
<td>Terminalia ivorensis</td>
<td>1.2</td>
<td>510</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Brazil, MG</td>
<td>Hibiscus rosa-sinensis</td>
<td>3.0</td>
<td>777</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Brazil, MG</td>
<td>Hibiscus rosa-sinensis</td>
<td>1.8</td>
<td>767</td>
<td>13.8</td>
<td>123</td>
</tr>
<tr>
<td>7</td>
<td>Brazil, MG</td>
<td>Citrus sp.</td>
<td>2.9</td>
<td>612</td>
<td>4.2</td>
<td>215</td>
</tr>
<tr>
<td>8</td>
<td>Brazil, MG</td>
<td>Citrus sp.</td>
<td>0.2</td>
<td>617</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Brazil, MG</td>
<td>Rhododendron sp.</td>
<td>0.3</td>
<td>603</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Brazil, MG</td>
<td>Carica papaya</td>
<td>6.0</td>
<td>563</td>
<td>7.5</td>
<td>174</td>
</tr>
<tr>
<td>11</td>
<td>Brazil, MG</td>
<td>Malpighia glabra</td>
<td>0</td>
<td>552</td>
<td>0</td>
<td>123</td>
</tr>
<tr>
<td>12</td>
<td>The Netherlands</td>
<td>Strongylodon macrobotrys</td>
<td>0</td>
<td>532</td>
<td>0</td>
<td>163</td>
</tr>
<tr>
<td>13</td>
<td>The Netherlands</td>
<td>Bauhinia grandiflora</td>
<td>0</td>
<td>705</td>
<td>0</td>
<td>198</td>
</tr>
<tr>
<td>14</td>
<td>Brazil, MG</td>
<td>Zingiber sp.</td>
<td>0</td>
<td>542</td>
<td>4.7</td>
<td>129</td>
</tr>
<tr>
<td>15</td>
<td>Brazil, MG</td>
<td>Hibiscus rosa-sinensis</td>
<td>0</td>
<td>499</td>
<td>4.2</td>
<td>118</td>
</tr>
<tr>
<td>16</td>
<td>Brazil, MG</td>
<td>Rhododendron sp.</td>
<td>0.2</td>
<td>671</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Brazil, MG</td>
<td>Rhododendron sp.</td>
<td>0.6</td>
<td>472</td>
<td>5.3</td>
<td>131</td>
</tr>
<tr>
<td>18</td>
<td>The Netherlands</td>
<td>Euphorbia xanthii</td>
<td>1.57</td>
<td>508</td>
<td>0.6</td>
<td>182</td>
</tr>
<tr>
<td>19</td>
<td>The Netherlands</td>
<td>Thevetia peruviana</td>
<td>6.72</td>
<td>506</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Brazil, SP</td>
<td>Citrus sp.</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**COI genotyping of mites**

We started with 75 isofemale lines which included representatives of each of the three species, *B. phoenicis*, *B. obovatus*, and *B. californicus*. Phylogenetic relationships were determined by sequencing part of the mitochondrial cytochrome oxidase I gene (*COI*). DNA was extracted using a modified CTAB (CTAB in water) extraction protocol (Doyle 1991). Per isofemale line 15 adult females were ground in 3 µl of CTAB buffer in a 0.5 ml tube. After grounding, another 97 µl CTAB was added. Tubes were then vortexed for 10 seconds and incubated at 55 °C for 1 hour. Next, 100 µl of chloroform : iso-amyl-alcohol (24:1) was added and tubes were shaken for 5 min. Then, tubes were centrifuged at 15,800g for 20 min. After spinning, 85 µl of supernatant was transferred to a clean tube. DNA was precipitated in the presence of 8.5 µl 5M NaCl and 170 µl ice-cold 96% ethanol. Tubes
were incubated at -20 °C for 1 hour and then centrifuged at 15,800g for 20 min at 4°C. All supernatant was removed and DNA pellet was air dried overnight. The following day, DNA was eluted in 20 µl sterile water and stored at -20°C. Part of the mitochondrial gene was amplified using primers 5'-TGA TTT TTT GGT CAC CCA GAA G-3’ and 5’-TAC AGC TCC TAT AGA TAA AAC-3’ (Navajas et al. 1996). In Brevipalpus these primers amplified a 410 bp fragment of the COI gene, excluding the primer annealing sites. PCR was performed in a total volume of 25 µl containing: 2.5 µl 10X Super Taq buffer (HT Biotechnology, Cambridge, U.K.), 1.25 µl bovine serum albumin (10 mg/ml), 1.5 µl MgCl$_2$ (25 mM) (additional to the buffer), 5 µl dNTP mix (1 mM of each nucleotide), 0.5 µl each primer (10 µM each), 0.2 µl of super Taq (5 u/µl) (HT Biotechnology), 12.55 µl water, and 1 µl of DNA extract. Cycling conditions were: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, concluded with a final extension at 72°C for 4 min. Products (2.5 µl) were checked by electrophoreses in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA, pH 8.0) on 1% agarose gels stained with ethidium bromide. Prior to sequencing, PCR products were purified using the DNA extraction kit (Fermentas, St. Leon-Rot, Germany). Cleaned products were cycle-sequenced in both directions using the same primers as used in the amplification and the ABI PRISM BigDye Terminator Sequence Kit (version 1.1, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) according to the manufacturer’s instructions but diluted 16 times. Sequences were run on an ABI 3700 automated DNA sequencer. The obtained sequences were aligned using ClustalX (Thompson et al. 1994) and a Neighbor-Joining tree was constructed using PAUP version 3.10b (Swofford 1998). Based on this tree, a subset of 19 isofemale lines was selected that covered most of the genetic variation present. A new phylogenetic tree was constructed using the COI sequences of the selected lines and two unknown sexual Brevipalpus species served as outgroup. Clade support for this tree was determined using a Neighbor-Joining bootstrap procedure with 10,000 replications.

Species identification
From each of the 19 isofemale line, adult female, nymph and, if available, male specimens were sent to Dr. G. de Moraes and N.C. Mesa (ESALQ, University of São Paulo, Brazil) for morphological species identification in order to link COI haplotypes with the classical species descriptions.
Abundance of males in natural populations

Five high-density field populations on three host plant species in Viçosa (Minas Gerais state, Brazil) were surveyed for the presence and abundance of males. From each population, several heavily infested leaves or fruits were brought into the laboratory. All adult mites were picked from the plant material using a microscope and transferred onto a single leaf placed on soaked cotton wool. Once all individuals were collected, the numbers of males and females were counted. By concentrating all individuals onto a single leaf, the gender of the mites can be accurately determined because then the shape of their abdomen, the main sexual dimorphism, can easily be compared among individuals.

Timing of male production

This experiment was designed to determine when sons are produced during the reproductive life of the female. The reproductive period, approximately 30 days, was divided into five-day intervals and the production of sons and daughters was determined for each interval. Three cohorts of equally aged females were created by allowing 50 females to oviposit on a leaf for one to three days. The resulting cohorts were reared to adulthood. Day 1 was the day the first egg was laid. All surviving females were transferred to clean leaves every fifth day until the last female had died. The resulting offspring on each of the leaves was reared to adulthood and the sex ratio was determined. This experiment was conducted with two isofemale lines of B. phoenicis, lines 1 and line 20. Unfortunately, shortly after the completion of this experiment line 20 was lost and could therefore not be genotyped at its COI and symbiont. However, microsatellite genotyping showed that it was closely related to line 2; the two lines differed at two out of 11 microsatellite loci, and both were different from line 1 at 6 out of 11 loci (data not shown).

Effects of antibiotic treatment

We determined male production of selected isofemale lines under controlled laboratory conditions and after antibiotic treatment. The frequency of males in a continuous culture of an isofemale line was determined by collecting and sexing all adult mites at a particular time. A minimum of about 500 individuals were counted per isofemale line. Eggs and juveniles were left to maintain the isofemale lines. In general, cultures contained fewer than 500 adult individuals at any point in time. Therefore, data collection was repeated several times and the results were summed.

A subset of the collected adult females – age was not determined - was exposed to antibiotics. They were placed on leaf disks (24 mm diameter) of common bean floating on cotton wool soaked in a 0.5% (wt/vol)
tetracycline hydrochloride (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) water solution. After two days, surviving female mites were transferred to clean seedlings and allowed to lay eggs. The antibiotic experiment was done twice. The first replicate was initiated with 40 females per isofemale line and survivors were allowed to lay eggs for five days, the second replicate was initiated with 30 females per isofemale line and survivors were allowed to lay eggs for seven days. Once the resulting offspring had matured, their sex ratio was determined as described above.

Presence and identification of Cardinium symbionts
The presence and identity of Cardinium symbionts in each of the 19 selected isofemale lines was determined with a specific PCR and sequencing of the gyrB gene. The primers used to amplify the gyrB were: 5’-GTT ACC GTA TAC CGA AAT GG-3’ and 5’-TGC TTT CCG RGC MGC TTG-3’. These primers were designed specifically to amplify the gyrB of Cardinium bacteria that infect Brevipalpus (Groot and Breeuwer unpub.). The same DNA extract that was used for the COI amplification was used to amplify gyrB. PCR conditions were also equal as for the COI amplification. The presence of an amplification product, and therefore the presence of Cardinium, was tested by electrophoresis of 2.5 µl of the PCR product in 0.5X TBE on 1% agarose gels stained with ethidium bromide. The products of all successful amplifications were sequenced as described for COI.

Screening for bacterial symbionts
Three isofemale lines, lines 11, 12, and 13, tested negative for the presence of Cardinium. To test if these contained other bacterial symbionts that might induce the thelytoky, we screened for the presence of bacteria using general bacterial 16S rDNA primers 27F (5’-AGA GTT TGA TCM TGG CTC AG-3’) and 1513R (5’-ACG GYT ACC TTG TTA CGA CTT-3’) (Weisburg et al. 1991). Three isofemale lines were screened in this way; lines 11 and 13 that tested negative for Cardinium using gyrB, and line 1 that tested positive. DNA was extracted using a chelex extraction protocol as described in Groot et al. (2005), but with a single modification; DNA was extracted from 10 pooled individuals instead of a single one. The 16S rDNA was amplified in a 25 µl reaction containing: 2.5 µl 10X Super Taq buffer (HT Biotechnology), 5 µl dNTP mix (1 mM of each nucleotide), 0.5 µl each primer (10 µM each), 0.2 µl of super Taq (5 u/µl) (HT Biotechnology), 11.3 µl water, and 5 µl of supernatant of the chelex extractions. PCR cycling conditions were identical to the COI PCR. After amplification, the entire PCR products were loaded on a 1% agarose in 1X TAE (40 mM Tris-acetate,
20 mM sodium acetate, 1 mM EDTA, pH 8.0) gel, excised, and cleaned using the DNA Extraction Kit (Fermentas). The cleaned products were ligated and transformed using the pGEM-T Easy Vector System and JM109 competent cells (Promega, Madison WI, U.S.). Plasmids were recovered from 13 colonies per sample, using minipreparation procedures (Sambrook et al. 1989). Plasmids were sequenced in one direction using the universal M13 forward primer, as described for COI. The identity of the obtained bacterial sequences was determined by performing a BLAST search in GenBank.

Flow cytometry
Flow cytometry was used to check whether females of the different isofemale lines are indeed haploid (Johnston et al. 2004). In short, DNA is stained with a fluorescent dye and subsequently fluorescent intensity of single cells is measured and a frequency distribution of the cells based on their fluorescent intensity is generated. Cells with equal DNA content will have similar intensity. Thirty adult females per isofemale line were crushed in 5 µl of DAPI staining solution (Cystain UV ploidy, Partec, Münster, Germany). After the mites were crushed, another 100 µl staining solution was added and the total volume was transferred onto a 50 µm CellTric filter (Partec, Münster, Germany). Another 1 ml of staining solution was poured onto the filter to wash all nuclei through it. Samples were incubated for five min at room temperature to allow the DAPI to bind to the DNA. Samples were then run on a Particle Analysing System (Partec, Münster, Germany). Males were used to determine the intensity of haploid nuclei, which corresponds to the intensity with the highest frequency. The fluorescent intensities of male and female cells were compared for one reference line, line 1. The frequency distribution of female cells of other isofemale lines was compared to the reference line. For the isofemale lines that produced sufficient males in the antibiotic treatments, these males were also run on the flow cytometer and compared to females of the same lines. We ran samples of haploid males and diploid females of Tetranychus urticae and Cenopalpus sp. mites as control to determine if our flow cytometry is a suitable method to establish ploidy level in these mites. In both T. urticae and Cenopalpus sp., male and female samples showed different frequency distributions, which confirm the validity of this technique to measure ploidy level in mites.
Results

Isofemale line haplotypes and morphology

COI haplotypes were determined for a total of 75 isofemale lines and a phylogenetic tree was constructed. Based on this tree, 19 lines were selected to represent all clades. The phylogenetic tree resulting from these 19 lines was well resolved with high bootstrap support for most nodes (Fig. 1). The tree contained three major clades; the first clade combined isofemale lines 1 to 10, the second lines 11 to 15, and the third lines 16 to 19. Based on morphological identification of these isofemale lines by Dr. G. Moreas and N. C. Mesa, two of the clades represent *B. phoenicis* and *B. californicus*. The third clade, represented by lines 11 through 15, contained two morphologically different taxa. Isofemale lines 14 and 15 were morphologically identified as *B. obovatus*, whereas lines 11, 12 and 13 were morphologically identified as *B. phoenicis*. There is a clear conflict between the original morphological species description (reviewed by Welbourn et al. 2003) and the molecular phylogeny of these mites (this paper), indicating that a taxonomic revision of this group is required. Awaiting this revision, we will name all five lines from this clade *B. obovatus* for the remainder of this paper.

Abundance of males in natural populations

The numbers of males that occurred in *Brevipalpus* field populations were variable (Table 2); some population contained up to 3.3 % males, others contained no males at all. This natural variation was not due to host plant species or variation in demography of natural populations, because a similar amount of variation was observed in isofemale lines reared under controlled laboratory conditions (Table 1).

<table>
<thead>
<tr>
<th>Host plant species</th>
<th>% males</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hibiscus rosa-sinensis</em></td>
<td>1.0</td>
<td>526</td>
</tr>
<tr>
<td><em>Malpighia glabra</em></td>
<td>0</td>
<td>455</td>
</tr>
<tr>
<td><em>Citrus</em> sp.</td>
<td>3.3</td>
<td>241</td>
</tr>
<tr>
<td><em>Citrus</em> sp.</td>
<td>1.0</td>
<td>403</td>
</tr>
<tr>
<td><em>Citrus</em> sp.</td>
<td>0</td>
<td>399</td>
</tr>
</tbody>
</table>
Figure 1. Neighbor-Joining phylogenetic tree of the 19 isofemale lines of *Brevipalpus* based on COI sequences. Values at the nodes indicate bootstrap support. Squares around the clades contain the species names used in this report. The table to the right summarizes the main results for each isofemale line; species status inferred from morphology, the type of symbiont (A or B) inferred from gyrB sequences, the percentage of males present in the cultures, and the average percentage of males observed in the two antibiotic treatments. Morphological species are abbreviated as: phoe = *B. phoenicis*, obov = *B. obovatus*, and calif = *B. californicus*. Genbank accession numbers for COI are provided in the figure, accession numbers for gyrB are DQ789574 and DQ789575 for type A and B respectively. Bar in the lower left corner shows the branch length corresponding to 1% genetic distance.

**Timing of male production**
Males were produced during the first five day period of the oviposition period (Table 3). After five days, all eggs developed into daughters and no males were observed. This indicates that young females are responsible for the male production in the population.
Table 3. The total number of males and females produced throughout the oviposition period of females of two *B. phoenicis* isofemale lines per 5 day interval. The production of three different cohorts is summed per isofemale line.

<table>
<thead>
<tr>
<th>Oviposition period</th>
<th>Line 1 Males</th>
<th>Line 1 Females</th>
<th>Line 20 Males</th>
<th>Line 20 Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5</td>
<td>6</td>
<td>228</td>
<td>5</td>
<td>140</td>
</tr>
<tr>
<td>6-10</td>
<td>0</td>
<td>431</td>
<td>0</td>
<td>144</td>
</tr>
<tr>
<td>11-15</td>
<td>0</td>
<td>357</td>
<td>0</td>
<td>107</td>
</tr>
<tr>
<td>16-20</td>
<td>0</td>
<td>385</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>21-25</td>
<td>0</td>
<td>94</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>26-30</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>31-35</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>1505</td>
<td>5</td>
<td>437</td>
</tr>
</tbody>
</table>

**Effects of antibiotic treatment**

Male production of isofemale lines was highly variable ranging from 0 % in some lines up to 6.7 % in line 19 (Table 1). Interestingly, the lines that did not produce males were identified as *B. obovatus* (Fig. 1). Thirteen isofemale lines were subjected to antibiotic treatments to test whether the removal of the symbiont would result in increased production of males.

Male production was significantly higher after antibiotic treatment of the females when tested over all isofemale lines [Friedman two-way analysis: $F_r=9.4$; $p=0.01$, (Siegel and Castellan 1988)]. Excluding the non-infected *B. obovatus* lines improved significance for the remaining infected lines slightly ($F_r=12.2$; $p<0.01$). Comparisons of groups showed that male production in the control was significantly lower than in both antibiotic treatment replicates ($q_{c, 0.01} = 12.48$, difference in rank sum = 13 and 14, respectively, excluding non-infected lines). The variation in male production between the two replicate treatments within lines was probably due to variation in the age of the females used and the amount of antibiotics ingested.

Female age distribution is similar between the control and treatment groups; both include females of all ages. In the control group, the young females are solely responsible for the production of males, which ceases after less than 5 days of oviposition (Table 3). Females in the treated group were on average three days older as a result of two days antibiotics treatment and one day recovery. Thus, male production in the control group would probably have been lower if we would have corrected for the three
day period. This would make the effect of antibiotics on male production even more pronounced.

Normal cultures of lines 14 and 15 did not produce any males. However they did produce males after antibiotic treatment. This suggests that in these lines a bacterium is responsible for the thelytoky, but that under normal circumstances symbiont transmission is very efficient. Lines 11, 12, and 13 did not produce any males after the antibiotic treatments. This suggests that either they harbor symbionts that are resistant to the antibiotics, or that thelytoky in these lines is not caused by a symbiont at all but rather is a heritable trait of the mite itself.

Screening for bacterial symbionts and identification of Cardinium
All isofemale lines were tested for Cardinium based on PCR assay for gyrB. The sequences of the obtained gyrB fragments revealed two types of Cardinium symbionts, type A and B (Fig. 1). The difference between the two types of Cardinium was considerably large; they differed in 17 out of 705 base pairs. Type A was found in all infected B. phoenicis and B. obovatus lines and two out of the four B. californicus lines (lines 18 and 19). Type B was only found in the other two B. californicus lines (lines 16 and 17). All lines tested negative for the presence of Wolbachia using specific primers for the Wolbachia wsp and ftsZ genes using Wolbachia-infected Bryobia mites as positive control (data not shown).

From three lines, line 11, 12, and 13, the gyrB could not be amplified (Fig. 1). To test if these lines contained a different kind of Cardinium or any other bacterial symbiont that might be responsible for the observed thelytokous reproduction, a survey of the entire bacterial community was performed using conserved 16S rDNA primers followed by cloning and sequencing of the resulting amplicons. Per isofemale line, 13 cloned amplicons were sequenced. From the Cardinium-infected line 1, all 13 clones contained the same insert that scored 99% similarity to the 16S rDNA from Cardinium (Weeks et al. 2001). No Cardinium sequences were found in cloned amplicons from the lines 11 and 13. Instead, these lines revealed sequences related to various bacteria that are associated with the digestive system. Most amplicons were 98 to 99% similar to Enterobacter, which is commonly found in the digestive tracts of arthropods (Hoy and Jeyaprakash 2004). A few amplicons were 98 to 99% similar to Streptococcus mitis, a bacterium that is commonly found in the mouths of humans (Diaz et al. 2006). It is unlikely that these bacteria manipulate the mode of reproduction of their hosts. This is corroborated by the fact that antibiotic treatment did not result in a change of the mode of reproduction of these lines (Table 1).
Flow cytometry
It was previously shown that *B. phoenicis* female embryos are haploid based on microscopic karyotyping (Pijnacker et al. 1980; Weeks et al. 2001). To determine the ploidy level of males from line 1 (*B. phoenicis*), fluorescence of male and female cells were compared in a flow cytometer running male and female samples separately and in mixed samples. Male and female cells showed the same frequency distribution with a single peak at the same fluorescent intensity. Thus, they have the same C-value, which is consistent with both genders being haploid. In addition, females from all other isofemale lines had frequency distributions that were similar to line 1, indicating that female haploidy is conserved in all parthenogenetic *Brevipalpus* species included in this study.

Discussion
In most *Brevipalpus* isofemale lines the production of males significantly increased after antibiotic treatments, except in lines 11, 12 and 13. This is a strong indication that *Cardinium* is responsible for thelytoky. We did not determine the infection status of the offspring of treated females in a molecular assay, because they were used for the flow cytometry experiments. Hence, there is no direct proof that the tetracycline has eliminated the bacterium in male but not in female offspring. However, PCR assays for *Cardinium* in natural occurring males were always negative (Groot unpublished data). In addition, two other studies have shown that males produced by antibiotic treated females were indeed uninfected (Chigira and Miura 2005; Weeks et al. 2001).

The amounts of males that were produced in the two treatments varied remarkably. Whereas some lines produced more males after the first treatment, others produced more males in the second (Table 1). This is probably due to a combination of factors: variation in the age distribution of the females taken from the cultures, the amount of antibiotics that is taken up by the females and the food quality. In maintaining the cultures, mites feed on the same seedling of the common bean for about one month before a fresh seedling is provided. This has two effects. Firstly, as seedling replacement is not synchronized across cultures, this will result in differences in age distribution between lines and replicates at the time of antibiotic treatment. This affects the efficiency of the antibiotic treatment and thus male production. For example, young females can produce males in the absence of antibiotics (Table 3). Secondly, the age of the seedling or
quality of the food plant may also have an effect on the health of the female and thus her susceptibility to antibiotics.

The increase in male production upon antibiotic treatment in our study was low compared to two other studies where females were treated with the same antibiotic (Chigira and Miura 2005; Weeks et al. 2001). This is probably caused by differences in the age of the females at the time of treatment. Both these studies used very young females that had not yet started oviposition whereas we used females of various ages. Again it suggests that timing of antibiotic treatment is an important determinant in male production.

All infected B. phoenicis and B. obovatus mites and two of the B. californicus mites were infected with the same symbiont, type A (Fig. 1). The other two B. californicus lines were infected with a different symbiont. This pattern suggests that Cardinium is capable of horizontal transmission. This observation is in line with the findings of our study on transmission patterns of Cardinium (Groot and Breeuwer unpublished). In that study, a large number of mites and their symbionts were genotyped and then compared revealing that the symbiont of B. phoenicis is highly mobile and has been transferred horizontally within B. phoenicis as well as to the other species. Despite the fact that most lines had the type A symbiont, there was a remarkable difference in the number of males they produced in the cultures. Most evident is the observation that males were absent from lines 14 and 15 whereas each of the 12 other lines with the same symbiont did contain males. For both mite and symbiont, males are a waste and therefore selection can be expected to favor optimal transmission. The fact that the same symbiont has different transmission efficiencies in different types of mites indicates that transmission efficiency is determined by the mite and not by the symbiont. Brevipalpus obovatus mites have the highest transmission efficiencies. This suggests that thelytoky has evolved in this clade first, and that therefore this species has had more time to optimize the transmission efficiency.

The experiment on the timing of the male production showed that females of these two B. phoenicis isofemale lines produced males only at the start of their reproductive lives. A likely explanation for this is that the number of endosymbiotic bacteria is rather low when they are still immature, but increases when egg production starts. When the very first eggs are produced, the number of bacteria might be too small to effectively feminize the genetically male offspring. This explanation is supported by our observation that antibiotic treatments are more effective in young mature females that have not laid eggs yet than in females that have already started oviposition (results not shown). Additionally, Pijnacker et al. (1981)
reported that for the 13 true males they observed in *B. obovatus*, they also found six inter-sex individuals that had both male and female reproductive organs. In these individuals feminization was incomplete, perhaps because the number of bacteria was insufficient. Similarly, in isopods partial feminization resulting in inter-sex individuals is correlated to *Wolbachia* densities (Rigaud et al. 2001), and the same dosage dependence affects the effectiveness of cytoplasmic incompatibility in *Nasonia* wasps (Breeuwer and Werren 1993). Finally, a remarkable difference was observed between *Cardinium* and *Wolbachia* induced thelytoky. In *Cardinium* infected *Brevipalpus* males are produced only in the earliest stages of a female’s reproductive life, whereas in *Wolbachia*-induced thelytoky males are produced towards the end of a female’s reproductive period (Stouthamer et al. 1999). This may indicate that the mode of action of *Cardinium* differs from that of *Wolbachia*.

Although we showed that *Cardinium* probably is causing thelytoky in most isofemale lines, the bacterium is absent from lines 11, 12, and 13, nor do these lines contain other bacteria that might induce thelytoky. We therefore conclude that in these lines thelytoky is most likely controlled by a genetic character of the mite itself. Remarkably, flow cytometry revealed that these females were haploid which indicates that the mechanism that causes thelytoky is the same as in the infected mites. How then did this genetic haploid thelytoky evolve? Because in all other clades thelytoky is caused by *Cardinium*, it is most parsimonious that thelytoky in this aberrant group has started from an infection with *Cardinium* too. When sexual behavior and function are no longer required for reproduction, selection on the maintenance of sexual functions is released (Carson et al. 1982; Muller 1949). This will result in the accumulation of deleterious mutations with the consequence that the asexual species becomes trapped in asexuality, unable to reproduce sexually even if the symbiont is lost. In this way, the host can become dependent on the symbiont for its reproduction (Dedeine et al. 2003). The fact that these lines are able to reproduce thelytokous without being infected requires that the host has somehow “learnt” to do all the tricks the symbiont would otherwise do. These tricks include the induction of pre-meiotic doubling to enable meiosis to take place (Pijnacker et al. 1981), and feminization of genetic males (Weeks et al. 2001). One intriguing possibility to how the mite might have “learnt” to perform these tricks is the transfer of the responsible genes from the symbiont genome to the genome of the mite. The same transfer of the feminizing genes from a symbiont to the host genome has been suggested for the isopod *Armadillidium vulgare* infected with *Wolbachia* (Rigaud et al. 1997). The transfer of symbiont genes to the host genome is common between
mitochondria and their hosts in both plants and animals (Bensasson et al. 2001; Palmer et al. 2000). Recently, the same type of gene transfer was also shown for a Wolbachia symbiont and its beetle host (Kondo et al. 2002).

In B. obovatus two types of asexual reproduction have been found; symbiont induced and genetic thelytoky. The two types are found in separate clades in the phylogenetic tree. This suggests that each mode of reproduction has only arisen only once in the ancestor of each clade. However, only four lines of B. obovatus have been tested for their mode of reproduction so far. It remains to be seen whether the phylogeny of B. obovatus will show separate clades for the reproductive types when more B. obovatus lines are included.

Finally, this study has revealed an important incongruity between the classical mite taxonomy based on morphological characters and the phylogeny based on molecular genetic markers. Hurst and Jiggins (2005) have argued that the use of mitochondrial markers might reveal ambiguous relationships if animals are infected with maternally transmitted symbionts. However, nuclear genetic markers too place the uninfected lines far from the B. phoenicis lines, showing that these are truly different groups (Groot, Breeuwer and Menken unpublished). What has been described as B. phoenicis based on morphology appears to be paraphyletic based on genetic data. Lines 11, 12 and 13 fall outside the other B. phoenicis lines in the first clade, and group in the second clade together with lines 14 and 15 which were identified as B. obovatus. This second clade thus contains two morphologically and biologically diverse groups. A revision of the species identification based on morphology is evidently needed.

In conclusion, we have shown that the variation in the number of males in natural Brevipalpus populations can be caused by at least two elements. First, the clonal composition of a population is important because some clones produce more males then others, and second, the population age structure is important because young females are more likely to produce males than old females. We could not distinguish between effects of symbiont type or host plant species on the number of males in a population. In respect to the evolution of thelytoky, our results suggest that B. obovatus was the first species to become thelytokous because of an infection with Cardinium. Because this species has been thelytokous for a long time, symbiont transmission has been perfected in some lines. In other lines, the cause of the feminization has shifted from the bacterial symbiont to a genetic character of the mite itself. We argued that this shift may be caused by gene transfer between symbiont and host. Finally, our results have shown that the morphological species descriptions of these species need to be revised.
Acknowledgements

We thank Dr. G. de Moraes and N.C. Mesa for morphologically identifying the mite species. We also thank the people at the Burger’s Bush tropical greenhouse for allowing us to collect mites and their help in doing so. We thank Prof. S. Menken for his remarks on an earlier draft in this manuscript. This study was supported by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), grant number W89-141.
Chapter 2

Adaptation in the Asexual False Spider Mite *Brevipalpus phoenicis*: Evidence for Frozen Niche Variation

Thomas V.M. Groot, Arne Janssen, Angelo Pallini and Johannes A.J. Breeuwer

Abstract

Because asexual species lack recombination, they have little opportunity to produce genetically variable offspring and can’t adapt to changes in their environment. However, a number of asexual species are very successful and appear to contradict this general view. One such species is the phytophagous mite *Brevipalpus phoenicis* (Geijskes), a species that is found in a wide range of environments. There are two general explanations for this pattern, the General Purpose Genotype (GPG) and Frozen Niche Variation (FNV). According to the GPG model, an asexual species consists of clones that can all survive and reproduce in all the different niches. Alternatively, the FNV model postulates that different clones are specialized to different niches. We have performed a test to distinguish between these models in *B. phoenicis*. Mites from three populations from three different host plant species (citrus, hibiscus and acerola) were transplanted to their own and the two alternative host plants and mite survival and egg production were measured. Additionally, the mite populations were genotyped using microsatellites. Fitness was seriously reduced when mites were transplanted to the alternative host plant species, except when the alternative host was acerola. We concluded that *B. phoenicis* clones are specialized to different niches and thus the FNV best describes the broad ecological niche of this species but that there is also some evidence for host plant generalization. This conclusion was strengthened by the observations that on each host plant species the native mite population performed better than the introduced ones, and that three microsatellite markers showed that the mite populations are genetically distinct.
Introduction

The inability of asexual species to adapt is a commonly used argument to explain their apparent rareness (Barton and Charlesworth 1998; Doncaster et al. 2000; Hurst and Peck 1996). In asexual species, fixed genotypes are inherited from parent to offspring, leaving no room for variation to arise. Although this fixed genotype might be well adapted to the current environment, the individual will be less fit when it moves to a different environment or when its environment changes. In contrast, new gene combinations are created every generation in sexual species with the possibility that at least some of the offspring are better adapted to any environment than their parents.

Thus, asexual species are expected to be rather unsuccessful and evolutionarily short-lived, an observation that is linked to the discussion on the evolution of sex (Rice 2002). However, there are a number of asexual species that do not obey this rule; they are successful, can reach high abundance, are spread over large geographical and ecological ranges and are evolutionarily long-lived. Well known examples are the bdelloid rotifers (Mark Welch and Meselson 2000), some ostracods (Van Doninck et al. 2002) and oribatid mites (Maraun et al. 2003).

Two evolutionary models explain how an asexual species can be adapted to different niches. The General Purpose Genotype model (GPG) explains the broad ecological niche of an asexual species by the species consisting of a few generalist clones that are ecologically tolerant throughout the entire species range (Lynch 1984). The Frozen Niche Variation model (FNV) postulates that the asexual species consists of many specialist clones, each adapted to a small and different part of the entire ecological range of the species (Vrijenhoek 1979). A general purpose genotype is expected to evolve as a consequence of temporal variation in the environment. Specialists will go extinct when their niche ceases to exist, and only generalist clones that are tolerant to different environments will survive (Lynch 1984; Parker and Niklasson 2000). Although these two hypotheses are theoretical opposites, they are not mutually exclusive, as FNV focuses on spatial heterogeneity and GPG on temporal variation (Vrijenhoek and Pfeiler 1997). At the same time, clones that are intermediate between generalists and specialists may exist and a clone that is a specialist in one trade can be a generalist in another (Vrijenhoek 1998).

In a changing environment, the evolution of generalists versus specialists depends upon the rate at which new clones arise. If this rate is relatively low, specialist clones might go extinct and generalists will predominate. If, on the other hand, this rate is relatively high, new clones
are continuously generated and generalist clones are likely outcompeted by superior new specialists (Vrijenhoek 1979; Vrijenhoek 1998). There are several ways in which new clones that are adapted to different niches can be created: 1. existing clones can change by mutations, 2. existing clones can occasionally go through sexual reproduction, and 3. entirely new clones can arise when sexual individuals become asexual.

Several studies have tested for GPG and FNV in a variety of species, and evidence has been found for both models. Some species seem to have a GPG, such as fish in the genus *Phoxinus* (Schlosser et al. 1998) and the snail *Melanoides tuberculata* (Müller) (Myers et al. 2000), whereas examples of FNV were found in the *Poeciliopsis* fishes (Gray and Weeks 2001; Lima 1998; Vrijenhoek 1979), in brine shrimp *Artemia* (Browne and Hoopes 1990) and others. Remarkably, some species show FNV in one part of their geographical range but show GPG in other locations. The snail *Potamopyrgus antipodarum* (Gray), for example, shows FNV in New Zealand where it is native (Fox et al. 1996; Jokela et al. 1999; Jokela et al. 1997), but a GPG was found in Europe where it has been introduced (Jacobsen and Forbes 1997).

Another species that does not fit the general expectation that asexuals will be evolutionarily unsuccessful is the mite *Brevipalpus phoenicis* (Geijskes). It has a worldwide distribution in the tropics and subtropics (Childers et al. 2003b; Kennedy 1995), it is extremely polyphagous, being reported from 486 plant species from 114 different plant families (Childers et al. 2003b), and it rapidly develops pesticide resistance (Campos and Omoto 2002; Omoto 1998).

We attempted to discriminate between GPG and FNV in the false spider mite, *B. phoenicis*. Weeks et al. (2001) discovered that asexuality in this species is induced by infection by a newly discovered intracellular bacterium in the genus *Cardinium* (Zchori-Fein et al. 2004). Curing the bacterial infection with antibiotics results in the production of males. Moreover, Weeks et al. (2001) showed that females are haploid. Since the only mode of sexual reproduction within the family Tenuipalpidae is arrhenotoky (Norton et al. 1993; Oliver 1971) and some sexual *Brevipalpus* species exist that are arrhenotokous (Helle et al. 1980), it is likely that haplo-diploidy is also the ancestral reproductive mode for the asexual *Brevipalpus* species. Combining these observations, Weeks et al. (2001) proposed that the bacterium feminizes genetic males and induces parthenogenesis. Sexual forms do not exist in this species, although males are sporadically produced, probably as a result of inefficient transmission of the bacterium. However, these males appear incapable of producing sperm and are therefore thought to be non-functional (Pijnacker et al. 1981).

51
The species of host plant is likely to be an important factor in the evolution of specialization in *B. phoenicis* because it completes its entire life cycle on a single host plant. If mites are generalists, they are expected to switch easily between host plant species with little reduction in fitness. If on the other hand the mites are specialists we expect a drop in fitness upon switching to a new host plant species. To distinguish between GPG and FNV in *B. phoenicis*, transplantation experiments were carried out that measured survival and egg production of three populations of mites that were transplanted onto different host plant species. In addition, the mite populations were genotyped using microsatellite markers.

**Material and methods**

**Collection of mites**

Mites were collected from three populations on and near the campus of the Federal University of Viçosa, Minas Gerais, Brazil. Each population occurred on a different host plant from a different plant family; citrus (*Citrus sinensis*, Rutaceae), hibiscus (*Hibiscus rosa-sinensis*, Malvaceae) or acerola (Barbados cherry, *Malpighia glabra*, Malpighiaceae). Mites were collected from March to May 2003, covering the transition from the rainy season to the dry season; mite populations were at their highest densities during this period.

On citrus, mites were mainly found on immature fruits whereas the mites abandon mature fruits. If found on the leaves, they occurred both on the under- and the upper sides and on leaves of all ages. On hibiscus, mites occurred on leaves of all ages as well, but almost exclusively on the undersides. On acerola, mites were mainly found on the undersides of leaves and rarely on the upper sides. The younger leaves of acerola contained many more individuals than the older leaves and young, green twigs also contained large numbers of mites.

We collected mites of the respective stages from the field on a daily basis to exclude possible adaptation and selection to laboratory breeding conditions. Teleiochrysalids (the last resting stage before adulthood) and adult females were freshly collected from fruits (citrus), leaves (hibiscus), or young branches (acerola) that were brought into the lab. Ideally one would like to determine the genotype of the individuals used in the transplantation experiments directly. This is not possible due to their small size and the fact that survival is one of the variables measured. Instead, we estimated the clonal variation among the mites used in the transplantation experiments by genotyping adult females that were collected simultaneously from the same
leaves and fruits and stored subsequently on 96% ethanol. Only the teleiochrysalids were used for the transplantation experiments. They were collected and placed in a small Petri dish (diameter 3.5 cm) containing moistened filter paper. The Petri dish was sealed with parafilm and placed in a climate chamber (24°C, 14h light) until adults emerged and the experiments were started.

Transplantation experiments
Upon emergence of adults, individual mites were placed in small arenas, consisting of a leaf disk (diameter 3 cm) placed on a layer of caraginine (25 g/L) in a Petri dish (diameter 3.5 cm). The agar-like caraginine was used to provide moisture to the leaf disk. The Petri dish was covered with parafilm with small holes punched into it to allow ventilation. Citrus leaf disks were placed with the upper sides up, leaf disks of hibiscus and acerola were placed with the undersides up. Arenas containing mites were incubated in a climate chamber (24°C, 14h light). In case the caraginine dried out or the leaf disk wilted, the mites were transferred to a new arena. Survival and egg production were scored daily for a period of 20 days. Mites from each of the three populations were tested on all three host plant species resulting in a total of nine treatments. Each treatment was replicated approximately 30 times. Survival data were used to calculate Kaplan-Meier survival curves. These curves were compared using STATISTICA (StatSoft Inc). If multiple comparisons of the three curves per host plant yielded a significant difference, additional pair-wise comparisons were performed (log-rank test) and p-values were Bonferroni corrected for multiple comparisons. Differences in egg production were not tested for significance. Instead, the data on both survival and egg production were combined to calculate a single reproductive value \( r_f \) using the Lotka-Euler equation (Carey 1993):

\[
l = \sum_{x=0}^{T} l_x m_x e^{-r_f (x+1)}
\]

with \( T \) being the time (days) since maturation (ranging from 0 to 20 days), \( l_x \) being the proportion of females surviving until day \( x \) and \( m_x \) the number of eggs produced per female at day \( x \). It is stressed that the reproductive value calculated here is a relative value only and not the absolute rate of increase. Since the egg hatching rate and the survival of the premature stages were unknown and not included, this reproductive value is larger than the actual rate of increase.
Although in general mites are quite robust, handling can easily damage teleiochrysalids. To exclude these accidental deaths, replicates were only included if mites survived for at least the first two days after maturation. Other premature causes of death were censored in the analyses and included mites drowned in condensation, mites trapped in caraginine and mites that escaped through ventilation holes.

Genetic analysis
About 24 individuals per population were genotyped for three microsatellite loci: Brev02, Brev03 and Brev07 (Weeks et al. 2001). DNA was extracted from single mites using a Chelex extraction procedure. Individual mites were placed in 3µl of proteinase-K (20 mg/ml) in a 0.5 ml microfuge tube and crushed. 50 µl of 5% (w/v) Chelex solution was added and tubes were incubated at 37°C for one hour. Tubes were vortexed prior to, halfway during, and directly after the incubation period. Finally, the tubes were heated to 96°C for 10 minutes to de-activate the proteinase-K. Before taking supernatant to use in PCR, tubes were vortexed again and centrifuged briefly. Supernatant (3 µl) was used as template per 10 µl reaction. Apart from the template the PCR mix contained 1X Super Taq buffer (Sphaero Q), 0.5 µg BSA, 15 nM MgCl\(_2\), 2 mM of each dNTP, 2 µM of each primer (one with an IRD-700 fluorescent label) and 0.4 units super Taq (Sphaero Q). Cycling conditions were: 4 min. at 94°C, then 35 cycles of 30 s at 94°C, 30 s at 54°C and 30 s at 72°C, and concluded with a final extension at 72°C for 4 min.

Results
Transplantation experiments
For the citrus population, survival curves on citrus and hibiscus were similar (Fig. 1a). Survival on acerola appeared to be higher than on the other host plants, but these differences were not significant after Bonferroni correction (Table 1). Egg production was highest on acerola and equal on the other hosts (Fig. 1d). Mites from hibiscus performed equally well on both hibiscus and acerola in terms of survival and egg production (Fig. 1b & 1e). On citrus, however, their survival was significantly reduced and they produced almost no eggs. Survival of mites from acerola was lowest on citrus, intermediate on hibiscus and highest on their own host plant species (Fig. 1c). All differences were significant. The same pattern was observed for egg production, with not a single egg produced on citrus (Fig. 1f).
Figure 1. Kaplan-Meier survival curves (a, b and c) and egg production (d, e and f) (number of eggs per surviving female per day) of the three populations on three host plants. Survival during the first two days was 1 by definition.

The reproductive values of citrus mites showed that they perform well on their own host plant ($r_f=0.19$), but even better on acerola ($r_f=0.28$). On hibiscus their performance was very poor ($r_f=0.05$). The hibiscus population showed relatively high reproductive values on both hibiscus and acerola ($r_f=0.28$). On citrus, however, the reproductive value is negative ($r_f=-0.20$). The population from acerola did not produce a single egg on citrus, which made it impossible to compute a reproductive value. The reproductive value was negative on hibiscus ($r_f=-0.01$) indicating that mites...
from acerola cannot establish on hibiscus. On their own host, acerola animals reached the highest reproductive value of all nine combinations ($r_f=0.36$).

**Table 1.** Comparisons of the Kaplan-Meijer survival curves on each of the host plants. If the differences between the treatments were significant (multiple comparison, log-rank test), additional pair-wise comparisons were made for all combination of host plants. P-values < 0.017 remain significant after Bonferroni correction.

<table>
<thead>
<tr>
<th></th>
<th>Multiple comparisons</th>
<th>Pair-wise comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chi-square</td>
<td>P</td>
</tr>
<tr>
<td>citrus animals</td>
<td>8.48</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hibiscus animals</td>
<td>41.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>acerola animals</td>
<td>29.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**Genetic analysis**

The genetic analysis revealed a total of six genotypes based on three microsatellite loci (Table 2). Because *B. phoenicis* is haploid, the genotype is synonymous with the haplotype. Because the latter term is normally used for mitochondrial DNA sequences we will use ‘genotypes’ here. The number of clonal genotypes found on citrus, hibiscus and acerola were three, two and one, respectively. No genotype was shared between populations from different host plant species. Furthermore, genotypes from different populations never shared more than one allele.

The populations from both citrus and hibiscus consisted of more than one genotype, which may perform differently on the various host plants. If they do, their survival and production cannot be combined to estimate population survival curves and reproductive values. To test whether different genotypes had different responses, we looked at the distribution of individual egg production and corrected for life span per treatment (Fig. 2). A normal distribution is expected if the different genotypes have the same response. In citrus animals we observed no significant departures from normality on citrus ($p = 0.33$) and acerola ($p = 0.95$) and a departure from normality on hibiscus ($p = 0.02$). The latter could be due to the fact that the citrus population consists of at least three clonal genotypes, which may be differentially adapted to hibiscus. Egg production of the hibiscus population
followed the normal distribution on both hibiscus (p = 0.99) and acerola (p = 0.81) and was too low to be tested on citrus (SPSS Inc.). Thus, survival and egg production of all individuals were combined for the estimation of population survival curves and reproductive values.

**Table 2.** Genotype diversity per population based on three microsatellite loci. Allele lengths are represented by a letter (S, T and R) which represents the length of the shortest encountered allele for that locus, followed by the number of dinucleotide repeats that each allele is longer than the shortest. N is the number of times the genotype was encountered, F its frequency within each host plant population.

<table>
<thead>
<tr>
<th>population</th>
<th>genotype</th>
<th>Allele length</th>
<th>N</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Brev02</td>
<td>Brev03</td>
<td>Brev07</td>
</tr>
<tr>
<td>citrus</td>
<td>A</td>
<td>S</td>
<td>T+8</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>S+12</td>
<td>T+1</td>
<td>R+14</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>S+12</td>
<td>T</td>
<td>R+14</td>
</tr>
<tr>
<td>hibiscus</td>
<td>D</td>
<td>S+9</td>
<td>T</td>
<td>R+5</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>S+13</td>
<td>T+1</td>
<td>R+6</td>
</tr>
<tr>
<td>acerola</td>
<td>F</td>
<td>S</td>
<td>T+11</td>
<td>R+5</td>
</tr>
</tbody>
</table>

**Discussion**

The transplantation experiments show that each mite population has low survival on at least one of the novel host plant species; the citrus population cannot survive on hibiscus leaves, hibiscus animals do not survive on citrus and acerola animals cannot survive on either of the other hosts. This suggests that the mites are incapable of switching between some hosts plant species, as predicted by the FNV, but other switches are possible, as is predicted by the GPG. Moreover, though the sampled populations were in close proximity (less than 10 km apart) and citrus and hibiscus are very common in the sampling area, providing possible stepping stones from one population to the other, all three populations are genetically different and do not share clonal genotypes. This indicates that there is neither migration nor genetic exchange between populations, which is consistent with the FNV model. At the same time, all populations have higher reproductive values on their own host plant than the other strains. This suggests that the mites have specific adaptations to their host plant that the other populations lack, which again is in agreement with the FNV model.
Figure 2. Distribution of the average number of eggs produced per day by the citrus and hibiscus animals. Animals that died a premature death because of the experimental set-up were excluded. Production of hibiscus animals on citrus is not shown since only very few animals were productive.

GPG and FNV predict opposing phenotypes with extreme wide or narrow niche breadth. In reality there may be a continuum in the niche breadth of different clones and therefore it might not be surprising that we find evidence supporting both models. The acerola mites are most specialized and may fit the FNV model best; they had the overall highest reproductive value on their own host plant and performed worst on the other hosts. On the other hand, citrus mites may be classified somewhere in between the two alternative models; specialization is least pronounced, survival appears to be equal on all hosts and it is the only strain with positive reproductive values on the alternative hosts.

It is interesting to note that the most specialized population (acerola) did not survive or oviposit on alternative host plant species, whereas the least specialized population (citrus) did survive and reproduce on all host plant species tested. This is consistent with the theoretical prediction that specialization comes with a cost, namely reduced performance on alternative hosts (Jaenike 1990; Kassen 2002). However, since we only tested three populations, it remains to be seen whether this is a general pattern in B. phoenicis.

Two general criticisms are possible on transplantation experiments: 1). confounding effects of the acclimation period, and 2). confounding effects of selection to laboratory conditions. The lower performance of transplanted mites could be due to the fact that adjustment to a new host
Adaptation in *Brevipalpus phoenicis*

Plant is costly in terms of energy and time, as has been observed in the two-spotted spider mite *Tetranychus urticae* Koch (Agrawal et al. 2002). However, if this is the case, we expect that mite performance on the novel plant host increases after an acclimation period. Such a recovery was not observed during the entire 20-day period (Fig. 1). In order to avoid the effects of laboratory growing conditions, we collected individual mites straight from the field for the transplantation experiments. To our knowledge, this is the first study in which the effects of laboratory selection are excluded in this way.

Reproductive values were lowest on citrus leaves, even for citrus mites. In the field, the majority of mites were found on citrus fruits, indicating that perhaps citrus leaves are an inferior food source, which is in agreement with results from Chiavegato (1986). Because citrus trees do not carry fruits throughout the year, the mites probably survive this period by feeding on leaf tissue. Alternatively, they may enter diapause or switch host plant species. Reproductive values of all populations were highest on acerola. This could facilitate colonization of acerola by mites from citrus and hibiscus. However, acerola mites are expected to outcompete mites from the other host plant species because their reproductive value on acerola is higher than that of the other two. Indeed, very high mite densities were observed on acerola (TVM Groot, pers. obs.).

Nevertheless, this study suggests that *B. phoenicis* consists of a collection of different specialist clones rather than a single generalist clone. Exactly how many different specialists are present depends on the niche width of each specialist. *Brevipalpus phoenicis* has already been reported from many different host plant species (Childers et al. 2003b), so we expect that the number of specialists will be high. This has consequences for the management of *B. phoenicis* as a pest. According to Childers et al. (2003b), pest management (mainly through the application of acaricides) should not only consider the crop plants, but should also take into account the high number of alternative hosts, because they might serve as sources from which crops are rapidly colonized after the pesticide has been applied. Our results suggest that these threats might be limited because the switch from one to the other host can be quite difficult and the number of alternative hosts might therefore not be as high as estimated earlier. For example, hibiscus is a common ornamental hedge plant along citrus orchards, but switching from hibiscus to citrus is unlikely. Clearly, more research is needed to determine the niche width of the different clones.

The many host plant species that are being used by *B. phoenicis* and the fact that we have found ecologically and genetically differentiated clones leads to another question: how is this variation generated in an
asexual haploid species? There are at least three possible explanations: First, new clones could arise from mutations that change existing clones. Second, asexual females might occasionally have sex. This could either be with males that are occasionally produced by asexual females, or with males of related sexual species that sometimes co-occur on the same host plant (Gonzalez 1975). Third, sexual females might become asexual when they become infected by a symbiont through horizontal transmission. In particular, if mites from an already diversified sexual species are converted to asexual reproduction, a broad array of specialized clones can be expected. Phylogenetic studies are needed to assess when and how often asexuality arose in this species.

Acknowledgements

We thank the collaborators of the Acarology Laboratory of the Federal University of Viçosa for their hospitality. The manuscript was improved by comments from J. Bruin, C. Childers and one anonymous reviewer. We also thank M. Hilbrant for his helpful comments on the design of the experiments. Finally, we thank A. and M. Grosman for their general support. This study was supported by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), grant number W89-141.
Chapter 3

Clonal Diversity and Host Plant Specialization in Asexual *Brevipalpus* Mites

Thomas V.M. Groot & Johannes A.J. Breeuwer

Abstract

Adaptation of asexual species is expected to be slow and limited. Nevertheless some asexual species have a very broad ecological niche and are widespread. Such species may be a collection of different clones each with a different niche or consist of a single clone with a broad niche. To test between these alternatives, asexual *Brevipalpus* mites were collected from various host plant species and locations in Brazil. Microsatellite diversity of clones within field samples ranged from 0 to 0.88. *COI* haplotypes were variable within samples too and occasionally more than one species was present. Four haplotypes were recovered from at least four host plants species, indicating that these are generalist clones. One subclade was a specialist on *Malpighia glabra*. Possible explanations for the coexistence of so many different clones on a single host plant are discussed, including mutation accumulation, occasional sex, migration and small scale niche differentiation.
Introduction

Issues concerning the evolution and maintenance of sexual reproduction have been a popular subject in evolutionary biology in the past decades (Bell 1982; Hurst and Peck 1996; Maynard Smith 1978; Rice 2002; West et al. 1999). Theories that explain the advantages of sexual reproduction are generally divided in two groups; mutation-based models and environmental, i.e., ecological, models. The latter group of theories states that asexual species are confronted with reduced rates of adaptive evolution. Whereas in asexual species genetic variation is generated by mutations only, in sexual species new genotypes are produced both by mutations and recombination of existing variation. Because the amount of variation generated by mutations only is less than the variation generated by the combination of recombination and mutations, the efficacy of selection is reduced in asexual species. Therefore, asexual species are expected to evolve slower than sexual species.

Nevertheless, there is a good number of asexual species that are quite successful. Some asexual species have persisted for millions of years and even radiated in the absence of recombination. Well known examples are the ancient asexual bdelloid rotifers (Mark Welch and Meselson 2000), darwinulid ostracods (Van Doninck et al. 2002), and oribatid mites (Maraun et al. 2003).

A second feature of successful asexuals is their broad ecological niche; many asexual plant and animal species have larger geographic and ecological ranges than their sexual relatives (Bell 1982; Hughes 1989; Lynch 1984; Vrijenhoek 1998). Two theoretical models explain how asexual species can obtain broad niches; the General Purpose Genotype (GPG) model and the Frozen Niche Variation (FNV) model. The GPG model (Baker 1965; Lynch 1984) predicts that only clones that can tolerate many different environmental conditions will survive over longer evolutionary times. The model assumes that an asexual species starts out with many different clones with variable niche widths, but also assumes that no new clones arise at later stages. Because of environmental changes over time, certain clones with narrow niches will go extinct because their niches cease to exist. Once enough time has passed and sufficient environmental changes have occurred, only the most generalist clones will have survived. The FNV model predicts clones to be selected that are highly specialized and have very narrow niches (Vrijenhoek 1979). The model assumes that many different clones originate from a sexual ancestor. Either these clones all originated at the same moment at the onset of asexual reproduction, or new clonal lineages are produced continuously. Each of these clones will
inherit a different suite of characters from the sexual ancestor and therefore will freeze a different part of the ancestor’s niche. Competition with the sexual ancestor and among different asexual clones will result in selection for clones that have highly specialized, narrow niches, which do not overlap between different clones. The GPG and FNV models thus operate on different time scales and make different assumptions on the production of new genotypes. The GPG model focuses on what happens over long periods of evolutionary time in a situation where no new clonal types are produced, whereas the FNV model considers short term clonal interaction at a spatial scale. Because of these differences, both models are not mutually exclusive (Vrijenhoek and Pfeiler 1997).

Distinguishing between the two models is difficult in practice and will depend upon the ecological traits under consideration. Clones may be generalist for one trait but specialist for the other. In addition, it is often so that clones vary in their niche breadth and thus makes it difficult to classify them as generalist of specialist. Empirical studies have revealed evidence for both models in a wide variety of animal species. The GPG model applies to the ostracod Darwinula stevensoni (Van Doninck et al. 2002), fish of the genus Phoxinus (Schlosser et al. 1998), the snail Melanoides tuberculata (Myers et al. 2000), and the sea anemone Nematostella vectensis (Pearson et al. 2002). Evidence for the FNV model has been found in Poeciliopsis fishes (Gray and Weeks 2001; Lima 1998; Vrijenhoek 1979) and in brine shrimp Artemia (Browne and Hoopes 1990). However, in other species evidence was found for both models, such as in the aphid Rhopalosiphum padi (Delmotte et al. 2002) and the snail Potamopyrgus antipodarum (Jacobsen and Forbes 1997; Jokela et al. 1999). Furthermore, other studies were only able to reject one model, but remained inconclusive for the alternative (Darling et al. 2004; Jensen et al. 2002; Robinson et al. 2002; Vorburger et al. 2003).

Here we investigate a group of asexual mite species that belong to the genus Brevipalpus. The three most common asexual species are B. phoenicis, B. obovatus, and B. californicus. In B. phoenicis, B. californicus and some clones of B. obovatus, parthenogenesis is induced by an intracellular bacterium of the genus Cardinium (Weeks et al. 2001; Zchori-Fein et al. 2004; Groot and Breeuwer in press). The Cardinium infection causes the haploid infected embryos to develop into females that remain haploid (Weeks et al. 2001). Parthenogenesis in some clones of B. obovatus is not caused by a bacterium, but seems to have a genetic origin (Groot and Breeuwer in press). They have an exceptionally broad niche, each of these species occurs worldwide throughout the (sub) tropical regions, and occur on hundreds of different host plant species (Childers et al. 2003b). Some
host plants harbor just one mite species, but many other plants are shared between two or all species.

Here we take a genetic approach to determine the niche width of *Brevipalpus* clones. To do so, a definition of a clone is required. A clone might be defined as all the descendants of a single transition from sexual to asexual reproduction. However, this definition cannot be applied to *Brevipalpus* since it is not known how many transitions there have been, nor if occasional sex and recombination have taken place since. Another definition of a clone is all the individuals that share the same genotype. However, this definition is crucially dependent on the resolving power of the marker used and may lead to the conclusion that the concept of a clone does not exist (Lushai and Loxdale 2002). Therefore, as a working hypothesis, we define a clone as all the individuals that are identical at their COI haplotype. We studied the niche width of clones, assuming that host plant species is the main factor determining the niches of *Brevipalpus* mites. This assumption is plausible because mites spend their entire life on the same host plant for many consecutive generations. We compared clonal genotypes for mites from the same and different host plant species collected from the same and different geographic locations. If the GPG model applies to *Brevipalpus*, clones have broad niches and therefore we expect to find the same genotype on different host plant species from the same location. Alternatively, if the FNV model applies, clones have narrow niches and therefore we expect to find relationships between certain genotypes and single host plant species independent of the location.

**Material and Methods**

**Collections**
Mites were collected at six different locations in the southern part of Brazil, representing 63 field samples (Fig. 1). A single field sample was defined as all the mites collected from a single plant, or on a group of physically connected plants of the same species. The number of mites within a field sample ranged from 1 to 45. The main collection area was the campus of the Federal University of Viçosa, Minas Gerais, and surroundings. Concentrating on four areas, all plants were checked for the presence of mites and field samples were taken from all infested plants. Further collections were made at variable distances from this main collection area; two locations, Ponte Nova and Araponga, at about 50 km, two locations, Santa Thereza and Piracicaba, at about 400 km, and finally one location, Pantanal, at 800 km. Two field samples collected in Arizona, USA, served
Figure 1. Map of Southern Brazil showing the sampling locations (Vi = Viçosa, Ar = Araponga, Pn = Ponte Nova and St = Santa Thereza).

as outgroup. These latter samples were from two unidentified sexual Brevipalpus species. To ensure that all plants that mites were collected from are true host plants, mites were only collected from plants that contained all developmental stages. Collected mites were either processed directly, or were preserved in ethanol (96%) or acetone (pure). Although acetone has been reported to be a better preservative of DNA than ethanol (Fukatsu 1999), no differences have been observed by us. All samples were collected before the first mites were genotyped, and thus without prior knowledge on haplotype diversity. Therefore our sampling represents an unbiased sample of the genetic diversity of Brevipalpus within and between natural field samples.

We took a two stage approach to assess overall clonal diversity. First clonal genetic variation within 22 field samples was determined by genotyping 3 to 45 individuals for three microsatellite loci. Subsequently for each field sample we sequenced part of the mitochondrial COI gene for one to five individual mites that had different microsatellite genotypes. For the other 41 field samples clonal genetic variation within the samples was not assessed because not enough material was available. Instead, a single individual per field sample was genotyped for its COI only.
DNA extractions
All DNA extractions were done on single individuals using a Chelex extraction procedure (Walsh et al. 1991). Fresh mites were placed directly in a 0.5 ml tube containing 2.5 µl of proteinase-K solution (20 mg/µl). Individuals that had been stored in acetone or ethanol were first placed on filter paper to allow them to dry and to check if the mites were not damaged. Mites were crushed and 50 µl of 5% (w/v) Chelex 100 (Sigma-Aldrich Chemie, Germany) water solution was added. The tubes were vortexed and incubated at 37°C for one hour. Directly after incubation, the tubes were vortexed again and boiled at 96°C for 8 minutes to de-activate the proteinase-K. Samples were either used immediately or stored at -20°C for a maximum of three weeks. Before taking supernatant to use in PCR, DNA samples were vortexed and centrifuged briefly.

Microsatellites
To study the variation within field samples, individual mites were genotyped at three microsatellite loci, brev02, brev03 and brev07 (Weeks et al. 2001). Each microsatellite locus was amplified separately in a PCR with a final volume of 10 µl containing: 1 µl 10X Super Taq buffer (Sphaero Q), 0.5 µl bovine serum albumine (10 mg/ml), 0.6 µl MgCl2 (25 mM) (additional to the buffer), 2 µl dNTP mix (1 mM of each nucleotide), 0.2 µl each primer (10 µM each) (one with an IRD-700 fluorescent label), 0.08 µl of super Taq (5 u/µl) (Sphaero Q), 2.42 µl water, and 3 µl supernatant of the Chelex extraction as template. Cycling conditions were: 4 minutes at 94 °C, then 35 cycles of 30 seconds at 94°C, 30 seconds at 54°C, and 30 seconds at 72°C, concluded with a final extension at 72°C for 4 minutes. Products were run on 6.5% denaturing polyacrylamide gels in a LI-COR DNA Sequencer (LI-COR, Westburg, The Netherlands) and scored manually. Because asexual Brevipalpus mites are haploid, their microsatellite genotype is a haplotype. However, to avoid confusion with the COI haplotypes, we will use the term genotype for microsatellites and reserve the term haplotype for the COI sequences.

COI amplification and sequencing
Part of the mitochondrial gene was amplified using primers designed by Navajas et al. (1996) (5’-TGA TTT TTT GGT CAC CCA GAA G -3’ and 5’-TAC AGC TCC TAT AGA TAA AAC -3’). In Brevipalpus these primers amplified a 410 bp fragment of the COI gene, excluding the primer annealing sites. PCR mixtures were the same as for the microsatellites, except that the reaction volume was increased to 25 µl and contained 5 µl template. Cycling conditions were: 4 minutes at 94°C, then 35 cycles of 1
Table 1. List of host-plant species (+ three letter code), the plant family names and the total number of field samples that have been collected and itemized by geographic location. Individuals indicated with an asterix are from field samples of sexual species collected in Arizona, USA, and serve as outgroup. (Vi = Viçosa, Pn = Ponte Nova, Ar = Araponga, St = Santa Thereza, Pi = Piricicaba, and Pa = Pantanal).

<table>
<thead>
<tr>
<th>Host plant species</th>
<th>Code</th>
<th>Family</th>
<th>Total</th>
<th>Ar</th>
<th>Pa</th>
<th>Pn</th>
<th>Pi</th>
<th>St</th>
<th>Vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrus sp.</td>
<td>Cit</td>
<td>Rutaceae</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Carica papaya</td>
<td>Car</td>
<td>Caricaceae</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Hibiscus rosa-sinensis</td>
<td>Hib</td>
<td>Malvaceae</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Coffea sp.</td>
<td>Cof</td>
<td>Rubiaceae</td>
<td>6</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Malpighia glabra</td>
<td>Mal</td>
<td>Malpighiaceae</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Passiflora edulis</td>
<td>Pas</td>
<td>Passifloraceae</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Inga sp.</td>
<td>Ing</td>
<td>Leguminosae</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Baccharis sp.</td>
<td>Bac</td>
<td>Compositae</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Vernonia sp.</td>
<td>Ver</td>
<td>Compositae</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Diospyros kaki</td>
<td>Dto</td>
<td>Ebenaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Ammonia reticulata</td>
<td>Ann</td>
<td>Ammonaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Rhododendron sp.</td>
<td>Rho</td>
<td>Ericaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Hedera helix</td>
<td>Hed</td>
<td>Araliaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Camellia japonica</td>
<td>Cam</td>
<td>Theaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ligustrum sp.</td>
<td>Lig</td>
<td>Oleaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Litchi sinensis</td>
<td>Lit</td>
<td>Sapindaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Vitis sp.</td>
<td>Vit</td>
<td>Vitaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Syzygium jambos</td>
<td>Syz</td>
<td>Myrtaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Cajanus cajan</td>
<td>Caj</td>
<td>Leguminosae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Duranta erecta</td>
<td>Dur</td>
<td>Verbenaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Pachira aquatica</td>
<td>Pac</td>
<td>Bombacaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Justicia sp.</td>
<td>Jus</td>
<td>Acanthaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Tecoma stans</td>
<td>Tec</td>
<td>Bignoniaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Anthurium sp.</td>
<td>Ant</td>
<td>Araceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Actinidia deliciosa</td>
<td>Kiw</td>
<td>Actinidaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Poncirus trifoliate</td>
<td>Pon</td>
<td>Rutaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Crotalearia sp.</td>
<td>Cro</td>
<td>Leguminosae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Sterculia chichi</td>
<td>Ste</td>
<td>Sterculiaceae</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Rhus trilobata</td>
<td>Rhu</td>
<td>Anacardiaceae</td>
<td>1*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pinus halapensis</td>
<td>Pin</td>
<td>Pinaceae</td>
<td>1*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

minute at 94°C, 1 minute at 48°C, and 1 minute at 72°C, concluded with a final extension at 72°C for 4 minutes. Products were analyzed by running 2.5 µl on 1% agarose gel in 0.5X TBE stained with ethidium bromide. If DNA yield was less than 5 ng/µl, the reaction product was diluted 50 times.
and PCR was repeated using 5 µl of the diluted product as template. To check for cross contamination, control PCR reactions with water were included at every eighth position. If these negative controls contained a PCR product, all reactions in that batch were discarded. Prior to sequencing, PCR products were purified using the method of Boom et al. (1989) or using a DNA extraction kit (Fermentas, The Netherlands). Cleaned products were cycle-sequenced in both directions using the same primers as used in the amplification and the ABI PRISM BigDye Terminator Sequence Kit (version 1.1, Applied Biosystems, The Netherlands) according to the manufacturer’s instructions but diluted 16 times. Sequences were run on an ABI 3700 automated DNA sequencer.

Data analysis
The sequences were aligned using Clustal-W with default settings (Thompson et al. 1994). The software of both PAUP* 4.0b10 (Swofford 1998) and Modeltest 3.6 (Posada and Crandall 2001) were used to select the optimal evolution model for the maximum likelihood analysis. Because COI is a protein coding gene, we tested if the likelihood of the selected models could be further improved by extending the model with specific rates for each of the codon positions (Shapiro et al. 2006). Under the selected model, parameters and tree topology were optimized using the successive approximations approach (Sullivan et al. 2005). The final tree was rooted using the two sexual species as outgroup.

Table 2. Clonal diversity within field samples based on three microsatellite loci and partial COI sequences. Genetic diversity based on multilocus genotypes is calculated as \( D = 1 - \Sigma P_i^2 \), where \( P_i \) is the frequency of the \( i \)-th genotype.

<table>
<thead>
<tr>
<th>Field sample</th>
<th>Host plant species</th>
<th>Location</th>
<th>Microsatellites</th>
<th>COI sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number of</td>
<td>Number of D</td>
<td>Number</td>
</tr>
<tr>
<td>1</td>
<td>Citrus sp.</td>
<td>17</td>
<td>7</td>
<td>0.79</td>
</tr>
<tr>
<td>2</td>
<td>Citrus sp.</td>
<td>16</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>Citrus sp.</td>
<td>15</td>
<td>2</td>
<td>0.63</td>
</tr>
<tr>
<td>4</td>
<td>Citrus sp.</td>
<td>45</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>Citrus sp.</td>
<td>13</td>
<td>2</td>
<td>0.45</td>
</tr>
<tr>
<td>6</td>
<td>Carica papaya</td>
<td>16</td>
<td>6</td>
<td>0.72</td>
</tr>
<tr>
<td>7</td>
<td>Carica papaya</td>
<td>11</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>8</td>
<td>Carica papaya</td>
<td>12</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>Hibiscus rosa-sinensis</td>
<td>24</td>
<td>2</td>
<td>0.44</td>
</tr>
<tr>
<td>10</td>
<td>Hibiscus rosa-sinensis</td>
<td>5</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>11</td>
<td>Coffea sp.</td>
<td>16</td>
<td>4</td>
<td>0.63</td>
</tr>
<tr>
<td>12</td>
<td>Coffea sp.</td>
<td>16</td>
<td>4</td>
<td>0.65</td>
</tr>
<tr>
<td>13</td>
<td>Malpighia glabra</td>
<td>9</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>14</td>
<td>Malpighia glabra</td>
<td>31</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>15</td>
<td>Malpighia glabra</td>
<td>18</td>
<td>3</td>
<td>0.29</td>
</tr>
<tr>
<td>16</td>
<td>Paspalum inflatum</td>
<td>15</td>
<td>6</td>
<td>0.78</td>
</tr>
<tr>
<td>17</td>
<td>Inga sp.</td>
<td>3</td>
<td>3</td>
<td>0.67</td>
</tr>
<tr>
<td>18</td>
<td>Baccharis sp.</td>
<td>16</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>19</td>
<td>Baccharis sp.</td>
<td>16</td>
<td>10</td>
<td>0.88</td>
</tr>
<tr>
<td>20</td>
<td>Dracunculus trilobus</td>
<td>4</td>
<td>2</td>
<td>0.38</td>
</tr>
<tr>
<td>21</td>
<td>Ammi reticulata</td>
<td>16</td>
<td>3</td>
<td>0.48</td>
</tr>
<tr>
<td>22</td>
<td>Rossellodendron sp.</td>
<td>16</td>
<td>3</td>
<td>0.23</td>
</tr>
</tbody>
</table>
Results

Collections
We collected in total 63 field samples (Table 1) from 28 different host plant species from 24 different plant families. At least one of the three species, *B. phoenicis*, *B. obovatus*, and *B. californicus*, has been previously reported from all but three of these plant species (Childers et al. 2003b). *Stercularia chicha*, *Crotalaria* sp., and *Actinidia deliciosa* (kiwi-fruit) were new for these species.

Clonal diversity within field samples
To assess the amount of clonal diversity, multiple individual mites from 22 field samples were genotyped for three microsatellite loci. Despite the limited number of loci, we found a wide range of genetic diversities within field samples (Table 2). A clear relation between diversity and host plant species was absent. Although some field samples from a particular plant consisted of a single genotype, in all cases we found other samples from the same host plant that contained multiple genotypes. This is illustrated by the field samples 18 and 19 collected from *Baccharis* plants. Field sample 18 was monomorphic, whereas field sample 19 contained ten different genotypes and had the highest diversity, $D = 0.88$, of all field samples tested.

The haplotype diversity within field samples was determined by sequencing part of the *COI* gene from up to five individuals with different genotypes (Table 2). We assumed that individuals with the same microsatellite genotype also have identical *COI* haplotypes, since microsatellite genotypes were more variable than *COI* haplotypes. Furthermore, no haplotype variation was found when five individuals with identical genotypes were sequenced for five different field samples. Out of the 16 field samples that contained more than one microsatellite genotype, 14 were variable at the haplotype level as well (Table 2). In six of these field samples the different haplotypes belonged to different clades, which indicated that different *Brevipalpus* species co-occur within field samples.

Clonal niche breadth
The *COI* sequences were obtained from 99 ingroup and two outgroup individuals. From the ingroup, 57 individuals were selected from field samples that were also genotyped with microsatellites. The remaining 42 individuals were single representatives from field samples with unknown genotypic variation. The final dataset, including the outgroup, contained 49 different haplotypes. Among the in-group sequences variation was observed
Figure 2. Maximum likelihood tree for all haplotypes. Each haplotype is numbered, followed by the location(s) / host plant species it was sampled from. The number of times a particular haplotype has been found is listed between parentheses. Locations are abbreviated as Vi = Viçosa, Ar = Araponga, Pn = Ponte Nova, St = Santa Thereza, Pi = Piracicaba and Pa = Pantanal. Host plant species codes are as in Table 1. Numbers at the nodes represent bootstrap values. For clarity, not every value higher than 50% is given. Bar at the bottom indicates branch length of 10% likelihood distance.

at 97 out of the 410 nucleotide positions (24%). All sequences consisted of non-interrupted reading frames and matched known mite COI sequences from GenBank. The sequences produced in this study can be retrieved from GenBank with accession numbers DQ450480 through DQ450528.

The preferred evolutionary model for tree reconstruction was GTR + G + I using Modeltest. However, a GTR model that assumes separate rates for the three coding positions improved the likelihood considerably (Akaike Information Criterion) and was therefore applied for parameter and tree topology estimation.
Table 3. The average maximum likelihood (GTR + separate rates for the three codon positions) distances between haplotypes within (diagonal) and between clades, and between clades and the outgroup sequences.

<table>
<thead>
<tr>
<th></th>
<th>Clade O</th>
<th>Clade P</th>
<th>Clade C</th>
<th>X01</th>
<th>Outgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade O</td>
<td>0.042</td>
<td>0.153</td>
<td>0.132</td>
<td>0.156</td>
<td>0.238</td>
</tr>
<tr>
<td>Clade P</td>
<td>0.019</td>
<td>0.141</td>
<td>0.134</td>
<td>0.242</td>
<td></td>
</tr>
<tr>
<td>Clade C</td>
<td></td>
<td>0.043</td>
<td>0.169</td>
<td>0.252</td>
<td></td>
</tr>
<tr>
<td>X01</td>
<td></td>
<td></td>
<td></td>
<td>0.249</td>
<td></td>
</tr>
</tbody>
</table>

The maximum likelihood tree showed that most ingroup samples were divided into three major clades (Fig. 2). The average maximum likelihood distance between haplotypes within clades ranged from 0.019 to 0.043 within clades and from 0.132 to 0.156 between clades (Table 3). These three clades, P, O, and C, represent the three most commonly reported species, *B. phoenicis*, *B. obovatus* and *B. californicus* respectively. However, because the biological species concept is rather difficult to apply to asexual species, we will use the term clade instead. With 27 haplotypes representing 62 individuals, clade P is the largest clade. Clade O was the second largest clade, containing 17 haplotypes representing 38 individuals. Clade C contained two haplotypes, each representing a single sample. Finally, a single haplotype, X01, could not be assigned to one of the three clades. This haplotype was retrieved from a single field sample collected from *Camelia japonica* in Piracicaba.

Both clades P and O contained individual mites from many different host plants and various locations indicating that they have broad niches at this phylogenetic level. A number of host plant species, such as *Coffea* sp. and *Citrus* sp., were shared by these clades. However, four other host plant species that were sampled extensively, namely *Carica papaya*, *Hibiscus rosa-sinensis*, *Passiflora edulis* and *Inga* sp., resulted in 30 individuals from clade P and only one from clade O. This indicated that the niche of clade P was broader than the niche of clade O. All the individuals in subclade Oa were collected from a single host plant species, *Malpighia glabra* (acerola), which indicated that this clade has a very narrow niche. Three of these *Malpighia glabra* plants were located in Viçosa and a fourth was located in Santa Thereza, 600 kilometers away. Subclade Oa may have a wider host plant range if it occurs on host plant species that were not sampled in this study. However, given that our sampling in the Viçosa area was extensive and random with respect to plant species, we conclude that this is unlikely.

Clade C contained two individuals, one taken from *Rhododendron* sp. (azalea) in Viçosa, and one from *Ligustrum* in Piracicaba. Finally, five individuals, comprising three basal haplotypes in clade O, were collected.
from a single field sample on *Baccharis* plants in Viçosa. This field sample had not only a remarkable position in the COI gene tree but also the highest within field sample clonal diversity based on microsatellites. Unfortunately, shortly after sampling all vegetation was cleared from this site making it impossible to further investigate the mites from these plants.

Most haplotypes were collected from one or two host plant species only (Fig. 2). Four haplotypes, numbered P06, P10, P11 and P13, were found on four or more host plant species each from different plant families, and therefore these could be regarded as generalists. All four haplotypes belonged to clade P. On the other hand, clade Oa appears to be specialized, because all mites were collected from *Malpighia glabra*, but from geographically well-separated field locations.

**Discussion**

The phylogenetic distribution of clones across host plant species shows that clades differ in their degree of host plant specialization (Fig. 2). For example, all clones of subclade Oa, although from different geographic origins, were found exclusively on *Malpighia glabra* suggesting that they are specialized on this host. This suggestion is consistent with previous results from transplantation experiments (Groot et al. 2005). Using mites from three different host plant species, the transplantations showed that the most specialized population was from *Malpighia glabra*. Mites from this population carried haplotype O16 (this study), which is in subclade Oa. Therefore, subclade Oa is likely a genuine host plant specialist.

Subclade Ob falls in between subclade Oa and clade P with respect to the number of host plant species its clones were found on. Its clones were found predominantly on *Citrus sp.*, *Coffea sp.* and *Baccharis* sp., which are also used by clones of clade P. This is the main overlap in host plant use of these two clades.

Clade P is the largest clade and its haplotypes were found on 23 different host plant species. Moreover, four clones (P06, P10, P11, and P13) were found on four or more different host plant species, each of these belonging to different plant families. These can be classified as host plant generalists relative to all other clones that were only found on one or two host plant species.

The above phylogenetic pattern in relation to host plant use indicates that in *Brevipalpus* some clones behave like generalists and others like specialists. How do we reconcile the observed pattern with the predictions of the alternative models? The GPG predicts that a generalist clone will
Clonal Diversity in *Brevipalpus*

survive over time in a fluctuating environment. The emphasis is on the unpredictability of niche stability, and the trait under selection is tolerance to niche instability. The FNV assumes a stable environment where competition among clones is the driving force. Competition within a niche will lead to survival of the best adapted and thus specialized clone. As Vrijenhoek en Pfeiler (1997) have argued, these two models are not mutually exclusive since competitive ability and tolerance to niche instability act on different scales and can be combined; a clone can be both a specialist in its niche but also tolerant to seasonal variation. As the importance of either trait may vary between host plant species, this is expected to result in clones that differ in the degree of host plant specialization.

Both GPG and FNV models are expected to result in a single clone per niche, although the underlying processes are different. However, we found a surprisingly high amount of clonal diversity within field samples; single host plants were often infested by mites with several different haplotypes. In six out of 22 field samples, these haplotypes even belonged to different clades. There are several not mutually exclusive explanations for the coexistence of different clones on a single host plant. These are: (1) mutation accumulation, (2) periodic sexual reproduction, (3) absent or weak competitive exclusion, (4) migration and, (5) small scale niche differentiation.

Mutation accumulation may explain existence of field samples that contain different clones that belong to the same clade because the genetic differentiation is only limited and may have originated within the population. However it is an unlikely explanation for the occurrence of clones from different clades in the same field sample because the genetic differences between them are large.

Occasional sexual reproduction may produce new clonal genotypes via hybridization with sexual relatives or spanandric males of other clones. Hybridization with sexual relatives seems unlikely because we have not found any sexual relatives in the same area. Occasional sex with spanandric males seems more plausible since these spanandric males were occasionally seen in the field.

Competitive exclusion may fail to operate because of the instability of the environment. In theory, competitive exclusion will operate when multiple species depend on the same resource in a stable environment (Armstrong and McGehee 1980). However, for the *Brevipalpus* mites the environment is not stable. A large number of the host plants species are deciduous while some others are annuals. Mites are completely absent from these plants at the end of the dry season. In fact, plants may need to be re-
colonized every season with mites from elsewhere. In theory, this will reduce the impact of competitive exclusion (Armstrong and McGehee 1980). This leaves migration and drift as important factors in determining clonal composition of field samples and may explain the high diversity within them.

Finally, different clones can coexist because they are ecologically distinct at a smaller scale than a single plant. Different clones may specialize in feeding on different parts of the host plant or differ in their susceptibility to pathogens or predators (Jokela et al. 2003; Sabelis and Bruin 1996). Such fine scale niche partitioning has been proposed to allow for the coexistence of different species on a single host plant in eriophyoid mites (Sabelis and Bruin 1996). The coexistence of different clones of the snail Potamopyrgus antipodarum is determined by density-dependent attack rates of trematode parasites (Jokela et al. 2003). We also observed that different laboratory clones of Brevipalpus reared on the same host plant Phaseolus vulgaris consistently use different parts of the leaf for feeding and oviposition. However, we have not recorded this for our field samples.

Bacterium-induced parthenogenesis in the genus Brevipalpus has resulted in extensive clonal variation. There is also a large amount of variation in the degree of specialization among clones, resulting in evidence for both GPG and FNV. Interestingly there appear to be phylogenetic constraints for the level of specialization; some clades are more specialized than others. This could be the result of competitive exclusion of the least adapted clones and specialization on single plant species (Malpighia glabra). On the other hand, the generalist clade P is often found in mixed field samples with clones from more specialized clades. The reason for this coexistence could be either niche partitioning within a host plant or seasonal recolonization. In the latter case, time may be too short for competitive exclusion to sort out between specialized and non-specialized clones. Additional fine scale sampling and temporal sampling is required to distinguish between these options.

Acknowledgements

Part of this work was done in the Plant Molecular Virology Laboratory at the Federal University of Viçosa, Brazil. We thank the staff from this lab, especially Murillo Zerbini and Renata Faier Calegario, for their hospitality and help. We also thank Arne Janssen and Steph Menken for their helpful comments on earlier drafts of this manuscript. This study was supported by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), grant number W89-141.
Chapter 4

Horizontal Transmission of Cardinium Symbionts Within and Between Closely Related Parthenogenetic Mite Species

Thomas V.M. Groot and Johannes A.J. Breeuwer

Abstract

The relationship between symbiotic bacteria and their arthropod hosts is largely determined by the mode of transmission of the symbiont. If transmission is vertical, mutualistic relationships are expected to evolve. However, if vertical transmission is by females only, symbionts can manipulate the reproduction of their host to enhance their own fitness in ways that reduce host fitness. If such reproductive parasites are also transmitted horizontally, parasites can become even more harmful to their hosts, while at the same time multiple infections might arise which facilitates recombination among symbiont lineages. In Wolbachia, horizontal transmission has been documented between both related and unrelated host species. Whether the same applies for the recently discovered Cardinium is yet unknown. We studied the mode of transmission of Cardinium within and between three closely related Brevipalpus species by comparing the phylogeny of the symbiont with the mitochondrial gene tree of its host. Although there was general congruence between the two phylogenies, incongruities were observed at various levels. It was unlikely that these incongruities resulted from lineage sorting. Instead, the incongruities suggested that horizontal transmission has occurred both within and between mite species. Especially the symbiont generally associated with B. phoenicis appeared to be quite mobile because it was also found in some B. obovatus and B. californicus individuals. Some other B. obovatus samples were infected by a second highly divergent type of Cardinium. This suggests that Brevipalpus has become infected at least twice from different sources. The mechanism of horizontal transfer of Cardinium is unknown. Symbionts may be transferred through sharing of the food source by differentially infected hosts. We conclude that Wolbachia and Cardinium have the same manipulative phenotypes and also similar transmission patterns.
Introduction

The relationship between a symbiotic bacterium and its host is to a large extent determined by transmission route of the symbiont. A symbiont that is transmitted vertically only, should not be harmful to its host as a reduced host fitness will reduce its own fitness as well (Lipsitch et al. 1995; Yamamura 1993). Instead, a vertically transmitted symbiont can enhance its fitness by improving the fitness of its host, and therefore mutualistic relationships are expected to evolve. Alternatively, since a vertically transmitted symbiont generally is transmitted by females only, it can also increase its fitness by manipulating the reproduction of its host (Werren and O'Neill 1997). Several processes have been described that increase the number of infected females relative to the uninfected females in a population. As these processes come at a cost to the host, symbionts that induce them have been named reproductive parasites (Werren et al. 1995b).

The occurrence of occasional horizontal transmission can have profound effects on the relationship between host and symbiont. Through horizontal transmission, even symbionts that have strong negative effects on host fitness can be maintained in a population (Lipsitch et al. 1995). Therefore, horizontal transmission can lead to parasitism. Additionally, horizontal transmission can lead to different symbionts infecting the same host. When this occurs, competition among the symbiont can result in increased virulence of the symbiont (Van Baalen and Sabelis 1995). In addition, multiple infections provide opportunities for genetic recombination among symbionts and this can increase the rate of adaptation of symbionts to their hosts as well as slow down the accumulation of deleterious mutations.

To make any prediction on the relation between host and symbiont is, knowledge about the mode of transmission is required. One way to indirectly study the mode of transmission is to compare the phylogeny of the symbiont with the phylogeny of the hosts. If transmission is strictly vertical, parallel divergence patterns are expected, whereas inconsistencies between the two phylogenies are indicative of horizontal transfer. This approach has been taken to unravel the evolutionary relationship between various bacterial symbionts and their hosts. In several cases congruence between both phylogenies, and therefore evidence for strict vertical transmission was found. Examples of such coevolving host-symbiont systems are aphids infected by *Buchnera* symbionts (Moran and Baumann 1994), psyllids infected by *Carsonella* (Thao et al. 2000), tsetse flies infected by *Wigglesworthia glossinidia* (Chen et al. 1999), and carpenter ants infected by *Blochmannia* (Sauer et al. 2000). Incongruous phylogenies
indicative of horizontal transfer have also been found, for example between aphids or psyllids and their secondary symbionts (Russell et al. 2003) and between whiteflies and their primary and secondary symbionts (Zchori-Fein and Brown 2002).

The same type of studies has been conducted for the reproductive parasite Wolbachia. These studies have shown that, on an evolutionary time scale, horizontal transmission occurs rather often and has been inferred to occur both between different host species (e.g. Kittayapong et al. 2003; Werren et al. 1995a) as well as within host species (e.g. Ahrens and Shoemaker 2005; Cordaux et al. 2004). To increase the frequency of infected females in a population, Wolbachia bacteria can manipulate the reproduction of their arthropod hosts in four different ways; 1) cytoplasmic incompatibility, 2) male-killing, 3) parthenogenesis induction, and 4) feminization (Stouthamer et al. 1999). Whereas these parasitic phenotypes are restricted to Wolbachia bacteria that infect arthropods, they are unknown for Wolbachia that infect filarial nematodes. Instead, these Wolbachia are believed to be beneficial to their nematode hosts (Fenn and Blaxter 2004). Interestingly, the phylogenies of the nematodes and the Wolbachia that infect them are congruent (Casiraghi et al. 2001).

Recently, another reproductive parasite has been discovered and was named Cardinium (Weeks et al. 2001; Zchori-Fein et al. 2004). Since its discovery, Cardinium has been shown capable of three different manipulations of host reproduction. It induces cytoplasmic incompatibility in Encarsia wasps (Hunter et al. 2003) and in the mite Metaseiulus occidentalis (Weeks and Stouthamer 2004), it induces parthenogenesis in again Encarsia wasps (Zchori-Fein et al. 2001; Zchori-Fein et al. 2004) and scale insects (Provencher et al. 2005), and it induces feminization in Brevipalpus mites (Chigira and Miura 2005; Weeks et al. 2001).

Brevipalpus phoenicis is the host species where feminization by Cardinium was shown (Weeks et al. 2001). The ancestral mode of reproduction in the genus Brevipalpus is probably sexual reproduction by arrhenotoky (Helle et al. 1980). The effect of feminization by Cardinium is that unfertilized, haploid eggs, develop into haploid females (Weeks et al. 2001). This species reproduces asexually because all females are infected and vertical transmission of the symbiont is nearly perfect. Recently Chigira and Miura (2005) showed that B. californicus too is infected with, and feminized by, Cardinium. Finally, also B. obovatus is reported to be infected with Cardinium (Weeks et al. 2003) and is entirely asexual (Pijnacker et al. 1980). However, not all B. obovatus clones are infected, in some clones asexuality appears to be a genetic trait of the mite itself (Groot and Breeuwer in press). The three afore mentioned Brevipalpus species are
closely related and morphological differentiation among them is very limited (Welbourn et al. 2003). All three species occur worldwide in tropical and subtropical areas and each has a remarkably broad range of host plant species, which partly overlap among the mite species (Childers et al. 2003b).

The fact the *Wolbachia* and *Cardinium* share a suite of manipulations of host reproduction suggests that both symbionts have similar patterns of vertical and horizontal transmission. On the other hand, two studies have screened for the presence of both symbionts in a total of 322 insect and mite species and showed that *Wolbachia* infects three times as many host species as *Cardinium* (22.7 % and 6.8 % infected, respectively) (Weeks et al. 2003; Zchori-Fein and Perlman 2004). This observation could suggest that *Cardinium* is transmitted horizontally less frequently. The screening of various species of insects and mites for the presence of *Cardinium* has shown that related bacteria occur in host species that belong to different orders, indicative of horizontal transmission at this level (Weeks et al. 2003; Zchori-Fein and Perlman 2004). Thus, at a higher taxonomic level *Cardinium* behaves similar to *Wolbachia*. Whether horizontal transfer of *Cardinium* also occurs at lower taxonomic levels, such as between closely related host species or within host species, has remained unstudied so far. Here we present the results of a study on the mode of transmission of *Cardinium* within and between three closely related asexual species of mites; *Brevipalpus phoenicis*, *B. obovatus*, and *B. californicus*.

**Material and Methods**

**Sampling**

The majority of the samples have been collected in southern Brazil as described in Groot and Breeuwer (chapter 3). In addition, mites have been collected in the USA (Tucson, Arizona), Spain (Valencia), Greece (Athens), Vietnam (O Mon), and the Netherlands (Arnhem, Burgers’ Bush tropical greenhouse). Adult mites were collected and then either used for DNA extraction immediately, or stored in 96% ethanol or acetone at ambient temperature. A list of all samples is provided in appendix 1.

**DNA extraction, PCR, and sequencing**

Mite DNA was extracted using chelex resin as described in (Groot et al. 2005). This method yields enough template for up to 15 PCR reactions from a single individual. From an individual mite a 450 bp fragment of the mitochondrial cytochrome oxidase I (*COI*) gene was amplified using the primers developed by Navajas et al. (1996) (5’- TGA TTT TTT GGT CAC
CCA GAA G -3’ and 5’- TAC AGC TCC TAT AGA TAA AAC- 3’). From the symbiont a 786 bp fragment of its DNA gyrase subunit B (gyrB) gene was amplified using primers: 5’- GTT ACC GTA TAC CGA AAT GG - 3’ and 5’- TGC TTT CCG RGC MGC TTG -3’. These primers were specifically designed to amplify gyrb in the symbionts of both B. phoenicis and B. obovatus based on sequences kindly provided by A. Weeks (CESAR, University of Melbourne). PCR cocktails for both genes were identical (except for primers) and contained 2.5 µl 10X Super Taq buffer (Sphaero Q), 1.25 µl bovine serum albumine (10 mg/ml), 1.5 µl MgCl₂ (25mM) (additional to the buffer), 5 µl dNTP mix (1mM of each nucleotide), 0.5 µl of each primer (10 µM ach), 0.2 µl super Taq (5 u/µl) (Sphaero Q), and 5µl of chelex extracted template, in a total volume of 25µl. Cycling conditions for COI were 4 minutes at 94˚C, then 35 cycles of 1 minute at 94˚C, 1 minute at 48˚C, and 1 minute at 72˚C, concluded with a final extension at 72˚C for 4 minutes. Cycling conditions were equal for gyrB, except that the annealing temperature was raised to 50˚C. Products were checked by running samples on 1% agarose gel in 0.5x TBE. If amplicon concentration was too low, the reaction product was diluted 50 times in sterile water and PCR was repeated using 5µl of the diluted product as template.

In the gyrB procedure, amplified fragments were separated from non-specific products by running the PCR product through a 1% agarose in 1x TAE gel and excising them. PCR products were purified using the method of Boom et al. (1990) or using the DNA Extraction Kit (Fermentas, St. Leon-Rot, Germany). Cleaned products were cycle-sequenced in both directions using the same primers as used for the amplification and the ABI PRISM BigDye Terminator Sequence Kit (version 1.1, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) according to the manufacturer’s instructions but diluted 16 times. Sequences were run on an ABI 3700 automated sequencer.

Phylogenetic analysis
The data set of each gene was aligned with Clustal-W (Thompson et al. 1994) and identical sequences were reduced to a single entry. Maximum likelihood analyses were performed in PAUP*4.0b10 (Swofford 1998). In addition, Modeltest version 3.6 (Posada and Crandall 2001) was used to select the optimal mutation models for maximum likelihood analyses. As COI and gyrB are both protein coding genes we tested if the likelihood of the models could be further improved by extending the selected model with specific rates for each of the codon positions (Shapiro et al. 2006). Under the selected models, parameters and tree topologies were optimized using the successive approximation approach (Sullivan et al. 2005). For the COI
tree we assessed bootstrap support by performing a Neighbor-Joining bootstrap (10,000 replicates) where the distances between sequences were calculated according to the selected maximum likelihood model.

Results

We amplified and sequenced both mite COI and symbiont gyrB from in total 112 individuals collected from different localities. The majority of *B. phoenicis* and *B. californicus* samples contained symbionts based on amplification of gyrB. In 10% of the *B. phoenicis* and 15% of the *B. californicus* mites, amplification of the symbiont gene failed whereas mite DNA was amplified successfully. This was probably caused by the low
amount of template DNA retrieved from ethanol conserved samples and because *Brevipalpus* mites are minute. However, we cannot rule out the possibility that some mites were uninfected. In contrast, the per cent of unsuccessful amplifications of the symbiont *gyrB* in *B. obovatus* was much higher reaching 65% of the samples where the mite *COI* did amplify successfully. Low symbiont densities and loss of DNA during conservation can only explain this partly. Most failures in *B. obovatus* were probably caused by the fact that this species is polymorphic for the infection. (Groot and Breeuwer in press).

**Table 1.** All variable nucleotide positions in the symbiont *gyrB* alignment. Dashes represent missing data.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Position 1</th>
<th>Position 2</th>
<th>Position 3</th>
<th>Position 4</th>
<th>Position 5</th>
<th>Position 6</th>
<th>Position 7</th>
<th>Position 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sample B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sample C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sample D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

BLAST searches of the obtained sequences in GenBank returned only *COI* and *gyrB* sequences, confirming the identity of our mite and bacterial amplicons, respectively. All obtained sequences had uninterrupted reading frames based on mtDNA and bacterial codon translation tables in GenBank; no evidence for pseudogenes was found. The optimal mutation models selected by model test were TrN+I+G and GTR for the *COI* and *gyrB* data sets, respectively. For both datasets, likelihood of the model was
improved by assuming separate mutation rates for the three codon positions (Akaike Information Criterion). Consequently, the resulting models became TrN + codon-specific rates for COI and GTR+ codon-specific rates for gyrB.

Sequencing COI from 112 individual mites revealed 47 different haplotypes; 26 were unique and 21 were found in more than one sample. The COI maximum likelihood tree shows three well supported clades representing the three species (Fig. 1). The most common species in our collection was B. phoenicis, with a total of 81 samples. Brevipalpus californicus was less common and was represented by 14 samples. Brevipalpus obovatus was intermediate in abundance, but only 17 samples were included in the final dataset because the symbiont could not be amplified from the others.

We found 28 different gyrB haplotypes among the 112 mites; 21 were unique and seven were found more than once (Table 1). The haplotypes could be assigned to three clades, Clade A, B and C (Fig. 3). To test if the gyrB haplotypes were from Cardinium, two representatives of each of these clades were compared to the sequences from Cardinium and related bacteria obtained from GenBank (Fig. 2). As the Brevipalpus symbiont sequences grouped with the known Cardinium sequences, their identity as Cardinium was confirmed. Clade A, represented by G01 and G02, is distantly related to the other two clades. The average nucleotide dissimilarity between clade A and the other clades was 0.18 (average uncorrected $P$ distance), which is equal to the difference between clade A and the symbionts of Encarsia wasps (average uncorrected $P$ distance = 0.17). Clades B and C were more related; the average dissimilarity between them was 0.04.

Clade A was the least common and contained three haplotypes from seven B. obovatus mites. The larger clade C contained five haplotypes and all came from 12 B. californicus mites. The clade B symbionts were the most widespread and mainly associated with B. phoenicis. The haplotype frequency in clade B distribution was highly skewed; haplotypes G10 and G20 were found in 67 and eight of the 112 samples, respectively, whereas all other haplotypes were found only once. These two main haplotypes differed at two nucleotide positions. They were at the basal positions within clade B and may represent a subdivision within clade B. Within B. phoenicis members of either part of this symbiont subdivisions were not randomly distributed. In general they were associated with monophyletic clades in B. phoenicis. Mites with haplotypes C12 to C28 all have G10 or derived symbionts, whereas mites with haplotypes C29 to C37 mostly had G20 or derived haplotypes. This suggests cocladogenesis of B. phoenicis and its symbiont.
Horizontal Transmission of Cardinium

Figure 2. Neighbor-Joining tree of representative types from the three symbiont clades from this study and several gyrB sequences from GenBank. Sequences from Brevipalpus symbionts obtained in this study are labeled with G and a number. All other sequences were retrieved from GenBank (accession numbers indicated after species names). The bar indicates the branch length that representing 10% genetic distance.

However, there were also a number of incongruities in the comparison of the two phylogenies (Fig. 3). Clade B was predominantly associated with B. phoenicis, but a few of its symbionts were also found in B. obovatus and B. californicus. In one case, mites with the same haplotype were infected by both symbionts of clades A and B. Brevipalpus obovatus mites with haplotype C01 were infected with three different symbionts; two from clade A (G01 and G02) and one from clade B (G04).

Incongruity was also found at the within species level in B. phoenicis. Roughly three monophyletic groups of mite haplotypes can be distinguished: C14-C28, C29-C33 and C34-C37 (Fig. 1). The first two groups contained G10–like and G20-like symbionts respectively. However, the monophyletic group that contained haplotypes C34-C37 harbored two different symbionts, G10-like or G20-like symbionts.
Chapter 4

**Figure 3.** Mite COI tree (cladogram) facing symbiont gyrB tree (phylogram). The names of the mite species and symbiont clades are followed by the number of samples that comprise to that species or clade. Haplotypes are numbered; haplotype numbers are preceded by a C for COI, and a G for gyrB. Each haplotype name is followed by the number of times the haplotype was encountered. Both trees are midpoint rooted. The colored lines connect individual mites with their symbionts. When several lines connect a single mite haplotype to various symbionts, this means that several mites with the same haplotypes were found to contain different symbionts. The bar with the gyrB tree indicates the branch length that represents 5% maximum likelihood distance.

**Discussion**

There is general congruence between the phylogenies of these closely related *Brevipalpus* mites and their *Cardinium* symbionts. *Cardinium* strains within a host species are generally very similar. Schilthuizen and Stouthamer (1997) observed a similar pattern at the species level in
Horizontal Transmission of Cardinum

*Trichogramma* wasps and their parthenogenesis inducing *Wolbachia*. This result is expected if vertical transmission of symbionts is the predominant mode of transmission. Especially for obligate mutualistic bacteria there is ample evidence that they cospeciated with their host (Chen et al. 1999; Moran and Baumann 1994; Sauer et al. 2000; Thao et al. 2000).

However, in contrast to the general concordance, there are a number of incongruities. The most obvious incongruities are found in the associations between symbionts of clade B and their hosts. Although this clade is the only infection found in *B. phoenicis*, some of its members were associated with the two other host species. Possible explanations for this pattern are lineage sorting of ancestral symbiont diversity, horizontal transmission or a combination of both.

There are several reasons why lineage sorting is not a likely explanation for the observed incongruities. Lineage sorting can only result in different haplotypes that contain similar symbiont types if the mitochondrial ancestor of the haplotypes has been infected with more than one type of bacterium, *i.e.* would have been multiply infected. Let us assume that the mitochondrial ancestral of the mite species indeed was infected with both clade A and B symbionts. Then one of the symbionts types, clade A, must have been lost in *B. phoenicis* lineage, but both A and B would have been maintained in *B. obovatus*. In addition, since identical symbiont *gyrB* sequences were found in *B. phoenicis* and *B. obovatus*, this scenario would also imply that during the divergence of the ancestral mite lineage in two daughter lineages, the symbionts of clade B did not diverge nor accumulate mutations at all. This is not very plausible.

Secondly, this scenario requires that multiple infections within individuals are maintained for relatively long periods of time; at least as long as needed for the host to speciate. We would expect then to find single mites that carry two or more symbiont types currently. So far we have not encountered mites that are doubly infected in our samples. It suggests that multiple infections are unstable. Indeed, most lineages still produce a few percent males indicating that symbiont transmission is not perfect (Groot and Breeuwer in press). Thus the major factor contributing to the discordance between the two phylogenies is probably horizontal transmission.

The incongruity between host and symbiont phylogenies depends to a certain extent on the particular phylogenetic trees that are selected, which in turn depend upon the genes being used. Gene trees do not necessarily represent species trees. In a recent study, Baldo et al. (2006) showed that recombination must occur frequently among *Wolbachia* lineages. Recombination may also occur between *Cardinium* strains and this could
mean that the \textit{gyrB} gene tree is different from the actual phylogeny the symbiont. This needs to be tested by the sequencing of other bacterial genes. In case the gene tree does not exactly match the species tree, the symbiont gene must have been horizontally transferred from one mite lineage to the other. Thus, the occurrence of recombination does not affect our conclusion that horizontal transmission of \textit{Cardinium} within and between \textit{Brevipalpus} species has occurred.

The genetic difference between the symbionts of clade A and clades B and C is large and comparable to the difference of either type to the symbionts of \textit{Encarsia}. This suggests that both types infecting \textit{Brevipalpus} have separate origins. Although we do not know what these origins are, \textit{Brevipalpus} probably has become infected twice by what may be regarded as horizontal transmission between unrelated species. This ability of \textit{Cardinium} to switch between unrelated hosts has been shown before (Weeks et al. 2003; Zchori-Fein and Perlman 2004). For example, Weeks et al. (2003) showed that the \textit{Cardinium} infecting the scale insect \textit{Aspidiotus nerii} clustered within a group of bacteria infecting parasitic wasps of the genus \textit{Aphytis}. Similar capacities to switch between unrelated host species are known from \textit{Wolbachia} (Stouthamer et al. 1999).

\textit{Brevipalpus obovatus} has a special position. In contrast to the other two species, we have found uninfected \textit{B. obovatus} females that still reproduce by thelytoky (Groot and Breeuwer in press). We do not know what the mechanism of thelytoky is in these uninfected lineages. It is possible that genetic material from the symbiont containing the genes responsible for the feminization has jumped to the host nuclear genome. A similar transfer of feminizing factors has been inferred between the genomes of \textit{Wolbachia} and their isopod hosts (Rigaud et al. 1997). Furthermore, symbiont to host gene transfer has been observed between \textit{Wolbachia} and their beetle host (Kondo et al. 2002), although in this case phenotypic effects on the host were absent. Based upon the existence of uninfected thelytokous \textit{B. obovatus}, we cannot exclude the possibility that infected lineages exist that do not require their symbiont for asexual reproduction. [So far symbiont induced parthenogenesis has only been determined in a limited number of \textit{B. obovatus} lineages (Groot and Breeuwer in press).] Such a lineage could be produced when an uninfected thelytokous line acquires a new infection through horizontal transmission.

Little is known about the mechanism of horizontal transmission of \textit{Cardinium} (Weeks et al. 2003; Zchori-Fein and Perlman 2004). Most evidence for horizontal transmission in the comparable \textit{Wolbachia}-system is indirect evidence that is based on phylogenetic comparisons of \textit{Wolbachia} and their hosts. Horizontal or infectious transmission may occur between
parasitoids and hosts, prey and predator or via shared food sources. There are only two studies that provide direct empirical evidence for horizontal transmission of *Wolbachia* between hosts. Heath et al. (1999) showed that the parasitic wasp *Leptopilina boulardi* can obtain a *Wolbachia* infection from its infected *Drosophila simulans* host. Huigens et al. (2000; 2004) demonstrated unequivocally that *Wolbachia* can be horizontally transmitted between infected and uninfected *Trichogramma* parasitoid wasps that develop within a single egg of their butterfly host.

*Cardinium* may exploit similar routes of horizontal transmission based on the observations that some hosts and parasitoids are infected with similar symbionts (Weeks et al. 2003; Zchori-Fein and Perlman 2004). In *Brevipalpus*, horizontal transmission is most likely to occur via a common food source; *Brevipalpus* is phytophagous and does not have parasitoids. Sintupachee et al. (2006) found that different insect species that feed on the same tissues of pumpkin plants were infected with similar *Wolbachia*. Insects and mites with piercing-sucking mouth parts often inject saliva into the food plants. If this saliva contains the bacteria, they might be ingested by other herbivores. *Cardinium* bacteria have been found in the salivary glands of the leafhopper *Scaphoideus titanus* (Marzorati et al. 2006). Whether the bacterium is also present in the saliva and can be transferred to, and taken up from, the plant remains to be tested. For *Brevipalpus* mites, that also feed by piercing-sucking plants cells, horizontal transfer via a shared food source is an attractive explanation, especially because we have shown that different mite lineages often feed from the same host plant (Groot and Breeuwer chapter 3).

In conclusion, the results of this study show that horizontal transfer of *Cardinium* probably has occurred at various phylogenetic levels. It is unclear, however, what the current rate of horizontal transmission is. In this respect, *Cardinium* behaves similar to *Wolbachia*, with which it also shares various reproductive manipulations. This is additional evidence of convergent evolution between these unrelated bacteria. Theory predicts that because of the horizontal transmission, *Cardinium* can become more parasitic in its relation to its host. In this respect it is of special interest that many *B. obovatus* mites are not infected. Somehow these lines have been able to resist the parasite. Other *B. obovatus* mites, however, do contain symbionts, which offer interesting opportunities to further investigate the relationship between host and symbiont.
Acknowledgements

We thank Vera Ros and Maarten Hilbrant for collecting Brevipalpus samples in Greece and Vietnam. Similarly, we are grateful to the staff of the Burgers’ Bush for allowing us to collect mites in their tropical greenhouses. Finally, we thank Murillo Zerbini and Renata Faier Calegario who allowed us to use their Plant Molecular Virology Laboratory at the Federal University of Viçosa, Brazil. This study was supported by the Netherlands Foundation for the Advancement Research (WOTRO), grant number W89-141.

Appendix 1. List of all the 112 samples indicating for each sample the COI haplotype number of the host and the gyrB haplotype number of the symbiont. Also stated are host plant species and sampling location. Country names are followed with abbreviations for states when relevant. These abbreviations are: MG = Minas Gerais, ES = Espírito Santo, SP = São Paulo, MS = Mato Grosso do Sul, AZ = Arizona.

<table>
<thead>
<tr>
<th>COI haplotype</th>
<th>gyrB haplotype</th>
<th>Host plant species</th>
<th>Plantfamilie</th>
<th>Country</th>
<th>City / Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>C01</td>
<td>G01</td>
<td>Baccharis sp.</td>
<td>Asteraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C01</td>
<td>G02</td>
<td>Baccharis sp.</td>
<td>Asteraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C01</td>
<td>G02</td>
<td>Baccharis sp.</td>
<td>Asteraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C01</td>
<td>G02</td>
<td>Baccharis sp.</td>
<td>Asteraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C01</td>
<td>G04</td>
<td>Baccharis sp.</td>
<td>Asteraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C02</td>
<td>G02</td>
<td>Malpighia glabra</td>
<td>Malpighiaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C02</td>
<td>G02</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C03</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C04</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, ES</td>
<td>Santa Thereza</td>
</tr>
<tr>
<td>C07</td>
<td>G06</td>
<td>Coffea sp.</td>
<td>Rubiaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C08</td>
<td>G10</td>
<td>Coffea sp.</td>
<td>Rubiaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C08</td>
<td>G10</td>
<td>Baccharis sp.</td>
<td>Asteraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C09</td>
<td>G10</td>
<td>Baccharis sp.</td>
<td>Asteraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C10</td>
<td>G07</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Spain</td>
<td>Valencia</td>
</tr>
<tr>
<td>C11</td>
<td>G12</td>
<td>unknown</td>
<td></td>
<td>Greece</td>
<td>Athens</td>
</tr>
<tr>
<td>C12</td>
<td>G10</td>
<td>Inga sp.</td>
<td>Leguminosea</td>
<td>Brazil, MS</td>
<td>Pantanal</td>
</tr>
<tr>
<td>C13</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C13</td>
<td>G10</td>
<td>Inga sp.</td>
<td>Leguminosea</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C14</td>
<td>G08</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C14</td>
<td>G09</td>
<td>Passiflora edulis</td>
<td>Passifloraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MS</td>
<td>Pantanal</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, SP</td>
<td>Piracicaba</td>
</tr>
<tr>
<td>Code</td>
<td>Genus</td>
<td>Species</td>
<td>Family</td>
<td>Location</td>
<td>City</td>
</tr>
<tr>
<td>------</td>
<td>-----------</td>
<td>---------------</td>
<td>------------</td>
<td>-------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, SP</td>
<td>Piracicaba</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Passiflora edulis</td>
<td>Passifloraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Passiflora edulis</td>
<td>Passifloraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C14</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C15</td>
<td>G10</td>
<td>Acalypha sp.</td>
<td>Euphorbiaceae</td>
<td>Vietnam, O Mon</td>
<td></td>
</tr>
<tr>
<td>C15</td>
<td>G10</td>
<td>Acalypha sp.</td>
<td>Euphorbiaceae</td>
<td>Vietnam, O Mon</td>
<td></td>
</tr>
<tr>
<td>C16</td>
<td>G10</td>
<td>Hedra helix</td>
<td>Araliaceae</td>
<td>Brazil, SP</td>
<td>Piracicaba</td>
</tr>
<tr>
<td>C17</td>
<td>G10</td>
<td>Hibiscus rosa-sinensis</td>
<td>Malvaceae</td>
<td>Brazil, SP</td>
<td>Piracicaba</td>
</tr>
<tr>
<td>C18</td>
<td>G10</td>
<td>Syzygium jambos</td>
<td>Myrtaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C18</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C18</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C18</td>
<td>G10</td>
<td>Pachira aquatica</td>
<td>Bombacaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C18</td>
<td>G10</td>
<td>Tecoma stans</td>
<td>Bignoniaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C18</td>
<td>G10</td>
<td>Actinidia deliciosa</td>
<td>Actinidaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C19</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C19</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C19</td>
<td>G10</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C20</td>
<td>G10</td>
<td>Hibiscus rosa-sinensis</td>
<td>Malvaceae</td>
<td>Brazil, MG</td>
<td>Ponte Nova</td>
</tr>
<tr>
<td>C20</td>
<td>G10</td>
<td>Passiflora edulis</td>
<td>Passifloraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C20</td>
<td>G10</td>
<td>Hibiscus rosa-sinensis</td>
<td>Malvaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C20</td>
<td>G10</td>
<td>Hibiscus rosa-sinensis</td>
<td>Malvaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C20</td>
<td>G10</td>
<td>Hibiscus rosa-sinensis</td>
<td>Malvaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C21</td>
<td>G13</td>
<td>Cajanus cajan</td>
<td>Leguminoseae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Litchi sinensis</td>
<td>Sapindaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Anthurium sp.</td>
<td>Araceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G10</td>
<td>Justicia sp.</td>
<td>Acanthaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G14</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C22</td>
<td>G15</td>
<td>Rododendron sp.</td>
<td>Ericaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>C23</td>
<td>G10</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
</tr>
<tr>
<td>Code</td>
<td>G10</td>
<td>Species</td>
<td>Family</td>
<td>Location 1</td>
<td>Location 2</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
<td>---------</td>
<td>--------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>C24</td>
<td>Passiflora edulis</td>
<td>Passifloraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
<td></td>
</tr>
<tr>
<td>C25</td>
<td>Passiflora edulis</td>
<td>Passifloraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
<td></td>
</tr>
<tr>
<td>C26</td>
<td>Hibiscus rosa-sinensis</td>
<td>Malvaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
<td></td>
</tr>
<tr>
<td>C27</td>
<td>Hibiscus rosa-sinensis</td>
<td>Malvaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
<td></td>
</tr>
<tr>
<td>C28</td>
<td>Hibiscus rosa-sinensis</td>
<td>Malvaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
<td></td>
</tr>
<tr>
<td>C29</td>
<td>Inga sp.</td>
<td>Leguminosea</td>
<td>Brazil, MS</td>
<td>Pantanal</td>
<td></td>
</tr>
<tr>
<td>C30</td>
<td>Coffea sp.</td>
<td>Rubiaceae</td>
<td>Brazil, MG</td>
<td>Ponte Nova</td>
<td></td>
</tr>
<tr>
<td>C31</td>
<td>Carica papaya</td>
<td>Caricaceae</td>
<td>Brazil, MG</td>
<td>Ponte Nova</td>
<td></td>
</tr>
<tr>
<td>C32</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MG</td>
<td>Ponte Nova</td>
<td></td>
</tr>
<tr>
<td>C33</td>
<td>Citrus sp.</td>
<td>Rutaceae</td>
<td>Brazil, MS</td>
<td>Pantanal</td>
<td></td>
</tr>
<tr>
<td>C34</td>
<td>Malpighia glabra</td>
<td>Malpighiaceae</td>
<td>Brazil, MS</td>
<td>Pantanal</td>
<td></td>
</tr>
<tr>
<td>C35</td>
<td>Coffea sp.</td>
<td>Rubiaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
<td></td>
</tr>
<tr>
<td>C36</td>
<td>Doxantha unguis-cati</td>
<td>Bignoniaceae</td>
<td>USA, AZ</td>
<td>Tucson</td>
<td></td>
</tr>
<tr>
<td>C37</td>
<td>Sterculia chichi</td>
<td>Sterculiaceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
<td></td>
</tr>
<tr>
<td>C38</td>
<td>Baccharis sp.</td>
<td>Asteraceae</td>
<td>Brazil, MG</td>
<td>Viçosa</td>
<td></td>
</tr>
<tr>
<td>C39</td>
<td>Phaseolus sp.</td>
<td>Leguminosea</td>
<td>Vietnam</td>
<td>O Mon</td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>Luecaena leucophala</td>
<td>Leguminosea</td>
<td>Netherlands</td>
<td>Arnhem</td>
<td></td>
</tr>
</tbody>
</table>

**Chapter 4**
Chapter 5

Male Function in the Asexual False Spider Mite
*Brevipalpus phoenicis*

Thomas V. M. Groot and Johannes A.J. Breeuwer

Abstract

In many asexual species males are occasionally found. If these males are capable of fertilizing the eggs of asexual females this could produce new clonal genotypes. In theory occasional sex and recombination can overcome most disadvantages of asexual reproduction and therefore occasional males may provide an explanation for the evolutionary success of asexual species. Here we studied the functionality of males in the asexual false spider mite, *Brevipalpus phoenicis* (Acari:Tenuipalpidae). Asexuality in this species is caused by the intracellular symbiont *Cardinium*. Males are regularly produced at low frequency, presumably when symbiont numbers are low, and these male readily mate. To test whether these matings result in fertilized eggs, reciprocal crosses were performed between two related isofemale lines with different microsatellite alleles. Naturally occurring males and males that result from antibiotic treated females were mated to normal infected females and antibiotic treated females. Female offspring was genotyped and the presence of the paternal alleles was tested. The offspring from all crosses showed only maternal genotypes. There are two possible explanations for this result; either males produce no sperm or fail to inseminate the female despite copulation, or females did not fertilize the eggs. We argue that the fact that apparently nonfunctional males are still regularly produced probably is an artifact of a sexual past, and therefore that origin of the asexuality in this species is relatively recent. Finally, we argue that the reason why females do not fertilize their eggs is because of a functional virginity mutation. Simultaneously, we propose an improved explanation for the relatively high fitness of this mutation.
Chapter 5

Introduction

A common feature of many asexual species is that females occasionally produce males, the so called spanandry (Lynch 1984). There are several possible explanations for the presence of these males that are not mutually exclusive: the production of males is adaptive, it is impossible to produce females without occasional males being produced as well, or the production of males is a remnant of past sexual reproduction and will cease somewhere in the future. With respect to the first possibility, several theoretical studies have shown that, if occasional males are capable of fertilizing the asexual females, this provides the asexual species with a valuable source of genetic variation (Green and Noakes 1995; Hadany and Feldman 2005; Hurst and Peck 1996). This genetic variation might enable the species to delay, or escape from, the disadvantages of asexual reproduction such as Muller’s ratchet (Muller 1964) and the low rates of adaptive evolution (Otto and Barton 1997). Therefore, occasional males might explain the persistence of an asexual species. Despite the apparent benefits that occasional sex can have, it has only been encountered in few animal species (Belshaw et al. 1999; D'Souza et al. 2004; Schneider et al. 2003).

A special case of asexual reproduction in arthropods is parthenogenesis induced by bacterial symbionts (Huigens and Stouthamer 2003). These symbionts are vertically transmitted by females only, and male hosts are a dead-end to them. To enhance their transmission, the symbionts manipulate the reproduction of the host and induce parthenogenesis. Parthenogenesis ensures that only females are produced and therefore that symbiont transmission is maximal. Although for this type of parthenogenesis the potential benefits of occasional sex and recombination to the host are evident, it is not clear whether the increased genetic variation is also beneficial to the symbiont. On one hand, the symbiont might benefit from the increased fitness of its host and therefore the symbiont should allow, or even promote, occasional sex. On the other hand, if the symbiont has specific adaptations to its host’s genotype, recombination of the host genotype may lead to maladaptions. In the most extreme case, the host might acquire genes that render complete resistance to the symbiont. In such cases, symbionts that prevent recombination of the host genotype are selected for.

One type of symbiont that induces parthenogenesis in a variety of arthropod species is *Wolbachia*. In most of these species, crosses between females and occasional males or males from closely related sexual species or populations, do not result in recombinant offspring (Arakaki et al. 2001; De Barro and Hart 2001; Pannebakker et al. 2005; Weeks and Breeuwer...
Male Function in *Brevialpus phoenicis* (Acari: Tenuipalpidae) occasional males have frequently been reported (Chigira and Miura 2005; Haramoto 1969; Kennedy 1995; Nagesha Chandra and Channabasavanna 1974; Oomen 1982; Pijnacker et al. 1980; Razoux Schultz 1961). Weeks et al. (2001) showed that asexuality in *B. phoenicis* is caused by an intracellular bacterium which was later named *Cardinium* (Zchori-Fein and Perlman 2004) that is unrelated to *Wolbachia*, but is similar in its phenotypic effects. Sexual species of the family Tenuipalpidae are arrhenotokous, where females develop from fertilized diploid eggs and males from unfertilized haploid eggs, and therefore the ancestral sexual mode of reproduction in asexual *Brevialpus* is probably arrhenotoky (Helle et al. 1980). The *Cardinium* in *B. phoenicis* induce asexuality by feminizing the unfertilized eggs resulting in haploid females (Weeks et al. 2001). Males account for up to 6 % of adult individuals in some of our laboratory cultures (Groot and Breeuwer in press) which indicates that symbiont transmission or expression is not complete. Although the occasional males in asexual mites are generally believed to be non-functional (Norton et al. 1993), *B. phoenicis* males do show stereotypic sexual behavior and copulation takes place readily, suggesting that they could be functional (TVM Groot, pers. obs.). Examples of typical male behavior displayed by these males are mate guarding behavior and fully functional mating behavior that results in copulation (Fig. 1a and 1b) [see Cone (1985) for a description of male sexual behavior of the related spider mites (genus *Tetranychus*).

Although adaptation is believed to be slow in species without sexual reproduction (Colegrave 2002; Doncaster et al. 2000), *B. phoenicis* is found in many different environments. It occurs worldwide throughout the tropics and subtropics and is highly polyphagous having been reported from 486 plant species from 114 different plant families (Childers et al. 2003b). In addition, it rapidly develops pesticide resistance (Campos and Omoto 2002; Omoto 1998).

If males are not functional, selection for the maintenance of male sexual traits will be relaxed. Genes that control sexual behavior will therefore accumulate deleterious mutations and the behavior will disappear (Carson et al. 1982; Muller 1949).
species do show typical sexual behavior could suggest that they are still functional. If they are, occasional sex might be the main explanation for the evolutionary success of this asexual species. Alternatively, if the males are not functional, the presence of typical sexual behavior would suggest that asexuality is relatively recent and it has not yet been lost due to deleterious mutations.

Here we present experiments on the functionality of males in B. phoenicis. We crossed mites from closely related isofemale lines and tested whether the offspring contained both maternal and paternal genotypes. Additionally, we tested if the Cardinium symbiont had an effect on the fertilization rate by eliminating the symbiont from the females by using antibiotic treatments.

Material and Methods

Origin of Brevipalpus phoenicis isofemale lines and rearing conditions

The B. phoenicis isofemale lines used in this study originated from collections made in citrus orchards in the Brazilian state of São Paulo in 1999. Isofemale lines have been maintained in the laboratory for at least four years prior to the first experiments. Mites were reared on detached leaves of the common bean (Phaseolus vulgaris) placed on soaked cotton wool. The rearing of the mites and all experiments were conducted in the same climate room with constant temperature (24 °C), relative humidity (70 %) and photoperiod (L 16 : D 8). In this study, two isofemale lines were used with identical mitochondrial COI haplotypes, but different multilocus microsatellite genotypes which enabled paternity analysis. Both lines were infected with the same symbiont based on identical gyrB sequences.

Crosses

To test whether mated B. phoenicis females fertilize their eggs, reciprocal crosses were performed. The males used in the crosses were either occasional males that occur naturally in these cultures [males are present in cultures of B. phoenicis with an abundance of maximally 6% (Groot and Breeuwer in press)] or artificial males that were obtained from antibiotic treated females. Four different types of crosses were made: 1) to study the natural situation, untreated females were mated with occasional males, 2) to study if the symbiont might have deleterious effects on male functionality, untreated females were mated with artificial males, 3) to study if the symbiont influences the acceptation of sperm by the female, antibiotic treated females were mated with occasional males, and 4) to study the effect
of the symbiont on both partners simultaneously, antibiotic treated females were mated with artificial males. Finally, as a control to crosses 3 and 4, additional groups of antibiotic treated females were left unmated.

Figure 1. Examples of typical male behavior. Figure 1a shows a pair of *Brevipalpus phoenicis* mites in *copula*. The male is located underneath the female, holding her with his two frontal pairs of legs. He has his abdomen bend over his back, with the tip touching the tip of the female’s abdomen. Figure 1b shows typical guarding behavior: males guard females in their final molting stage (teleiochrysalid) before they molt into adults. This way the male ensures that he is the first to mate when she matures. The male sits on top of the female. The individual on the right is an adult female.
Male mites mate preferentially with females immediately after emergence from the teleiochrysalid stage (the last molting stage, in-between deutonymph and adult). As a matter of fact, males generally guard teleiochrysalid females to ensure that they are the first to mate with the female. Therefore, mass matings were set-up with deutonymph or teleiochrysalid females and adult males with sex-ratios of female : male between 2 : 1 and 1 : 1. For crosses 1 and 2, about 50 teleiochrysalid females were placed on a detached leaves together with males. Crosses 3 and 4 were initiated with 36 treated females in the deutonymph or teleiochrysalid stage. The unmated control group consisted of the same number of females.

**Antibiotic treatment**
Artificial males for crosses 2 and 4 were obtained from groups of five to ten adult females that were placed on leaf discs (24 mm diameter) of common bean floating on cotton wool soaked in a 0.5% tetracycline solution. After two days, surviving females were transferred and maintained on clean leaves as described under normal rearing conditions above. The sons produced by these treated females were used in crosses 2 and 4. Antibiotic treated females for crosses 3 and 4 received the same treatment at the deutonymph stage, which is the last nymphal stage before adulthood.

**Genotyping**
To test whether the offspring produced after the matings developed from fertilized eggs, F1 females were genotyped at two microsatellite loci. If females fertilized their eggs, we expected to find heterozygous offspring with both the maternal and the paternal alleles. Alternatively, if they did not fertilize their eggs we expected to find the maternal genotype only. DNA extraction, PCR amplification, and fragment analysis were conducted as described in (Groot et al. 2005). The two microsatellite loci used were Brev05 (Weeks et al. 2001) and the undescribed Brev10 with primer sequences: Brev10-F 5’- TTT CCA CAA TCT TCT GGG -3’ and Brev10-R 5’- GTA CAA GTC GGT CCA ATG AG -3’. For crosses 3 and 4, every F1 females was genotyped. In crosses 1 and 2 the number of female offspring was too large to genotype them all. Instead, offspring was sampled at regular intervals throughout the entire female’s reproductive life.
Table 1. Genotypes of F1 *Brevipalpus phoenicis* females from crosses 1 and 2 using untreated females. In cross 1 males were used that occur naturally in the cultures, in cross 2 males were used that were produced after females have received an antibiotic treatment. The F1 sex ratio was 98.9% and 98.3% female for crosses 1 and 2 respectively.

<table>
<thead>
<tr>
<th>Parents (F x M)</th>
<th>Cross 1</th>
<th>Cross 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 2 x line 1</td>
<td>Maternal 192</td>
<td>Paternal 0</td>
</tr>
<tr>
<td>Line 1 x line 2</td>
<td>Maternal 326</td>
<td>Paternal 0</td>
</tr>
</tbody>
</table>

Results

Although no exact measurements were done on male behavior, no obvious differences in sexual activity were observed between occasional males and artificial males. Both types of males performed guarding behavior and copulations were readily observed in all four crosses. Nevertheless, in each cross all F1 females, 989 in total, had both maternal alleles (Tables 1 and 2) and none had the paternal alleles. We therefore conclude that the crosses were ineffective in producing fertilized eggs, despite the frequently observed copulations. As the different antibiotic treatments had no effect on fertilization rates, it is unlikely that the ineffectiveness of these crosses is caused by direct action of the symbiont.

In the crosses 1 and 2, respectively 98.9 and 98.3 percent of the F1 individuals were female. These values fall within the range of what is generally observed for this species under laboratory conditions (chapter 1). In the crosses 3 and 4, the sex ratio produced by the mated groups, 41% females on average, appeared to be lower than the sex ratio produced by the control group which was on average 45% females. However, this difference was not significant (one way ANOVA, p = 0.14, Table 2). If the mating would have resulted in fertilized eggs, the percentage females would have been higher in the mated treatments. The fact that this is not the case again showed that the crosses are ineffective.

In all crosses, females of line 1 produced more offspring than line 2. This is probably caused by a difference in how well the lines accept bean leaves as an alternative food source. Although both lines originated from citrus, line 1 females produced more eggs on bean leaves and lived longer because they less frequently walked from the leaf (mites that leave the bean leaf die from drowning in the soaked cotton wool). In crosses 3 and 4 the control groups were more productive than the mated groups. Summed for both lines, the unmated individuals produced a total of 335 offspring, whereas the mated groups produced on average 142.5 offspring. This
difference may be caused by the fact that the females in the mated groups were frequently harassed by males attempting to mate.

### Table 2

Per cent females and genotypes (females only) of F1 from crosses 3 and 4 using antibiotic treated females. In cross 3 males were used that occur naturally in the cultures, in cross 4 males were used that are produced after females have received an antibiotic treatment. An equally seized group of females were left unmated as a control for the sex-ratio.

<table>
<thead>
<tr>
<th>Parents (F x M)</th>
<th>% Females</th>
<th>Genotype</th>
<th>% Females</th>
<th>Genotype</th>
<th>% Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cross 3</td>
<td></td>
<td>Cross 4</td>
</tr>
<tr>
<td>Line 2 x line 1</td>
<td>40%</td>
<td>12 0</td>
<td>47%</td>
<td>8 0</td>
<td>47%</td>
</tr>
<tr>
<td>Line 1 x line 2</td>
<td>33%</td>
<td>37 0</td>
<td>45%</td>
<td>57 0</td>
<td>42%</td>
</tr>
</tbody>
</table>

### Discussion

Results show that the matings between these two closely related *B. phoenicis* clones do not result in offspring bearing paternal genotypes. Therefore, it is highly unlikely that the presence of males and the retention of typical male behavior is an adaptive trait to gain the benefits that occasional sex has in the production of clonal variation. The presence of males might still be adaptive if males would be able to fertilize females of related sexual species. We have not tested this possibility, but we have never observed sympatric sexual and asexual *Brevipalpus* species despite extensive sampling of many populations in Southern Brazil (but see Gonzalez 1975).

There are two possible non-adaptive reasons that explain why males are still produced. Either it is mechanistically impossible for these mites to produce 100% females, or the production of males is a remnant of a recent sexual past. An argument against a structural impossibility to produce 100% females is the observation that no males were produced in two *B. obovatus* isofemale lines that are infected with, and feminized by, exactly the same *Cardinium* that infects the *B. phoenicis* isofemale lines used in this study (Groot and Breeuwer in press). Apparently, *B. obovatus* is capable to produce 100% females with the same symbiont. In favor of the second reason, the same report also showed that males are only produced by very young females. It is possible that in very young *B. phoenicis* females the number of bacteria is too low to effectively feminize all offspring. The fact that the young *B. obovatus* we studied did not produce males indicates that vertical transmission is more effective in this species, perhaps because it has been asexual for a longer period of time than *B. phoenicis*. The observation that *B. phoenicis* males still display typical male behavior too favors the explanation of males as a remnant of a relatively recent sexual past. As soon
as males lose their function, purifying selection on the maintenance of male specific behavior will be released and this behavior will eventually be lost. The fact that it has not been lost yet, thus indicates that asexuality in *B. phoenicis* probably originated relatively recently. The reasoning above leads to the expectation that *B. obovatus* males that can be produced with antibiotic treatments will display less sexual behavior than *B. phoenicis* males. This prediction has not been tested yet.

Crosses 3 and 4 were aimed at studying the possibility that the Cardinium bacteria somehow prevent or inhibit egg fertilization. Table 2 shows that in these crosses and their controls females were still produced and therefore that Cardinium was not entirely eliminated. However, the fact that more than half the offspring was male indicates that symbiont numbers were severely reduced to a level that they often were unable to induce feminization. Although it is unknown whether this implies that they are also unable to inhibit fertilization, it is unlikely that the symbiont would inhibit the fertilization of an egg that it cannot feminize, because it would then result in a male which is a dead-end for the symbiont. Considering the arguments above, we conclude from crosses 3 and 4 that the failure to fertilize the eggs is most likely not due to by the symbiont, but rather is a characteristic of the mite.

Which of the two sexes is responsible for the failure to reproduce sexually? Pijnacker et al. (1981) have studied the production of sperm by males of the related *B. obovatus* using electron microscopy. They reported that sperm production was low compared to the spider mite *Tetranychus urticae*, and that the studied individuals were unable to transfer sperm. However, lifetime egg production in *Brevipalpus* is naturally much smaller than in *Tetranychus* and therefore one might expect that sperm production is less too. In addition, the fact that *B. obovatus* males are unable to transfer sperm does not necessarily mean that the same applies for *B. phoenicis*. The observation that *B. phoenicis* produces more males than *B. obovatus* (Groot and Breeuwer in press) and probably has become asexual more recently (see above), both indicate that male function might be less degraded in *B. phoenicis* than it is in *B. obovatus*. Although the possibility that both sexes are unable to reproduce sexually remains, we will show below that females are likely to lose their sexual functions before males do.

Despite the potential advantages of occasional sex, our study as well as others have shown that females of species where asexuality is induced by bacterial symbionts have lost the ability to reproduce sexually (Arakaki et al. 2001; Arakaki et al. 2000; De Barro and Hart 2001; Jeong and Stouthamer 2005; Pannenbakker et al. 2005; Pijls et al. 1996; Weeks and Breeuwer 2001; Zchori-Fein et al. 1994; Zchori-Fein et al. 1992). This suggests that there
could be other, more powerful, selective forces against the maintenance of sexual reproduction.

The ability to reproduce sexually might be lost due to random mutations. When reproduction becomes fully asexual, traits that are associated with sexual reproduction are no longer maintained by natural selection (Carson et al. 1982; Muller 1949). As the genes encoding these traits gradually accumulate random mutations, the species might become locked in asexuality, unable to return to sexual reproduction even if the symbiont is lost. Besides degradation of the traits involved in sexual reproduction due to mutation accumulation, their may also be active selection against these traits. This will mainly affect females and not males, resulting in a more rapid decay of female than male function. Pijls et al. (1996) suggested that female sexual traits are selected against because they are costly when expressed in individuals that reproduce asexually. Males however are not produced, or at least are not required for reproduction. Therefore, male function is not selected against but will decay as a neutral marker, and thus more slowly than female function does.

Huigens and Stouthamer (2003) suggested an alternative explanation for the difference in decay rate between the two sexes which is applicable to cases where parthenogenesis is induced by a bacterial symbiont. Recognizing that all known cases of parthenogenesis inducing bacteria occur in arrenhotokous species, they introduced the ‘functional virginity mutation’ (FVM). Females that carry a FVM do not fertilize their eggs, which in the absence of the symbiont results in the production of sons. When a parthenogenesis inducing bacterium spreads through the population, the population becomes female-biased. If asexual females do fertilize their eggs, sexual females can gain a higher fitness through the production of sons instead of females. Sons will have a higher fitness than daughters because the sons can mate with both sexual and the asexual females. In this situation a FVM that causes asexual females to produce sons, even if sperm is available, will have a selective advantage and spread. The mutation can spread from the sexual into the asexual population when sexual males mate with asexual females, but it can also move back into the sexual populations when infected females produce uninfected offspring. In the end, the mutation goes to fixation and the sexual population is lost when all asexual females have produced sons only. However, this explanation depends on the existence of mixed populations containing both sexual and asexual females, which have not been found in B. phoenicis. What if the infection rapidly goes to fixation before a functional virginity mutation arises?

We argue that there is another fitness advantage for a FVM. This advantage is equivalent to what is referred to as the ‘cost of genome
dilution’ (Williams 1975). This is the cost of sex paid by sexual species since their offspring shares only half their genome with each of their parents. Sexual species cannot escape this cost, because they have to reduce their genome into a gamete that subsequently fuses with another gamete to develop into a new individual. In asexual species the fusion of gametes is not necessary for reproduction. Therefore, asexual females can escape the cost of genome dilution by not fertilizing their eggs. A daughter from an unfertilized egg shares all her genes with her mother, whereas a daughter from a fertilized egg shares only half her genes with her mother. From the point of view of the mothers’ genes, this means that the fitness from each egg is doubled when fertilization is prevented. This way, the FVM has a large fitness benefit over alleles of the same gene that do not prevent fertilization, and therefore it will spread through the population. This explanation does not require the presence of sexual females, but does require that some males are present, which is typically the case in *B. phoenicis*. In addition, in most species where asexuality is induced by bacterial symbiont males are produced at low frequencies. At the same time, if occasional males are also produced by females that already carry the FVM, they will inseminate females that do not yet carry the FVM thereby further increasing the rate at which the FVM will spread through the population. We have run simulations that showed that this kind of FVM will quickly go to fixation, even if the transmission efficiency of the symbiont is nearly 100%. Only when the transmission efficiency is exactly 100%, the mutation will not spread. In this particular case there are no males, and therefore a FVM will have no effect because females can not fertilize their eggs anyway.

In conclusion, we have shown that copulations between males and asexual females of *B. phoenicis* do not lead to recombinant genotypes, perhaps because a functional virginity mutation has spread to fixation. Thus, occasional sexual reproduction is not a likely explanation for clonal diversity in these mites. The observation that males are still relatively common and behavioral normal has lead to the hypothesis that asexuality in this species is of relatively recent origin.

**Acknowledgements**

We thank Jan van Arkel who skillfully produced the pictures of the minute mites in Fig. 1. We also thank Martijn Egas for his help in modeling the effects of a virginity mutation. Steph Menken provided valuable comments that improved earlier drafts of this manuscript. This study was supported by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), grant number W89-141.
Chapter 6
Recombination and Intragenomic Variation in Asexual Brevipalpus Mites

Thomas V. M. Groot, Johannes A.J. Breeuwer and Steph B.J. Menken

Abstract

Although there are various costs associated with sexual reproduction, most animal species reproduce sexually. To explain this paradox of sex, various theories have been proposed that describe advantages of sexual reproduction. Recently, an increasing number of studies have shown that sexual reproduction is indeed advantageous, prompting the question why asexual species still exist. Asexual species may exist because new asexual lineages are constantly produced from the sexual ancestor. Alternatively, asexual species may persist because they occasionally reproduce sexually. Both these explanations may apply for asexual Brevipalpus mites where asexuality is induced by intracellular Cardinium symbionts. To test if asexual Brevipalpus species have reproduced strictly clonally for a long period of time, we compared the phylogenetic signal inferred from mitochondrial COI sequences with the phylogenetic signal of nuclear AFLP fingerprints for 75 isofemale lines representing three different mite species. As an additional nuclear marker, ITS1 sequences were determined for 26 isofemale lines. The amount of intragenomic variation of ITS1 copies was surprisingly large which may be explained by reduced levels of gene conversion caused by the haploid state of the mites. At the species level mitochondrial and nuclear markers were incongruous indicating that recombination has occurred after the divergence of the species. Within B. phoenicis and infected B. obovatus both markers are incongruous suggesting that recombination has occurred recently. Therefore, the asexuality in these two species is of recent origin, or they still go through sexual reproduction occasionally. Within B. californicus and uninfected B. obovatus COI and AFLP produced congruent phylogenies indicating that these species have reproduced strictly asexually for a considerable period of time. The analysis of ITS1 sequences also suggested a strictly clonal history for B. californicus and the uninfected B. obovatus based on the presence of intragenomic ancestral polymorphisms that are maintained in the genomes. We conclude that asexuality has arisen independently in each species and at different times.
Introduction

Although sexual reproduction is costly (Crow 1999), 99% of all animal species reproduce sexually and only one percent asexually (White 1978). Several theories have been proposed to explain this paradox of sex by ascribing various advantages to sexual reproduction (Barton and Charlesworth 1998; Hurst and Peck 1996). Mathematical models have been widely applied to show that these advantages indeed can explain why sex is so common (Doncaster et al. 2000; Green and Noakes 1995; Hadany and Feldman 2005; Otto and Lenormand 2002; Otto and Nuismer 2004; Pound et al. 2004; Wilke 2004), especially if multiple advantages act simultaneously (West et al. 1999). Recently, a number of experimental studies have demonstrated that sexual reproduction is indeed advantageous (Butlin 2002; Colegrave 2002; Cutter and Payseur 2003; de Visser and Rozen 2005; Fischer and Schmid-Hempel 2005; Goddard et al. 2005; Grimberg and Zeyl 2005; Jokela et al. 2003; Paland and Lynch 2006; Rice 2002; Rice and Chippindale 2001; Xu 2004). With this evidence for sexual reproduction being more advantageous than asexual reproduction, one may wonder why asexual reproduction still exists.

There are two possible explanations for the persistence of asexual reproduction. The first explanation is that asexual species may still exist because new clonal lineages are continuously being produced from sexual ancestors. In the short run, a clonal lineage can be quite successful; it is equally well adapted and has the same mutational load as the sexual ancestor it originated from, but it does not have to pay the costs of sex. In the long run, however, the disadvantages of not having sex will accumulate, eventually driving the clonal lineage to extinction. Hence, the existence of asexual species may be the result of continuous production and extinction of short-lived asexual lineages. The second explanation is that asexual species may escape the disadvantages of asexual reproduction by having occasional sex. Mathematical models have shown that very limited amounts of sex and recombination are sufficient to overcome the disadvantages of asexual reproduction (Green and Noakes 1995; Hadany and Feldman 2005; Hurst and Peck 1996). In many asexual species males are produced at low frequency (Lynch 1984) that could enable this occasional sex. Alternatively, asexual females may mate with males from sexual populations or with males from related sexual species.

A special type of asexuality that is relatively common in certain groups of insects and mites is parthenogenesis induced by intracellular symbionts such as Wolbachia (Huigens and Stouthamer 2003) and Cardinium (Groot and Breeuwer in press; Weeks et al. 2001; Zchori-Fein et
In this specific type of asexual reproduction both explanations for the persistence of asexual reproduction may apply. New clonal lineages may continuously be produced when symbionts are transmitted horizontally to sexual individuals that become asexual upon infection. Based on comparisons of symbiont and host phylogenies horizontal transmission of *Wolbachia* and *Cardinium* infections is relatively common (Ahrens and Shoemaker 2005; Cordaux et al. 2004; Groot and Breeuwer chapter 4; Kittayapong et al. 2003; Werren et al. 1995a). Evidence that asexual lineages can have occasional sex comes from parthenogenesis inducing *Wolbachia* and *Trichogramma* wasps, where asexual infected females fertilize their eggs when sperm is available (Stouthamer and Kazmer 1994; Stouthamer and Luck 1993; Stouthamer et al. 1990).

The asexual species of the mite genus *Brevipalpus* are an example of symbiont induced parthenogens. The three most common asexual *Brevipalpus* species, *B. phoenicis*, *B. obovatus* and *B. californicus*, are widely distributed (Childers et al. 2003b). Each of these species occurs worldwide in sub-tropical and tropical areas. In addition, each species is found on hundreds of different host plant species, many of which are shared between the species (Childers et al. 2003b). In *B. phoenicis*, *B. californicus*, and some *B. obovatus* clones, asexuality is induced by the intracellular bacterium *Cardinium* (Zchori-Fein et al. 2004). The ancestral sexual mode of reproduction is most likely arrhenotoky, where fertilized eggs develop into diploid females and unfertilized eggs develop into haploid males. In case of an infection, the *Cardinium* feminizes the unfertilized eggs which then develop into haploid females. When all the individuals in the populations are infected, the species is entirely haploid (Weeks et al. 2001). Some *B. obovatus* clones are not infected by a bacterium and in these clones asexuality appears to have a genetic origin (Groot and Breeuwer in press). These uninfected asexual females are also haploid.

In *Brevipalpus*, there is no evidence that occasional sexual reproduction between asexual females and rare males plays an important role in the maintenance of asexual reproduction despite normal mating behavior (Groot and Breeuwer Chapter 5). However, it is possible that egg fertilization is extremely rare. Alternatively, conversion of sexuals into asexuals by horizontal (or infectious) transmission of *Cardinium* is also not a very likely mechanism. Asexual *Brevipalpus* species from a monophyletic group and closely related sexual species have not been found so far (Groot and Breeuwer Chapter 3). Thus sexual reproduction in *Brevipalpus* appears to be absent.

Phylogenetic comparison of mitochondrial and nuclear markers can be used to determine whether there has been long-term absence of sex and
recombination (Crease et al. 1989). If reproduction has been strictly asexual long enough for mutations to accumulate, both types of markers will show the same phylogenetic relations among individuals. In contrast, if recombination took place, both markers will show different phylogenies. This approach provided evidence that some parthenogenetic species, such as among aphids and parasitoid wasps, have occasional sex (Belshaw et al. 1999; Delmotte et al. 2001, Schneider et al. 2003). The ostracod *Cyprinotus incongruens*, on the other hand, has probably been strictly asexual for five million years based on congruence between nuclear and mitochondrial markers (Chaplin and Hebert 1997).

In this study we seek answers to the following questions: Is there evidence for long-term absence of sex and recombination in three asexual *Brevipalpus* species, and is the evolutionary pattern the same for all three species? We do so by comparing the phylogenetic signal inferred from a mitochondrial marker (*COI* sequences) with that from two nuclear markers (AFLPs and ITS1 sequences).

### Table 1. Overview of the *Brevipalpus* isofemale lines used in this study. The last column shows for which isofemale lines ITS1 was sequenced. Names of Brazilian states are abbreviated as MG = Minas Gerais and SP = São Paulo.

<table>
<thead>
<tr>
<th>Isofemale line</th>
<th>Clade</th>
<th>Hostplant species</th>
<th>Location</th>
<th>Symbiont</th>
<th>ITS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>B. phoenicis</em></td>
<td>Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2</td>
<td><em>B. phoenicis</em></td>
<td>Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td><em>B. phoenicis</em></td>
<td><em>Carica papaya</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>4</td>
<td><em>B. phoenicis</em></td>
<td><em>Hibiscus rosa-sinensis</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>5</td>
<td><em>B. phoenicis</em></td>
<td>Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td><em>B. phoenicis</em></td>
<td>Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td><em>B. phoenicis</em></td>
<td><em>Hibiscus rosa-sinensis</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>8</td>
<td><em>B. phoenicis</em></td>
<td><em>Carica papaya</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>9</td>
<td><em>B. phoenicis</em></td>
<td><em>Carica papaya</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><em>B. phoenicis</em></td>
<td>Citrus sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td><em>B. phoenicis</em></td>
<td>Citrus sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td><em>B. phoenicis</em></td>
<td>Citrus sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td><em>B. phoenicis</em></td>
<td><em>Hibiscus rosa-sinensis</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>14</td>
<td><em>B. phoenicis</em></td>
<td>Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td><em>B. phoenicis</em></td>
<td><em>Hibiscus rosa-sinensis</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>16</td>
<td><em>B. phoenicis</em></td>
<td>Passiflora nigriadenia</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td><em>B. phoenicis</em></td>
<td><em>Hibiscus rosa-sinensis</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td><em>B. phoenicis</em></td>
<td><em>Hibiscus rosa-sinensis</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td><em>B. phoenicis</em></td>
<td>Passiflora nigriadenia</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td><em>B. phoenicis</em></td>
<td><em>Malpighia glabra</em></td>
<td>The Netherlands</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>21</td>
<td><em>B. phoenicis</em></td>
<td><em>Carica papaya</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>22</td>
<td><em>B. phoenicis</em></td>
<td><em>Carica papaya</em></td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>23</td>
<td><em>B. phoenicis</em></td>
<td><em>Persea americana</em></td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td><em>B. phoenicis</em></td>
<td><em>Persea americana</em></td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td><em>B. phoenicis</em></td>
<td><em>Thevetia peruviana</em></td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td><em>B. phoenicis</em></td>
<td><em>Terminalia ivorenensis</em></td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>No.</td>
<td>Host Species</td>
<td>Location</td>
<td>Recombination</td>
<td>Intragenomic Variation</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>------------------------------</td>
<td>----------------</td>
<td>---------------</td>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>B. phoenicis Pavonia multiflora</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>B. phoenicis Anthurium sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>B. phoenicis Monodora crispata</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>B. phoenicis Terminalia ivorenensis</td>
<td>The Netherlands</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>B. phoenicis Anthurium sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>B. phoenicis Malpighia sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>B. phoenicis Dioscorea sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>B. phoenicis Monodora crispata</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>B. phoenicis Strongylodon macrobotrys</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>B. phoenicis Strongylodon macrobotrys</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>B. phoenicis Inga sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>B. phoenicis Citrus sp.</td>
<td>Brazil, SP</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>B. phoenicis Citrus sp.</td>
<td>Brazil, SP</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>B. phoenicis Citrus sp.</td>
<td>Brazil, SP</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>B. phoenicis Citrus sp.</td>
<td>Brazil, SP</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>B. californicus Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>B. californicus Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>B. californicus Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>B. californicus Rhododendron sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>B. californicus Euphorbia xanthii</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>B. californicus Euphorbia xanthii</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>B. californicus Baccharis sarathroides</td>
<td>The Netherlands</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>B. californicus Baccharis sarathroides</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>B. californicus Parkinsonia aculeata</td>
<td>The Netherlands</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>B. californicus Thevetia peruviana</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>B. californicus Leucaena leucophala</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>B. obovatus + Carica papaya</td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>B. obovatus + Zingiber sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>B. obovatus + Zingiber sp.</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>B. obovatus + Hibiscus rosa-sinensis</td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>B. obovatus + Hibiscus rosa-sinensis</td>
<td>Brazil, MG</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>B. obovatus + Hibiscus rosa-sinensis</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>B. obovatus - Malpighia glabra</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>B. obovatus - Duranta erecta</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>B. obovatus - Strongylodon macrobotrys</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>B. obovatus - Duranta erecta</td>
<td>Brazil, MG</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>B. obovatus - Malpighia glabra</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>B. obovatus - Dioscorea sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>B. obovatus - Dioscorea sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>B. obovatus - Dioscorea sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>B. obovatus - Plumeria acuminate var.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>B. obovatus - Beaumontia grandiflora</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>B. obovatus - Strongylodon macrobotrys</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>B. obovatus - Citrus sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>B. obovatus - Citrus sp.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>B. obovatus - Strongylodon macrobotrys</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>B. obovatus - Duranta repens</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>B. obovatus - Plumeria acuminate var.</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>B. obovatus - Beaumontia grandiflora</td>
<td>The Netherlands</td>
<td>X</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Material and Methods

Sampling and production of isofemale lines

*Brevipalpus* mites were collected from six different host plant species in Brazil, in the states of São Paulo and Minas Gerais, and from 20 different host plant species in the Netherlands, in the tropical greenhouse of Burger’s Bush (Table 1). The collections from São Paulo were made in 1999, the collections from Minas Gerais and the Netherlands were made in 2004. It is not possible to identify *Brevipalpus* mites at the species level without fixing them on microscopic slides or genotyping. Given the broadly overlapping distribution ranges and host plant spectra, all field collections can be considered as being random samples in relation to mite species. From these field collections, isofemale lines were initiated by placing individual adult females on detached leaves of the common bean (*Phaseolus vulgaris*) placed on soaked cotton wool. All mites were kept in a climate room with constant temperature, relative humidity, and photoperiod (24°C, 70%, and 16L:8D). In this way, a total of 141 isofemale lines were produced. To assess the number of different genotypes that resulted from each field collection, all isofemale lines were genotyped at three microsatellite loci using methods described in Groot et al. (2005). Based on the microsatellite genotypes, a subset of the isofemale lines was selected for more detailed genotyping. For each field collection, isofemale lines were selected in such a way that all observed genotypes were represented at least once, and that no genotype was represented more than twice.

DNA extraction

From the selected 75 isofemale lines DNA was extracted using a modified CTAB extraction protocol (Doyle 1991). Per sample 15 adult females were crushed in 3 µl of CTAB buffer (2% CTAB w/v, 2% PVP-40 w/v in 100mM Tris-HCl [pH8], 20mM EDTA, and 1.42M NaCl) in a 0.5 ml tube. After crushing, another 97 µl CTAB buffer was added. Tubes were then vortexed for 10 seconds and incubated at 55 °C for 1 hour. Next, an equal volume of chloroform:iso-amyl-alcohol (24:1) was added and tubes were shaken for 5 minutes. DNA was precipitated in the presence of 1/10 volume 5M NaCl and 2 volumes ice-cold 96% ethanol. Tubes were incubated at -20 °C for 1 hour and then centrifuged at 15,800 g for 20 minutes at 4°C. All supernatant was removed and the DNA pellet was air dried overnight. The following day, DNA was eluted in 20 µl sterile water and stored at -20°C.
Mitochondrial genotyping: COI sequences
As mitochondrial marker, part of the gene for cytochrome oxidase subunit 1 (COI) was sequenced for all 75 isofemale lines. DNA was amplified using the primers 5'-TGA TTT TTT GGT CAC CCA GAA G-3' and 5'-TAC AGC TCC TAT AGA TAA AAC-3' (Navajas et al. 1996). In our Brevipalpus species these primers amplified a 410 bp fragment of the COI gene, excluding the primer annealing sites. PCR was performed in a total volume of 25 µl containing: 2.5 µl 10X Super Taq buffer (HT Biotechnology, Cambridge, U.K.), 1.25 µl bovine serum albumin (10 mg/ml), 1.5 µl MgCl₂ (25 mM) (additional to the buffer), 5 µl dNTP mix (1 mM of each nucleotide), 0.5 µl each primer (10 µM each), 0.2 µl of super Taq (5 u/µl) (HT Biotechnology), 12.55 µl water, and 1 µl of DNA extract. Cycling conditions were: 4 minutes at 94˚C, followed by 35 cycles of 1 minute at 94˚C, 1 minute at 54˚C and 1 minute at 72˚C, concluded with a final extension at 72˚C for 4 minutes. Products (2.5 µl) were checked by electrophoresis in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0) on 1% agarose gels stained with ethidium bromide. Prior to sequencing, PCR products were purified using the DNA extraction kit (Fermentas, St. Leon-Rot, Germany). Cleaned products were cycle-sequenced in both directions using the same primers as used in the amplification and the ABI PRISM BigDye Terminator Sequence Kit (version 1.1, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) according to the manufacturer's instructions but diluted 16 times. Sequences were run on an ABI 3700 automated DNA sequencer.

Screening for Cardinium infection
Each isofemale line was tested for infection with Cardinium symbionts with PCR using Cardinium-specific primers for the gyrase subunit B (gyrB) gene, 5'-GTT ACC GTA TAC CGA AAT GG-3' and 5'-TGC TTT CCG RGC MGC TTG-3' (Groot and Breeuwer chapter 4). PCR mix and cycling conditions were similar to the amplification of COI. The presence of an amplification product, and therefore the presence of Cardinium, was tested by electrophoresis of 2.5 µl of PCR product in 0.5X TBE on 1% agarose gels stained with ethidium bromide. Successful amplification results in a product of 786 bp, including the primer annealing sites.

Nuclear genotyping: AFLP fingerprints
As general nuclear marker AFLP fingerprints were made for all 75 isofemale lines. The restriction-ligation and pre-amplification steps were performed with the AFLP Template Preparation Kit (LI-COR, Westburg, The Netherlands). The manufacturer’s protocol was followed using 9 µl of
DNA extract. Three primer combinations were used in separate end-amplification reactions; Eco-AC in combination with Mse-CAC, Mse-CAT and Mse-CCA. End-amplifications were performed in 10 µl reactions containing: 1.0 µl 10X Super Taq buffer (HT Biotechnology), 2.0 µl dNTP mix (1 mM of each nucleotide), 0.5 µl Mse primer (50 ng/µl), 0.5 µl Eco primer (1 µM, IRD-700 labeled), 0.04 µl of super Taq (5 u/µl) (HT Biotechnology), 1.16 µl water, and 5.0 µl diluted pre-amplification product (60 times). Cycling conditions were: initial denaturation at 94˚C for 1 minute, then 12 cycles of denaturation at 94˚C for 30 seconds, annealing at 65˚C decreasing with 0.7˚C per cycle for 30 seconds and extension at 72˚C for 1 minute, followed by 24 cycles of denaturation at 94˚C for 30 seconds, annealing at 56˚C for 30 seconds and extension at 72˚C for 1 minute, concluded by an final extension at 72˚C for 5 minutes. Products were run on 6.5% denaturing polyacrylamide gels in a LI-COR DNA Sequencer (LI-COR) and presence (1) and absence of bands (0) were scored manually.

**Figure 1.** Maximum likelihood phylogram of *Brevipalpus COI* haplotypes. The tree is midpoint rooted. On the right the infection status of the haplotypes is given. The bar indicates the branch length representing 5 % maximum likelihood distance.
Nuclear genotyping: ITS1 sequences

In addition nuclear ribosomal internal transcribed spacer 1 (ITS1) sequences were determined for a subset of the isofemale lines (Table 1). These 26 isofemale lines were a representative sample from the mitochondrial phylogeny (Fig. 1). The ITS1 was amplified using the primers 5’- GTC GTA ACA AGG TTT CCG TAG G-3’ and 5’- TGG CTG CGT TCT TCA TCG-3’ (Hinomoto and Takafulji 2001) that prime within the 18S and 5.8S coding regions. The PCR were performed in a total volume of 25 µl containing: 2.5 µl 10X Super Taq buffer (HT Biotechnology), 5 µl dNTP mix (1 mM of each nucleotide), 0.5 µl each primer (10 µM each), 0.2 µl of super Taq (5 u/µl) (HT Biotechnology), 15.3 µl water, and 1 µl of DNA extract. Cycling conditions were: 4 minutes at 94˚C, followed by 35 cycles of 1 minute at 94˚C, 1 minute at 48˚C and 1 minute at 72˚C, concluded with a final extension at 72˚C for 4 minutes. The entire PCR products were loaded and run in 1X TAE (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0) on a 1% agarose gel, excised, and cleaned using the DNA Extraction Kit (Fermentas). The size of the amplicon varied between the *Brevipalpus* species and isofemale lines of *B. californius* consistently amplified two fragments which were excised and cleaned separately. The cleaned products were ligated and transformed using the pGEM-T Easy Vector System and JM109 competent cells (Promega, Madison WI, U.S.). To check whether plasmids contained an insert of the correct size, a PCR was performed using the universal M13 primers that prime on the plasmid at either side of the insert. After the bacteria had grown overnight, colonies were picked from the plates and suspended in 30 µl of water. The PCR was performed in 25 µl containing 2.5 µl 10X Super Taq buffer (HT Biotechnology), 2.5 µl dNTP mix (1 mM of each nucleotide), 0.25 µl each primer (10 µM each), 0.05 µl of super Taq (5 u/µl) (HT Biotechnology), 15.3 µl water and 8.5 µl of bacteria suspension. Cycling conditions were: 2 minutes at 94˚C, followed by 35 cycles of 45 seconds at 94˚C, 40 seconds at 55˚C and 45 seconds at 72˚C, concluded with a final extension at 72˚C for 2 minutes. Products (2.5 µl) were checked by electrophoreses on 1% agarose gels in 0.5X TBE buffer stained with ethidium bromide. Products of expected size were first cleaned using the method described by Boom et al. (1990) and then sequenced using the M13 primers and protocols as described for COI. For each *B. phoenicis* and *B. obovatus* isofemale line eight clones were sequenced, and for each *B. californicus* isofemale line eight clones were sequenced for either length fragment.
DNA data analyses
All obtained sequences were edited in BioEdit (www.mbio.ncsu.edu/bioedit). Sequence alignments were created using ClustalX (Thompson et al. 1994) with occasional manual adjustment for ITS1. A maximum likelihood tree was constructed based on the COI sequences. The optimal model was selected using Modeltest 3.6 (Posada and Crandall 2001) and PAUP version 3.10b (Swofford 1998). Because COI is a protein coding gene, it was investigated whether the likelihood of the model could be improved by allowing separate rates for the three codon positions (Shapiro et al. 2006). Under the selected model, parameters and tree topology were optimized using the successive approximations approach (Sullivan et al. 2005). Bootstrap support was assessed by performing a Neighbor joining bootstrap (10,000 replicates) where the distances between sequences were calculated according to the selected maximum likelihood model.

For the AFLP data, distances were calculated from the 1 and 0 matrix using the distances for restriction sites by (Nei and Li 1979) implemented in PAUP. These distances were used in UPGMA clustering. Support for the obtained clustering was obtained from 10,000 bootstrap replicates.

Per isofemale line the eight ITS1 clones (or seven for isofemale lines 48 and 57) were compared to see if they contained variation. Each different ITS1 copy was labeled with the isofemale line number, a specific roman number, and the number of times it occurred within that isofemale line. Clones were considered different copies only if they differed at more than two nucleotide positions based on a PCR and cloning error rate of 3x10^{-3} (Peijnenburg et al. 2004). This was done to prevent that minor changes induced by PCR and cloning errors would result in different copies. Therefore, our estimate of the variation within individuals is a conservative estimate. The positions of the 18S and 5.8S were determined by comparing the copies from all species and all subsequent analyses were performed on the ITS1 only. The spacer sequences were too divergent to compile a single alignment for all isofemale lines. Instead, four separate alignments were created for B. phoenicis, B. californicus, B. obovatus with symbiont, and B. obovatus without symbiont. Within the alignments all indels were reduced to a single position and gaps were treated as a fifth base. The large indel in the B. californicus isofemale lines was treated similarly. Dissimilarity (D) was determined in PAUP and the average D among the ITS1 copies within each isofemale line was computed. The average D among the long ITS1 copies of B. californicus was calculated without removing the indel. Neighbor joining trees were constructed in PAUP and clade support was determined from 10,000 bootstrap replicates.
Figure 2. Phylogram based on UPGMA clustering of the AFLP data. The + and – signs with *B. obovatus* indicate the infected and uninfected isofemale lines, respectively. The bar indicates the branch length representing 5 % Nei & Li distance.
Results

**COI**
The 75 isofemale lines contained a total of 17 different COI haplotypes. Of these, seven were retrieved from a single isofemale line and ten were found more than once. The model used for maximum likelihood tree estimation was a GTR model with separate rates for the three codon positions. The resulting tree contained three distinct clades corresponding with *B. phoenicis*, *B. californicus* and *B. obovatus* (Fig. 1). *Brevipalpus phoenicis* was the most abundant species with 41 isofemale lines and ten haplotypes. The least common species was *B. californicus* with 11 lines representing just two haplotypes, and intermediate in abundance was *B. obovatus* with 23 isofemale lines representing five haplotypes. The *B. obovatus* clade was split in two separate clades corresponding with infection status; the clade representing isofemale lines 53 to 58 was infected with *Cardinium*, whereas the remaining isofemale lines 59 to 75 were uninfected. All *B. phoenicis* and *B. californicus* isofemale lines were infected with *Cardinium*.

**AFLP**
The three primer combinations used yielded a total of 148 markers. The UPGMA tree based on the distance matrix contained four major clusters (Fig. 2). Two clusters grouped all *B. phoenicis* and all *B. californicus* isofemale lines. The infected and uninfected *B. obovatus* isofemale lines clustered separately in the remaining two clusters. Remarkably, *B. obovatus* was not monophyletic; all the infected isofemale lines were in a single cluster that was sister to *B. phoenicis*, whereas all the uninfected isofemale lines were in a separate cluster that was sister to *B. californicus*.

**COI-AFLP comparison**
To test if reproduction has been strictly clonal the phylogenetic relationships inferred from the mitochondrial COI sequences and the nuclear AFLP fingerprints were compared. Both markers were expected to show similar phylogenies if reproduction has been strictly clonal, whereas incongruities were taken as evidence for recombination. To prevent that inferences were made on weakly supported relationships, this comparison was made on majority rule consensus trees from the bootstrap analyses (Fig. 3). At a higher taxonomic level, both markers were congruent in assigning all isofemale lines to the same of each of the four clades, including the three *Brevipalpus* species plus the infected and uninfected *B. obovatus* as separate clades. Thus the clades were well defined. The relationships among these four clades, however, were different; based on the mitochondrial marker
Figure 3. Majority consensus cladograms of both the mitochondrial COI and nuclear AFLP fingerprints. Lines between the two trees connect individual isofemale lines. The branches of the four different clades have been given different colors. The + and – signs with *B. obovatus* indicate the infected and uninfected isofemale lines, respectively.
B. californicus was sister to B. phoenicis, whereas based on the AFLP phylogeny the infected B. obovatus was sister to B. phoenicis (crossing lines in Fig. 3). This difference between mitochondrial and nuclear markers suggests that the divergence of the clades did not take place under strict asexuality.

At a lower taxonomic level the degree of congruence within the clades was variable. Within the clades of B. californicus and uninfected B. obovatus, the phylogenies inferred from both markers were congruent, suggesting that recombination has been absent in these clades. Within the clade of infected B. obovatus there was an incongruity; isofemale lines 55, 56, 57, and 58, were grouped together based on the AFLP markers but had two different mitochondrial haplotypes. Although bootstrap values were low, this may indicate recombination between clones of this species. Incongruities supported by higher bootstrap values were observed within the B. phoenicis clade. A number of isofemale lines had markedly different relations based on COI and AFLP. For example, isofemale line 02 shared its COI haplotype with isofemale lines 03 to 05 but clustered with isofemale line 01 based on AFLP. Another clear example was isofemale line 14 that had the same mitochondrial haplotype as isofemale lines 15 to 18 but was placed in a different group based on AFLP. These incongruities suggest that recombination has occurred several times within the B. phoenicis clade.

ITS1 sequences
In contrast to limited COI sequence variation, the ITS1 sequences were highly different for the various clades. In addition, the ITS1 sequences retrieved from most isofemale lines also revealed extensive intra-individual variation between copies of the multicopy ITS1. In the alignment of all cloned PCR products, only the 18S and 5.8S parts flanking ITS1 could be aligned, for all other positions homology could not be ascertained. The 18S + 5.8S alignment revealed four clear groups that were congruent with the four clades. Therefore, the ITS1 alignments and further analyses were made for each species separately.

Brevipalpus phoenicis
The length of the ITS1 in B. phoenicis varied between 806 and 813 bp. The number of different ITS1 copies retrieved from a single isofemale line varied from one (all clones were similar) to seven (Table 2). The neighbor joining tree contained three groups (Fig. 4). In the lower two groups of the tree (copies 04 II to 13 II), all ITS1 copies from a within isofemale lines were more similar to each other than to copies from other isofemale lines. In other words, ITS1 copies within isofemale lines are monophyletic.
Table 2. Diversity of ITS1 sequence types within isofemale lines. Frequency of types denotes the number of times the respective types were encountered, for example 7,1 indicates that type I was encountered 7 times and type II once. The average \( D \) between types within an isofemale line was calculated not taking into account the number of times each type occurred. An * indicates isofemale lines with only seven clones sequenced. For \( B. \) californicus the number and frequency of types are given for the long fragment first and separated from the same values for the short fragment by a slash. The + and – sign with \( B. \) obovatus indicate infected and uninfected isofemale lines, respectively.

<table>
<thead>
<tr>
<th>Isofemale line</th>
<th>Clade</th>
<th>Number of different ITS1 copies</th>
<th>Frequency of different ITS1 copies</th>
<th>Average ( D ) between within long within short</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>( B. ) phoenicus</td>
<td>2</td>
<td>7,1</td>
<td>0.004</td>
</tr>
<tr>
<td>03</td>
<td>( B. ) phoenicus</td>
<td>2</td>
<td>7,1</td>
<td>0.005</td>
</tr>
<tr>
<td>04</td>
<td>( B. ) phoenicus</td>
<td>2</td>
<td>7,1</td>
<td>0.009</td>
</tr>
<tr>
<td>07</td>
<td>( B. ) phoenicus</td>
<td>1</td>
<td>8</td>
<td>0.001</td>
</tr>
<tr>
<td>08</td>
<td>( B. ) phoenicus</td>
<td>3</td>
<td>4.3,1</td>
<td>0.023</td>
</tr>
<tr>
<td>13</td>
<td>( B. ) phoenicus</td>
<td>2</td>
<td>7,1</td>
<td>0.004</td>
</tr>
<tr>
<td>15</td>
<td>( B. ) phoenicus</td>
<td>2</td>
<td>7,1</td>
<td>0.003</td>
</tr>
<tr>
<td>20</td>
<td>( B. ) phoenicus</td>
<td>2</td>
<td>6.2</td>
<td>0.034</td>
</tr>
<tr>
<td>21</td>
<td>( B. ) phoenicus</td>
<td>1</td>
<td>8</td>
<td>0.000</td>
</tr>
<tr>
<td>22</td>
<td>( B. ) phoenicus</td>
<td>3</td>
<td>6.1,1</td>
<td>0.022</td>
</tr>
<tr>
<td>30</td>
<td>( B. ) phoenicus</td>
<td>2</td>
<td>7,1</td>
<td>0.006</td>
</tr>
<tr>
<td>38</td>
<td>( B. ) phoenicus</td>
<td>7</td>
<td>1,1,1,1,2,1,1</td>
<td>0.021</td>
</tr>
<tr>
<td>40</td>
<td>( B. ) phoenicus</td>
<td>6</td>
<td>1,2,1,2,1,1</td>
<td>0.029</td>
</tr>
<tr>
<td>42</td>
<td>( B. ) californicus</td>
<td>3 / 1</td>
<td>2.5,1,1 / 8</td>
<td>0.019</td>
</tr>
<tr>
<td>44</td>
<td>( B. ) californicus</td>
<td>1 / 1</td>
<td>8 / 8</td>
<td>0.028</td>
</tr>
<tr>
<td>48</td>
<td>( B. ) californicus</td>
<td>1 / 3</td>
<td>8.4,1,2,1*</td>
<td>0.025</td>
</tr>
<tr>
<td>50</td>
<td>( B. ) californicus</td>
<td>3 / 4</td>
<td>6.1,1,1,1</td>
<td>0.025</td>
</tr>
<tr>
<td>53</td>
<td>( B. ) obovatus +</td>
<td>5</td>
<td>1,2,1,2,2</td>
<td>0.018</td>
</tr>
<tr>
<td>54</td>
<td>( B. ) obovatus +</td>
<td>4</td>
<td>5.1,1,1</td>
<td>0.016</td>
</tr>
<tr>
<td>56</td>
<td>( B. ) obovatus +</td>
<td>8</td>
<td>1,1,1,1,1,1,1,1</td>
<td>0.023</td>
</tr>
<tr>
<td>57</td>
<td>( B. ) obovatus +</td>
<td>6</td>
<td>1,1,1,2,1,1*</td>
<td>0.022</td>
</tr>
<tr>
<td>59</td>
<td>( B. ) obovatus -</td>
<td>3</td>
<td>6,1,1</td>
<td>0.043</td>
</tr>
<tr>
<td>60</td>
<td>( B. ) obovatus -</td>
<td>2</td>
<td>6,2</td>
<td>0.009</td>
</tr>
<tr>
<td>62</td>
<td>( B. ) obovatus -</td>
<td>3</td>
<td>5,2,1</td>
<td>0.019</td>
</tr>
<tr>
<td>64</td>
<td>( B. ) obovatus -</td>
<td>2</td>
<td>7,1</td>
<td>0.054</td>
</tr>
<tr>
<td>65</td>
<td>( B. ) obovatus -</td>
<td>2</td>
<td>7,1</td>
<td>0.048</td>
</tr>
<tr>
<td>74</td>
<td>( B. ) obovatus -</td>
<td>3</td>
<td>6.1,1</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Isofemale lines in the upper group of the tree (copies 22 III to 40 I) contained more variation. The different ITS1 copies of this group of isofemale lines were divided in two sub-groups (copies 22 III to 38 VI and copies 21 I to 40 I). Most isofemale lines had ITS1 copies that belonged to each subgroup. In other words ITS1 copies within an isofemale line were paraphyletic. The presence of ITS1 copies from different subgroups in a single isofemale line could be the result of past recombination and thus sex between isofemale lines with different ITS1 copies. Some of the isofemale lines within this sub-group contained only one copy.
Figure 4. Neighbor joining phylogenies of the ITS1 types for each of the four *Brevipalpus* clades. Labels contain the isofemale line number, followed by the sequence type number in roman numerals and the number of times that the type was encountered. For *B. californicus* labels also indicate S or L for the short and long fragments respectively. All trees are unrooted. The bars indicates the branch length that representing 1% uncorrected distance.
There is little congruence between the ITS1 tree (Fig. 4) of *B. phoenicis* and the mitochondrial *COI* tree (Fig. 1). Isofemale lines with identical mitochondrial haplotypes had ITS1 copies that belonged to highly divergent monophyletic groups. For example, isofemale lines 21 and 30 both had mitochondrial haplotype but were placed in different sub-groups of the ITS1 phylogeny. Examples of the same mitochondrial haplotype showed that there was no relation between haplotype and the amount of variation between ITS1 copies; isofemale lines 21 and 30 contained little variation, whereas isofemale lines 20 and 22 contained much (Table 2).

*Brevipalpus californicus*

The PCR for ITS1 on all the *B. californicus* isofemale lines resulted in two fragments with different length. These fragments were cloned and sequenced separately. The number of different copies varied from two in isofemale line 44 (a single long and a single short copy), to seven in isofemale lines 50. The size of the shorter ITS1 fragment varied from 500 to 508 bp, whereas the longer fragment varied from 778 to 780 bp in length. The difference in length is due to a 247 bp indel. The indels in the long ITS1 fragments of all isofemale lines were clearly homologous, because the point of insertion was identical and sequence similarity was more than 90 per cent.

The average *D* between copies from the same isofemale line varied from 0.019 to 0.028, excluding the indel. Most of this dissimilarity was due to differences between the long and the short copies which ranged from 0.027 to 0.031, excluding the indel. Finally, the indel was more variable than the flanking regions of the ITS1 with on average 9.5 % and 3.2 % variable nucleotide positions respectively. The presence of short and long ITS1 copies in all *B. californicus* isofemale lines, suggests that the length mutation occurred in the common ancestor of all isofemale lines.

There was congruence between the ITS1 and mitochondrial *COI*; ITS1 copies retrieved from samples with identical mitochondrial haplotypes formed monophyletic groups (Fig. 4). Although only two haplotypes were present among these isofemale lines, it was consistent with the congruence between COI and AFLP trees.

*Brevipalpus obovatus*

The ITS1 copies obtained from the infected and uninfected *B. obovatus* could not be reliably combined in a single alignment due to the large amount of sequence variation. However, the sequences fell into two classes based on infection status. The length of the ITS1 copies in the infected *B. obovatus* isofemale lines varied between 551 and 567 bp. The length of the
ITS1 fragment in the uninfected *B. obovatus* isofemale lines varied between 532 and 541 bp. All analyses were therefore done separately.

**Infected *Brevipalpus obovatus***

The number of different copies retrieved from these isofemale lines varied between four and eight and dissimilarity ranged from 0.018 to 0.023. The large number of different ITS1 copies retrieved by sequencing only eight clones suggests that not all variation has been revealed yet and sequencing additional clones would probably result in more copies (Table 2).

The neighbor joining tree based on the ITS1 sequences did not reveal clear clades based on the isofemale lines they were retrieved from (Fig. 4). Neither did the tree contain any structuring based on the two mitochondrial haplotypes that were present in the infected *B. obovatus* isofemale lines. Likewise, the average $D$ between the different ITS1 copies of isofemale lines with different mitochondrial haplotypes was 0.021, whereas the average $D$'s for the ITS1 sequences from the two groups of isofemale lines with identical mitochondrial haplotypes were 0.022 and 0.017. In addition, almost identical ITS1 copies were found that originated from isofemale lines with different haplotypes, for example copies 53 IV and 57 II.

**Uninfected *Brevipalpus obovatus***

The patterns observed in uninfected *B. obovatus* and infected *B. obovatus* were different. Uninfected isofemale lines contained on average fewer copies compared to infected isofemale lines; two to three versus four to eight copies (Table 2). In addition, the difference between copies from the same uninfected isofemale line were generally much higher (intra-individual $D$ varied from 0.009 to 0.054 in uninfected lines versus 0.016 to 0.023 in infected lines). Uninfected *B. obovatus* had the highest average $D$ of all species. This is inconsistent with recombination. Recombination is expected to prevent divergence of lineages and consequently divergence between ITS1 copies within and between individuals.

Another difference compared to the infected *B. obovatus*, was the skewed recovery rate of the different ITS1 copies among the eight cloned fragments that were sequenced per uninfected isofemale line (Table 2). The ITS1 tree contained a basal split with copies in the upper group that were found several times in the different isofemale lines, and ITS1 copies in the lower group that were only found once (Fig. 4). It is unlikely that in the presence of recombination the various isofemale lines will have identical frequency distributions of their ITS1 copies.
The ITS1 phylogeny had a basal split in single and multiple copies. This split probably represents an ancestral polymorphism in copy type and frequency, similar to the indel polymorphism observed in *B. californicus*. The topologies of each group on either side of this basal split were congruent with the mitochondrial *COI* tree indicating that both ancestral copy types evolved in parallel with the mitochondrial marker. This is consistent with the congruence between AFLP and *COI* phylogenies (Fig. 3). The exceptions was isofemale line 60, which groups with isofemale line 59 based on ITS1, but with the other isofemale lines based on *COI*. This suggests that recombination has occurred in this isofemale line. Nevertheless, the overall pattern suggests a general lack of recombination among uninfected *B. obovatus* isofemale lines.

**Discussion**

**Evidence for recombination**

We have tested for the occurrence of recombination in three species of *Brevipalpus* mites by comparing the phylogeny inferred from mitochondrial and two nuclear markers. If the results show that recombination is absent, the favored interpretation is that reproduction has been strictly asexual since the moment the lineages started to diverge. When the results indicate that recombination has occurred, there are two not mutually exclusive explanations: (1) reproduction is not strictly clonal and occasionally sex occurs, or (2) reproduction is currently strictly clonal but has been sexual in the recent past.

It is clear from the phylogenetic comparisons that the three species have a different evolutionary history. *Brevipalpus phoenicis* and infected *B. obovatus* have incongruous AFLP and *COI* phylogenies. In addition, the ITS1 phylogeny shows that the different copies within isofemale lines are not monophyletic and do not mirror the *COI* branching pattern. These are signs of recombination.

However, *B. phoenicis* and infected *B. obovatus* differ in the degree of intragenomic variation. Whereas *B. phoenicis* isofemale lines have variable number of ITS1 copies, the infected *B. obovatus* isofemale lines had the highest number of different ITS1 copies per genome of all. Among these *B. obovatus* copies, similar ITS1 copies are found in isofemale lines with different mitochondrial haplotypes. This can only be the result of recombination.

Within *B. phoenicis* isofemale lines differ in the amount of intragenomic variation. A number of isofemale lines contained ITS1 copies
that belong to different sub-groups which suggests that they are the result of sex between mites that belong to these sub-groups. Other *B. phoenicis* isofemale lines contained only one copy or two highly similar copies. There are several explanations for the latter. Firstly, this may indicate that not all individuals have a hybrid origin. Secondly, it is possible that recombination was not detected because they had sex with mites that carried identical ITS1 copies. Finally, it is possible that these monotypic isofemale lines do contain divergent copies of ITS1, but that these were missed when eight random clones were sequenced. However, none of these explanations are incompatible with the observation of that some isofemale lines are recombinant.

In conclusion, both *B. phoenicis* and uninfected *B. obovatus* show evidence for recombination indicating that there is occasional sex or that asexuality has only recently evolved. In the latter case, the signs of recombination are a remnant of their sexual ancestry. The large difference in the number of different ITS1 copies per genome (isofemale line) between *B. phoenicis* and uninfected *B. obovatus* is probably due to the frequency of occasional sex or the age of asexuality.

A very different picture emerges from phylogenetic comparisons in *Brevipalpus californicus* and uninfected *B. obovatus*; nuclear and mitochondrial phylogenies are congruent. In addition, both contained evidence for ancestral polymorphism in ITS1 in isofemale lines; *B. californicus* is polymorphic for an indel and the uninfected *B. obovatus* is polymorphic for a basal split between frequent and rare ITS1 copies. Fixation of an ancestral polymorphism in multicopy DNA is difficult to maintain when sex occurs, because recombination will break up these fixed polymorphisms. Interestingly, asexual reproduction in uninfected *B. obovatus* is genetically determined and no males have been found (Groot and Breeuwer in press). This is consistent with the explanation that this group is strictly clonal. Thus, congruent phylogenies and the fixed ancestral polymorphism in both groups indicate that sexual reproduction and recombination has been absent since the different isofemale lines in either group started to diverge.

The difference in evolutionary patterns between three species suggests that asexuality has arisen several times in the genus *Brevipalpus*. Asexuality in *B. phoenicis* and infected *B. obovatus* is probably of more recent origin than in *B. californicus* and uninfected *B. obovatus*, because they show several signs of recombination. Asexuality probably has independent origins in *B. californicus* and uninfected *B. obovatus* too. This is supported by two arguments. Firstly, the ancestral polymorphisms in *B. californicus* and uninfected *B. obovatus* are different. Secondly, if they
would have had an asexual common ancestor, the mitochondrial and AFLP phylogenies at the species level should be congruent. This is not the case (Fig. 3). Therefore, asexuality has probably evolved after divergence of the sexual ancestor of the genus.

Intragenomic variation

The amount of ITS1 variation we retrieved from the *Brevipalpus* isofemale lines was unexpectedly large. The closest relatives of *Brevipalpus* for which data on ITS sequences are available are spider mites of the genus *Tetranychus*. Both genera belong to the superfamily Tetranychoidea. In *Tetranychus* species intragenomic variation was absent (Navajas and Boursot 2003; Navajas et al. 2001; Navajas et al. 1998; Osakabe et al. 2002) or very limited (Hinomoto and Takafuji 2001). Even among individuals at the species level the variation is limited to a few positions (or in some cases entirely absent). In other mite species intragenomic variation is variable (De Rojas et al. 2002; Fenton et al. 2000; Murrell et al. 2001; Navajas et al. 1994; Navajas et al. 1999; Noge et al. 2005; Ochs et al. 1999; Rees et al. 2003; Rich et al. 1997; Shaw et al. 2002; Vargas et al. 2005; Webster et al. 2004; Zahler et al. 1999) but the intragenomic variation reported here for *Brevipalpus* is among the highest. The question then arises what is causing this high amount of intragenomic variation? Asexual reproduction itself is insufficient to explain it as has become clear from the ostracod species *Darwinula stefensoni* (Gandolfi et al. 2001; Schon et al. 1998). Although this species is believed to be asexual for 25 million years, intragenomic variation is very limited. The reason for the intragenomic variation in *Brevipalpus* species might lie in the fact that they are haploid. The two main processes that drive concerted evolution of the multicopy ribosomal genes are unequal crossing over and gene conversion (Hillis and Dixon 1991). Both processes can occur during meiosis in *Brevipalpus* species after pre-meiotic doubling (Pijnacker et al. 1981). In normal diploid species, gene conversion can also occur at mitosis, and the rDNA is a known hot spot for mitotic recombination (Thomas and Rothstein 1991). In *Brevipalpus*, however, mitotic recombination cannot take place because it requires diploidy. Hence, the large intragenomic variation observed in *Brevipalpus* is probably due to reduced gene conversion because of haploidy.
Chapter 6

Acknowledgements

We thank B. Voetdijk who did the major part of the lab work to clone and sequence ITS1. We thank P. Kuperus for his advice on the AFLP procedures. The staff of the Burgers’ Bush is acknowledged for kindly allowing us to collect mites and their assistance in doing so. This study was supported by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), grant number W89-141.
General Discussion

Thomas V.M. Groot

Clonal diversity and the persistence of asexual *Brevipalpus*

In the general introduction I provided three explanations for the persistence of asexual species. Here I will discuss the likelihood of each explanation based on the results of the various chapters of my thesis. The first explanation was that asexual species might be characterized by specific adaptations that compensate for not having sex and recombination, such as very low mutation rates or highly efficient DNA repair. Although I have not specifically tested for such adaptations, I have also not found evidence for it. To the contrary, the mutation rate does not appear to be very low; within and between *Brevipalpus* species the amount of genetic variation at the mitochondrial *COI* gene is comparable to, if not higher than that of *Tetranychus* species at the same taxonomic level (Hinomoto and Takafuji 2001; Navajas et al. 1997; Navajas et al. 1998) Ideally, to determine the mutation rate, one would compare the genetic variation within the asexual *Brevipalpus* with that of its closest related sexual relative. At this point, *Tetranychus* is the most closely related genus for which data are available; both genera belong to the superfamily Tetranychoidae and have a similar biology. Furthermore, DNA repair is expected to be less efficient in asexual *Brevipalpus* than in sexual species because the asexual *Brevipalpus* females are haploid. Haploid species lack the “spare” copy which may act as a template for certain types of DNA repair mechanisms. Additionally, gene conversion processes that may restore deleterious mutations are probably slowed down, as has been shown in chapter 6.

The second explanation was that new clonal lineages arise constantly from the sexual ancestor. This explanation is more plausible for the persistence of asexual *Brevipalpus*. For new clonal lineages to arise in *Brevipalpus*, two conditions are required. Firstly, the symbiont needs to be transmitted horizontally. In chapter 4 we showed that this requirement is met; the *Cardinium* symbionts are transmitted horizontally among *Brevipalpus* lineages and *Brevipalpus* has become infected at least twice. Secondly, it requires the presence of sympatric sexual individuals that can be infected by the symbiont. It is not clear if this second requirement is currently met. During my extensive fieldwork in Brazil I have never observed sexual populations of the same species; in all observed populations
males were absent or very rare (less than 5%). Moreover, during a sampling trip to Arizona we did not find closely related sexual species even though these have been described from this area (Helle et al. 1980). However, sympatric sexual and asexual individuals may exist elsewhere. One such population might have been described by Gonzalez (1975) who observed *B. phoenicis* and *B. phoenicoides*, which are at least morphologically highly similar, occurring sympatrically. Gonzales observed no males of the former species, but did describe males for the latter. The problem however with this and other species descriptions (see for example De Leon 1960; 1961) is that they only described males as being present or absent without mentioning the proportion of each gender in the population or the actual mode of reproduction. Regardless of whether sympatric sexual and asexual populations currently exist, it is conceivable that they have existed in the past when not yet all individuals were infected. Therefore, the persistence of asexual *Brevipalpus* might be explained because asexuality arose multiple times in various sexual individuals. In addition, a recent origin of asexuality is one of the two explanations for the recombination observed in *B. phoenicis* and infected *B. obovatus* in chapter 6.

The third explanation was that clonal lineages escape from the disadvantages of asexuality by having occasional sex. This explanation is plausible for *B. phoenicis* and the infected *B. obovatus*. Occasional sex is one of the two explanations for the recombination observed in *B. phoenicis* and infected *B. obovatus* in chapter 6. For *B. phoenicis*, this observation seems to be in conflict with the results of chapter 5 where attempts to cross different isofemale lines failed. These crosses were made with two isofemale lines only and the possibility that other lines are more easy to cross can not be excluded. However, in chapter 6 one of the lines in the crosses (line 1) was shown to contain ITS1 copies that belong to different sub-groups (line 40) suggesting that this lineage has had some sex in the recent past. The failure to get fertilized eggs in chapter 5 was ascribed to a Functional Virginity Mutation (FVM). Assuming that a FVM indeed is responsible, the evidence for recent recombination suggests that the mutation occurred only recently, or alternatively, that it is not 100% effective. Occasional sex may not seem possible in the infected *B. obovatus* because these lines do not have males in the cultures (chapter 1). However, these results were based on only few isofemale lines and under constant laboratory conditions. Males may be produced by other lines and/or under more variable conditions in the field. For the uninfected *B. obovatus* and *B. californicus* occasional sex cannot explain the persistence of the asexual lineages. Recombination in these species is very limited or absent, although some gene conversion might occur among multicopy genes within genomes.
In conclusion, a recent origin of asexuality and/or recent occasional sex is the most likely explanation why *B. phoenicis* and the infected *B. obovatus* do not suffer from the disadvantages of asexual reproduction. The results of chapter 6 show that the other two species have been strictly clonal for some period of evolutionary time. The question, however, is whether this period of clonality has been too long for the short-term advantages of asexual reproduction to explain their persistence. Theory predicts that asexual species are at a disadvantage in the long run, but it is unknown at what exact time scale this will be. Neither have I been able to assess the exact moment that these species became strictly asexual. Therefore it is not possible to determine whether a recent origin of asexuality and/or recent occasional sex can explain the persistence of these species. However, two observations may make it plausible that not yet enough time has passed for *B. californicus* and uninfected *B. obovatus* to really suffer from being asexual. Firstly, chapter 6 revealed that the phylogenetic relations among the four species based on mitochondrial *COI* and nuclear AFLPs were incongruous. From this it was concluded that strict clonality arose after the split between the species and therefore is of relatively recent origin. Secondly, most cytological mechanisms of thelytoky result in the asexual lineage becoming homozygous for their entire genome (Suomalainen et al. 1987). When heterozygocity is lost, the asexual lineage will suffer from the expression of recessive deleterious mutations. In this respect, asexual species that have arrhenotokous ancestors are at an advantage. Because males are haploid in arrhenotokous species, all deleterious mutations are expressed and therefore effectively purged from the population. Thus the asexual descendants start with a genome that is free of deleterious mutations and will endure longer before the mutational load becomes a real problem.

The chapters 5 and 6 have studied the possibility of occasional sex. In chapter 5 experiments to show occasional sex failed, whereas chapter 6 showed that it may occur in *B. phoenicis* and infected *B. obovatus*. In theory occasional sex is highly advantageous because it allows a species to benefit from the advantages of sex at a minimal cost. Indeed, one may wonder why not more species reproduce sexually only occasionally. However, some species clearly do have occasional sex; the cyclic parthenogens such as aphids and daphnia (Innes and Singleton 2000; Vorburger et al. 2003). These species reproduce sexually in autumn when the environment turns unfavorable and the future uncertain. It makes sense to reproduce sexually when the future environmental conditions are uncertain. Sexual reproduction will ensure genetic variation among the offspring enhancing the chance that at least some of them will survive in the changed environment. If the same would apply to *Brevipalpus*, sexual reproduction
would be inducible by stress. This may explain the failure to observe sexual reproduction in chapter 5 because all experiments were performed in a climate room with stable temperature, humidity, and light regime. On the other hand, the females were exposed to other kinds of stress; they were fed on a different host plant species than they were originally collected from, some females were treated with antibiotics, and females were harassed by the large number of males present. It is therefore unlikely that occasional sex in *Brevipalpus* is stress-induced.

The results of both chapter 1 and chapter 6 led to suggestions about the relative age of the asexuality in the mite species. In chapter 1, I suggested that asexuality in the infected *B. obovatus* is older than in *B. phoenicis* and *B. californicus*. This was based on the observations that symbiont transmission is most efficient in the infected *B. obovatus* compared to the other infected species. Provided that males have no function in fertilizing the eggs of infected females, producing males is wasteful for both host and symbiont. The fact that no males are produced in *B. obovatus* suggests that the mite and bacterium have had more time to adapt to each other to maximize transmission. In Chapter 6, I suggested that the relative age of strict clonality of the uninfected *B. obovatus* and *B. californicus* is older than in the other two species. This suggestion is based on the observation that there is no evidence for recombination between clonal lineages in these species, whereas there is in *B. phoenicis* and the infected *B. obovatus*. The suggestions made in both chapters do not necessarily conflict because the moment a lineage becomes infected may differ from the moment the lineage becomes strictly asexual. This is because after becoming infected, lineages may have occasional sex for different periods of time before clonality becomes fixed. Unfortunately, it is impossible to distinguish between recombination through obligate sexual reproduction or through occasional sex. Because of this and because of the horizontal transmission of the symbiont, it is impossible to reconstruct the order of events that turned the sexual ancestor of these mites into the current group of asexual lineages.

In chapter 5, it was shown that males of *B. phoenicis* still display typical male sexual behavior such as mating and guarding behavior. Theory predicts that traits associated with male sexual behavior will not be maintained by selection when males are no longer functional (Carson et al. 1982; Muller 1949). The notion that *B. phoenicis* males still display this behavior although they were non-functional in the crosses, has led to the suggestion that the asexuality in this species is of recent origin. The results of chapter 6 are inline with this suggestion; the evidence for recent recombination indicated that asexuality could be of recent origin. The
phylogenetic congruence between mitochondrial and nuclear markers suggested that *B. californicus* has been asexual for a longer period of time than *B. phoenicis*. Chapter 1, however, revealed that the isofemale lines of both species produced comparable amounts of males. Combining these observations with theory, one would expect that the males of *B. californicus* will show less pronounced male sexual behavior than *B. phoenicis* males. This expectation still needs to be tested.

**Relation between host and symbiont**

Based on the evolutionary scenario that I will sketch below, *Cardinium* can be considered as a parasite of its mite host. When the bacterium first infects a sexual mite, that mite will then become a new parthenogenetic lineage. The symbiont will give the infected lineage a fitness benefit because the infected individuals produce relatively more females than non-infected individuals as these still invest in males. In other words, the symbiont relieves the host from paying the cost of males. Because of this benefit, the symbiont may initially be regarded as a mutualist. If the mite has occasional sex, it can escape from the disadvantages of asexual reproduction and the mutualistic relation will be maintained. However, it is questionable if this situation is evolutionarily stable. As was argued in chapter 5, when sex is not required for reproduction, selection on maintaining the traits necessary for sexual reproduction is relaxed. As a consequence, these traits will accumulate deleterious mutations and the possibility to reproduce sexually will eventually be lost. Moreover, this possibility is lost even more rapidly if a functional virginity mutation arises. Either way, the mite will become locked in strict asexuality. When this happens, it will begin to suffer from the long term disadvantages of asexuality and eventually go extinct. Thus the symbiont can be considered a parasite, in the long run, because it will drive its host extinct. Before the host goes extinct, the symbiont will have to reach a new host through horizontal transmission. Horizontal transmission thus is the escape route for *Cardinium*, otherwise it would go extinct with its host. Chapter 4 has revealed that *Cardinium* is indeed capable of horizontal transmission. Because the host loses the ability to reproduce asexually, as described above, a remarkable situation arises. Although the symbiont is a parasite, the host becomes dependent on the parasite for its reproduction. Therefore, the host will benefit from maximizing the transmission of the symbiont because failure to transmit the symbiont results in males that do not add to host fitness. Thus, selection favors mites that are good hosts to the symbionts that in the long run will drive them extinct. In other words, it pays to be good to the parasite.
Notwithstanding the parasitic role of the symbiont in the long run, it may well have beneficial effects too. The *Cardinium* infecting the mite *Metaseiulus occidentalis* induces cytoplasmic incompatibility, but has also a positive effect on the fecundity of its host (Weeks and Stouthamer 2004) and *Wolbachia* bacteria can also have positive effects on the fitness of their host (Bordestein and Werren 2000; Vala et al. 2003). Bacterial endosymbionts can also have specific beneficial effects on their hosts. For example, various secondary symbionts of aphids are known to make the host resistant to parasitoids, improve host tolerance to heat stress or allow the host to feed on specific plant species (Montllor et al. 2002; Oliver et al. 2003; Tsuchida et al. 2004). Perhaps the *Cardinium* strains that infect *Brevipalpus* provide similar services to their hosts. If so, clonal *Brevipalpus* might have the possibility to adapt to changes in their environment without recombination, but by acquiring different symbionts. This intriguing possibility might be tested using isofemale lines of the uninfected *B. obovatus*. As discussed in chapter 4, it is conceivable that these lines can re-obtain *Cardinium*. If so, mites with and without this secondary infection can be compared to test the effect on fitness, independent from the effects on feminization and parthenogenesis.

**Specialists and generalists**

Chapters 2 and 3 have shown that asexual *Brevipalpus* clones can be generalists or specialists. Chapter 3 shows that there is a relation between mite species and the level of specialization. The *B. phoenicis* clones are best described as generalists whereas the *B. obovatus* clones are more specialists. Note that in chapter 3 it was not possible to discriminate between infected and uninfected *B. obovatus* because the mitochondrial COI marker that was used can not discriminate between the two, see also below. In chapter 6 the relative age of the clones of these species is determined; clones in *B. phoenicis* are younger than the uninfected clones of *B. obovatus*. The combined results of these chapters are in conflict with the theory of GPG and FNV. The specialist clones of FNV are expected in situations where clones are of recent origin and arise frequently, as is the case in *B. phoenicis*. The generalist clones of GPG are expected in situations where fixed clonal types have survived for a longer period, as is the case for the uninfected *B. obovatus* clones. Thus, the expected distribution of generalists and specialists is the opposite of what is observed. An explanation for this phenomenon is lacking. Unfortunately, the degree of specialization of *B. californicus* could not be reliably assessed because in chapter 3 only few *B. californicus* individuals were sampled.
Mite phylogeny and species descriptions
A recurring problem in studies of *Brevipalpus* species is the general lack of a robust phylogeny. Chapter 1 shows that there is incongruity between the classical, morphological species descriptions and the phylogeny based on mitochondrial COI sequences. Isofemale lines 11 to 13 were morphologically identified as *B. phoenicis* but based on the mitochondrial genetic marker they grouped with the isofemale lines that were morphologically identified as *B. obovatus*. The more extensive mitochondrial phylogeny in chapter 3 showed that there was no clear separation between the “real” (infected) *B. obovatus* and the ambiguous samples, and therefore I provisionally named them *B. obovatus* too. Chapter 1 showed that the reproductive biology between the two groups of *B. obovatus* is rather different; the “real” *B. obovatus* mites were infected and mites from the ambiguous group were uninfected. Consequently, I named both groups infected *B. obovatus* and uninfected *B. obovatus*. The results of chapter 6 showed that nuclear markers of both types are quite different. It can therefore be concluded that the infected and uninfected *B. obovatus* are indeed different species and should be described as such. Morphological differentiation between the uninfected *B. obovatus* and *B. phoenicis* is possible based on the reticulation patterns of the hysterosoma (N.C. Mesa, pers. comm.). Besides the observation that there may be cryptic species among the thelytokous *Brevipalpus* species [shown here for the uninfected *B. obovatus*, and already predicted by (Childers et al. 2003b)], another factor that may explain why robust species descriptions are lacking is the frequently reported high level of intra-specific morphological variation [reviewed by (Welbourn et al. 2003)]. One potential cause of this variation might be that inter-specific variation is interpreted as intra-specific variation. When species are described, it is often assumed that all individuals in a single sample, for example from one host plant individual, belong to the same species. Chapter 3 has shown that this assumption is not correct; the mites that occur in a single sample frequently belong to more than one species. To disentangle intra-specific from inter-specific variation, it would be best if species description would be made on isofemale lines. This procedure would then also allow the combination of both morphological and molecular genetic characterization.

The phenotype of *Cardinium* in *Brevipalpus*
The effect of a symbiont on its host is generally described as its phenotype and in the case of reproductive manipulations these can be either parthenogenesis induction, feminization, male-killing, or cytoplasmic incompatibility. Generally, a symbiont induces only one of these phenotypes
in a certain host. The situation of Cardinium in Brevipalpus appears to be more complex. One effect of the symbiont is feminization, because all females are genetically male and removal of the symbiont results in male offspring. However, Cardinium appears to have a second effect by also inducing parthenogenesis. This can be concluded from the observation that feminization alone does not necessarily result in asexuality; genetic males of Armadillidium vulgare are feminized by Wolbachia, but they remain sexual (Rigaud et al. 1997). The cytological mechanism of asexual reproduction is peculiar. Pijnacker et al. (1981) showed that the amount of DNA is doubled before meiosis. Presumably, this so-called premeiotic doubling is necessary to allow for meiosis to take place in the germline of otherwise haploid females. It may therefore be concluded that Cardinium is active at two different moments: prior to meiosis to induce pre-meiotic doubling and shortly after meiosis to feminize the genetic males. This two-stage activity seems redundant because if the symbiont would induce premeiotic doubling in a diploid female, the resulting egg would be diploid and feminization would no longer be necessary. Another peculiar thing is the fact that when the infection is cured with antibiotics, females produce haploid sons. How do these females go through meiosis if the symbiont is not inducing pre-meiotic doubling? To resolve these questions, more work on the cytology of meiosis in both infected and uninfected Brevipalpus is clearly needed.

Acknowledgements

I thank Steph Menken, Vera Ros, Arne Janssen, Hans Breeuwer and Maus Sabelis for giving valuable feedback on earlier drafts of the general discussion.
References


References


References


References


Hurst, L. D., and J. R. Peck. 1996. Recent advances in understanding of the evolution and maintenance of sex. TREE 11:46-52.


References


References


References


References


Dankwoord

Als eerste wil ik mijn dank betuigen aan mijn co-promotor en dagelijks begeleider Hans. Inclusief mijn stage hebben we nu zo’n 5 jaar prettig samen gewerkt. De directe en ontspannen contact heeft zeker een belangrijke bijdrage geleverd aan het snel afronden van mijn promotie. Veel dank ook voor mijn beide promotores. Steph, jij hebt misschien een meer officiële rol gehad, bijvoorbeeld met voortgangsgesprekken en dergelijke. Je hebt me verschillende keren gedwongen om overzicht te houden met wat ik allemaal aan het doen was en vooral waarom, en dat was nodig. Je commentaar op mijn manuscripten heb ik ook zeer gewaardeerd, niet alleen vanwege de vele kleine spel- en vormfauten die je verbeterde, maar vooral ook omdat je ze bekeek met de frisse blik van iemand die niet tot over zijn oren in het endosymbionten vakgebied zit. Maus, met jouw enthousiasme en ideeën ben je een inspirator voor het mijten onderzoek in het algemeen. Elk van onze gesprekken leverde weer een grote hoeveelheid nieuwe ideeën, vragen en mogelijkheden op.

The *Brevipalpus* project was based on the work of Andrew Weeks. When I started the project I inherited a whole lot of ideas, techniques, primers, and even mites! Thanks for them, end for taking the time to answer all my emails.

Dan Vera, met jouw komst heb ik toch een ‘sister in arms’ gekregen. Ineens was ik niet meer de enige AIO die aan parthenogenetische mijten en hun symbionten werkte en ik heb in verschillende opzichten veel aan je gehad. In praktische zin als het ging om de uitvoering van experimenten, maar ook in theoretische zin als klankbord voor mijn ideeën. Dan Egbert, vierjaar zijn wij kamergenoten geweest maar bij elkaar hebben we elkaar maar een paar maanden gezien. Toch was het altijd gezellig als je er onverwacht weer was en heb ik een heel belangrijk ding van je geleerd: als een proefschrift niet binnen vier jaar af is, dan kan het heel lang duren voordat het wel af komt. Dat is zeker een motivatie geweest om mijn proefschrift binnen de 4 jaar af te ronden.

Bedankt zeg ik ook aan de andere leden van de EB groep, Peter, Aletta, Katja, Saskia, Cecile en anderen. Het was heel nuttig om wat ideeën en resultaten te kunnen presenteren toen de eigen EB lunch praatjes nog bestonden. Nu is daar de wekelijkse koffie voor in de plaats gekomen. Dank ook aan die andere groep, de populatie biologen. Om zo maar enkele name te noemen; Martijn, Paulien, Michiel, Merijn, Marin, Arne, Nicola, Mathias,
Roos, Sara, Tessa, Amir, Martha, Maus, Iza, Karen, Andre, Maarten en de anderen. Hoewel ik misschien niet helemaal officieel lid was van jullie groep, was ik er toch wel erg mee verbonden. Dank voor de goede ideeën na de praatjes die ik voor de groep heb mogen geven, maar vooral ook voor de gezelligheid op de gang.

Veel heb ik mogen leren tijdens de wekelijkse bijeenkomsten van de molecular evolution discussion group. De kennis en ervaring die in deze bijeenkomsten gedeeld werd was anders moeilijk te vergaren. De samenstelling van deze groep veranderde doorlopend, maar een aantal constantere personen wil ik toch even noemen; Katja, Dirk, Patrick, Marc, Vera, Cecile, Pieternella, Hans. Omdat het veelal dezelfde personen betreft wil ik hier meteen de collega’s in het moleculaire lab bedanken. Met name de analisten, Wil, Peter en Betsie. Vooral Peter is erg behulpzaam geweest in discussie over verschillende technieken (moet je nu wel of niet in je PCR spuwen?) en Betsie heeft veel werk uit handen genomen door de ITS1 monsters te kloneren en sequencen.

Voor dit onderzoek zijn een groot aantal bonenplanten nodig geweest, ongeveer 400 bakken! Ludek, Harold en anderen, bedankt voor de constante productie van mijtenvoer.

Jan van Arkel was verantwoordelijk voor de foto’s die dit proefschirft sieren. Zijn foto’s waren bovenverwachting goed; er zijn vaak meer details zichtbaar op zijn foto’s dan dat ik ooit door een binoculair gezien heb.

Ik ben ook dankbaar voor de aanwezigheid van de snoep en frisdrank automaten in gebouw 1. Na 14:00 uur zijn dit de enige bronnen van koolhydraten op de Anna’s Hoeve en omgeving. Niet zelden hebben ze mij door de middag en avond heen geholpen.

Part of the work was done in Brazil and a number of people there need to be acknowledged. First of all the people of the acarology lab where I did the transplantation experiments are thanked for their hospitality. I wish to thank especially Angelo who, among other things, got the paperwork to export living mites done, and João for the good times drinking some ‘biertje’ at Moreiras. The molecular work was done in pant virology lab where I was again received with great hospitality. I wish to thank two people in particular in this lab, Murillo and Renata.

Although I didn’t do much work there, I have had a good contact with several colleagues in Piracicaba. I wish to thank Gilberto, Nora and Elliot for kindly sharing their knowledge on parts on the *Brevipalpus* biology that I am still mostly ignorant about: their morphology and their role in vectoring viruses.
Het waren niet alleen maar Brazilianen in Brazilië. Voorbereidende pilot-studies waren al gedaan door Maarten, die mij zo flink op weg heeft geholpen. Verder heeft Arne zo’n beetje als begeleider gefunctioneerd in Brazilië, omdat Amsterdam soms toch wel erg ver weg was. Maar bovenal ben ik de familie Grosman-Fisser heel erg dankbaar, voor heel veel praktische zaken, maar vooral ook voor hun vriendschap toen ik dat heel hard nodig had.

Dan vooral ook veel dank aan mijn ouders, familie en vrienden om me heen. Te veel om ze allemaal te noemen, dus om niemand uit te sluiten noem ik geen namen. Dank voor al de gezelligheid, goede gesprekken, maar vooral heel veel slap gelul. Het contrast was vaak erg groot; door de weeks wetenschap op de faculteit, in het weekend pilsen in de kroeg of voetbal kantine. Toch hecht ik juist veel waarde aan dit contrast, de perfecte manier om even de gedachten te verzetten. Als ik niet de mogelijkheid had gehad om met jullie af te kicken in de weekenden, dan had ik door de weeks dit wetenschappelijke resultaat nooit kunnen bereiken.

Dan Esther, mijn vriendinnetje gedurende het grootste gedeelte van de afgelopen vier jaar. Je hebt me de ruimte gegeven de dingen te doen die ik wilde doen. Je toonde begrip en geduld en bent bovenal steeds een grote steun geweest.
Curriculum Vitae

Thomas Volkert Marie Groot werd geboren op 24 december 1978 in het Medisch Centrum Alkmaar. Onder de naam Tom was hij de middelste in een gezin van vijf kinderen. Hij is opgegroeid in een jaren 70 vinexwijk in het Westfriese Heerhugowaard. Zijn basisschool periode bracht hij door op de Montessorischool. Op deze school heeft hij slechts weinig gedaan van het leerwerk wat van hem verwacht werd, maar daarentegen heeft hij wel een groot deel van de boeken in de schoolbibliotheek verslonden. Hardwerken was wel nodig in de brugklas van het Murmellius gymnasium in Alkmaar. Hier werd ook geconstateerd dat Tom dyslectisch was, net als zijn moeder, broer, zus, broertje en zusje. Vanwege deze handicap werd het Murmellius na het eerste jaar vervuild voor het Han Fortmann college in Heerhugowaard waar na vijf jaar in 1997 het VWO diploma werd behaald. Het plan was eerst om diergeneeskunde te gaan studeren, maar de numerus fixus computer besliste anders. De tweede keus, algemene biologie in Amsterdam, beviel na het eerste jaar zo goed dat Tom bij deze studie is gebleven. Na een stage aan de ecologie van planten in tropische berg nevelwouden in Costa Rica en een stage aan parthenogenese in tijgerpythons werd de studie in succesvol 2002 afgerond. Direct aansluitend werd in september begonnen met het promotie onderzoek aan Brevipalpus waarvan deze thesis het tastbare resultaat is.

Al op jonge leeftijd was de interesse voor biologie duidelijk aanwezig. Dit leidde niet alleen tot lastige vragen toen zijn moeder zwanger was, maar ook tot een slaapkamer vol terraria en een periode van 7 jaar als vrijwilliger op de Heerhugowaardse kinderboerderij. Veel is er tot op heden niet veranderd; de terraria zijn er nog steeds maar staan nu op een aparte kamer, en de lastige vragen zijn er ook nog getuige deze thesis. Wanneer Tom niet met zijn beesten en planten aan het rommelen is, dan rent hij graag een rondje door de polder, speelt een spelletje bij vrienden of drinkt een biertje in de voetbalkantine of kroeg.