

Ministério da Saúde
Fundação Oswaldo Cruz
Instituto Oswaldo Cruz
Programa de Pós-Graduação em Biologia Celular e Molecular

**Análise da diferenciação molecular entre populações brasileiras
de *Anopheles (Kerteszia) cruzii* utilizando uma abordagem
multilocus (Diptera: Culicidae)**

Luísa Damazio Rona Pitaluga

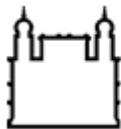
Orientador: Dr. Alexandre Afranio Peixoto (IOC / Laboratório de Biologia Molecular de Insetos)

Rio de Janeiro, Setembro de 2009.

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**Tese apresentada como requisito à obtenção do título de Doutor em
Biologia Celular e Molecular, com área de concentração em Entomologia
Molecular.**

Orientador: **Dr. Alexandre Afranio Peixoto** (IOC / Laboratório de Biologia Molecular de Insetos)

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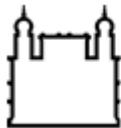
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Tese de Doutorado em Biologia Celular e Molecular, área de concentração em Entomologia Molecular.

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1 . *Anopheles cruzii* 2. análise *multilocus* 3. especiação 4. complexo de espécies



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Análise da diferenciação molecular entre populações brasileiras de *Anopheles (Kerteszia) cruzii* utilizando uma abordagem *multilocus* (Diptera: Culicidae)

Tese submetida à coordenação do curso de Pós-Graduação em Biologia Celular e Molecular do Instituto Oswaldo Cruz como parte dos requisitos para obtenção de grau em Doutor em Biologia Celular e Molecular, área de concentração: Entomologia Molecular.

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Pedro e a minha mãe Rosalba.

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RESUMO

Anopheles (Kerteszia) cruzii (Diptera: Culicidae) é vetor primário de malária humana e simiana no sul e sudeste do Brasil. A distribuição deste mosquito segue a costa da Mata Atlântica. Estudos anteriores têm sugerido que *An. cruzii* é um complexo de espécies crípticas.

Neste estudo, um fragmento do gene *timeless*, um *locus* envolvido no controle dos ritmos circadianos, foi utilizados como marcador molecular para avaliar a diferenciação genética entre seis populações de *An. cruzii* (Florianópolis, Cananéia, Juquitiba, Itatiaia, Santa Teresa e Itaparica). Os resultados indicaram que *An. cruzii* de Itaparica constitui um grupo altamente diferenciado comparado com as outras cinco populações do sul e sudeste do Brasil, e diferenças genéticas significativas também foram observadas entre estas últimas. Além disso, a análise *multilocus* revelou valores extremamente altos de F_{ST} e diferenças fixas entre Florianópolis e Itaparica nos seis *loci* analisados, independente da função. Esta análise também indicou que estas duas populações não trocam migrantes desde sua separação há aproximadamente 2,4 milhões de anos.

Um fragmento do gene *cpr*, um *locus* envolvido na resistência à inseticida e no olfato em insetos, foi também usado para analisar a divergência entre as cinco populações de *An. cruzii* do sul e sudeste do Brasil citadas acima. O gene *cpr* revelou valores extremamente altos de F_{ST} e diferenças fixas entre Itatiaia e as outras quatro populações analisadas (Florianópolis, Cananéia, Juquitiba e Santa Teresa). Os dados também sugerem uma evidência preliminar da ocorrência de duas espécies crípticas simpátricas em Itatiaia.

Nossos resultados confirmam que este vetor de malária é de fato um complexo de espécies crípticas.

ABSTRACT

Anopheles (Kerteszia) cruzii (Diptera: Culicidae) has long been known as the primary vector of human and simian malaria parasites in southern and south-eastern Brazil. The distribution of this mosquito follows the coast of the Brazilian Atlantic forest. Previous studies have suggested that *An. cruzii* is a complex of cryptic species.

In the current study, a fragment of the *timeless* gene, a locus involved in the control of circadian rhythms, was used as a molecular marker to assess the genetic differentiation between six *An. cruzii* populations (Florianópolis, Cananéia, Juquitiba, Itatiaia, Santa Teresa and Itaparica). The results indicate that *An. cruzii* from Itaparica constitutes a highly differentiated group compared with the other five populations from south and south-east Brazil, and significant genetic differences were also observed among some of the other populations. In addition, a multilocus analysis revealed very high F_{ST} values and fixed differences between Florianópolis and Itaparica in all six loci, irrespective of their function. This analysis also indicates that these two populations have not exchanged migrants since their separation around 2.4 million of years ago.

A fragment of the *cpr* gene, a locus involved in metabolic insecticide resistance and odorant clearance in insects, was also used to analyze the divergence between the five *An. cruzii* populations from south and south-east Brazil listed above. The *cpr* gene revealed very high F_{ST} values and fixed differences between Itatiaia and the other four populations studied (Florianópolis, Cananéia, Juquitiba and Santa Teresa). The data also provided preliminary evidence for the occurrence of two sympatric siblings in Itatiaia.

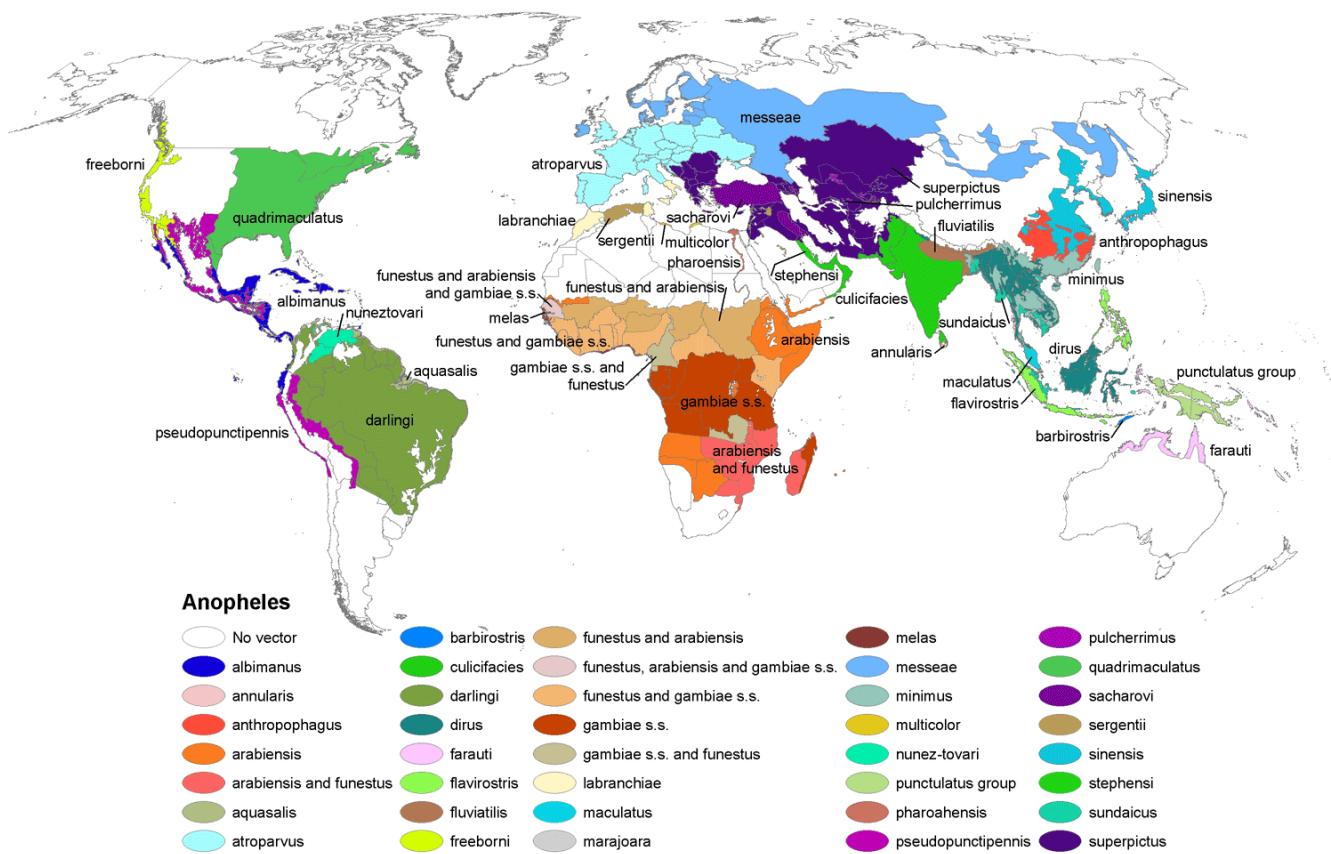
Overall, our results confirmed that this malaria vector is indeed a complex of cryptic species.

INTRODUÇÃO

Malária

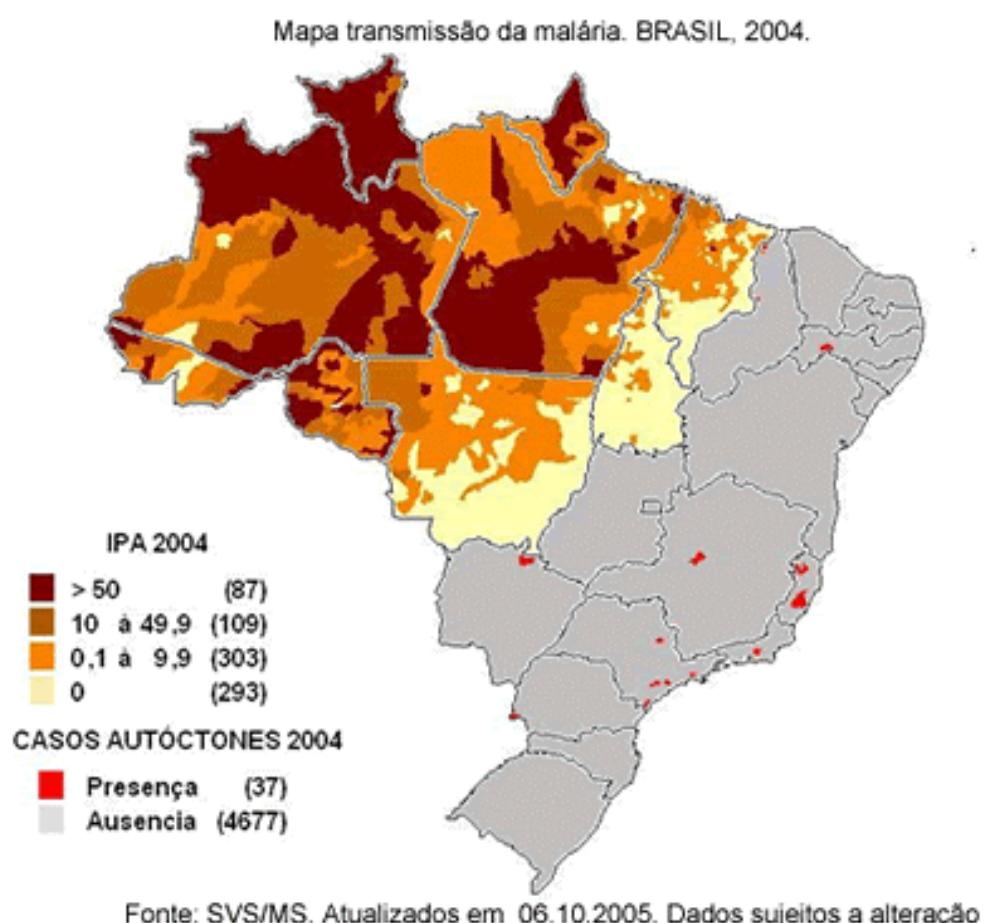
A malária é uma doença causada por parasitas do gênero *Plasmodium* (*Plasmodium falciparum*, *P. vivax*, *P. ovale* e *P. malariae*), transmitidos pela picada de fêmeas de várias espécies de mosquitos do gênero *Anopheles*, que picam principalmente entre o pôr-do-sol e o amanhecer. É uma doença freqüente e muitas vezes fatal em áreas tropicais e subtropicais e é atualmente endêmica em mais de 100 países da Ásia, África, América Central e do Sul e na Oceania. Aproximadamente 3.3 bilhões de pessoas estão em áreas de risco e todo ano são registrados 250 milhões de casos da doença sendo 1 milhão de mortes. É a principal parasitose tropical e uma das mais frequentes causas de morte em crianças nesses países. A Figura 1 mostra a distribuição das principais espécies de *Anopheles sp.* transmissores do *Plasmodium sp.* no mundo (WHO, 2009).

Figura 1. Distribuição das principais espécies de *Anopheles* sp. transmissores do *Plasmodium* sp.



No Brasil, a malária é um grave problema de saúde pública, principalmente na região da Amazônia Legal, onde aproximadamente 99,5% dos casos da doença são registrados. A alta incidência ocorre principalmente nos estados do Acre, Amapá, Amazonas, Pará, Rondônia, Roraima, Tocantins, Mato Grosso e Maranhão. A Figura 2 mostra a situação da malária no Brasil (MS, Ministério da Saúde). Na Região Sudeste, embora ocorram surtos de malária introduzida, os casos autóctones são raros (Carvalho *et al.*, 1988; Azevedo, 1997; Branquinho *et al.*, 1997).

Figura 2. Situação da malária no Brasil.



O gênero *Anopheles*

O gênero *Anopheles* comprehende aproximadamente 500 espécies espalhadas em todos os continentes, com exceção da Antártica, e distribuídas em seis subgêneros: *Kerteszia*, *Nyssorhynchus*, *Stethomyia*, *Lophopodomyia*, *Anopheles* e *Cellia* (Krzywinski & Besansky, 2003). Destes, os quatro primeiros habitam a América do Sul, *Cellia* é encontrado apenas no Velho Mundo e o subgênero *Anopheles* é cosmopolita (Consoli & Lourenço de Oliveira, 1994; Krzywinski & Besansky, 2003).

No Brasil, *Nyssorhynchus* é responsável, principalmente, pela manutenção da malária na região Amazônica, enquanto *Kerteszia* destaca-se pela transmissão do *Plasmodium* em áreas ricas em Mata Atlântica (Consoli & Lourenço de Oliveira, 1994).

Subgênero *Kerteszia*

Os mosquitos do subgênero *Kerteszia* utilizam recipientes naturais como criadouros, principalmente bromeliáceas, com exceção de *Anopheles bambusicolus* que é a única espécie do subgênero que utiliza o bambu como habitat para suas larvas (Consoli & Lourenço de Oliveira, 1994; Marrelli *et al.*, 2007).

Este subgênero possui 12 espécies: *Anopheles auyantepuiensis*, *Anopheles gonzalezrinconesi*, *Anopheles rollai*, *Anopheles pholidotus*, *Anopheles boliviensis*, *Anopheles lepidotus*, *Anopheles homunculus*, *Anopheles neivai*, *Anopheles laneanus*, *Anopheles bambusicolus*, *Anopheles cruzii* e *Anopheles bellator*, sendo que as oito últimas têm registro de ocorrência no Brasil (Zavortink, 1973; Marrelli, *et al.*, 2007).

***Anopheles cruzii*: biologia e importância epidemiológica**

O subgênero *Kerteszia* agrupa alguns vetores primários de malária na região sul e sudeste do Brasil, como *An. cruzii* (Figura 3). Essa espécie é encontrada desde o Rio Grande do Sul até Sergipe, nas matas mais ricas em gravatás, como a Mata Atlântica, e numa variedade enorme de habitats, desde as áreas costeiras até as mais montanhosas. A Figura 4 mostra a distribuição da Mata Atlântica no país. Esta espécie cria-se apenas em bromélias, com preferência àquelas situadas em locais sombreados onde a evaporação da água contida nessas plantas é mais lenta, por isso *An. cruzii* pode se desenvolver em plantas de qualquer tamanho (Consoli & Lourenço-de-Oliveira, 1994). A Figura 5 mostra o local onde as amostras de Florianópolis, Estado de Santa Catarina, utilizadas neste trabalho (Capítulos 1, 2 e 3) foram coletadas, evidenciando a mata fechada e bem sombreada onde *Anopheles cruzii* é encontrado em alta densidade.

Figura 3. Fêmea de *Anopheles cruzii* (Fonte: Luísa Damazio Rona Pitaluga).

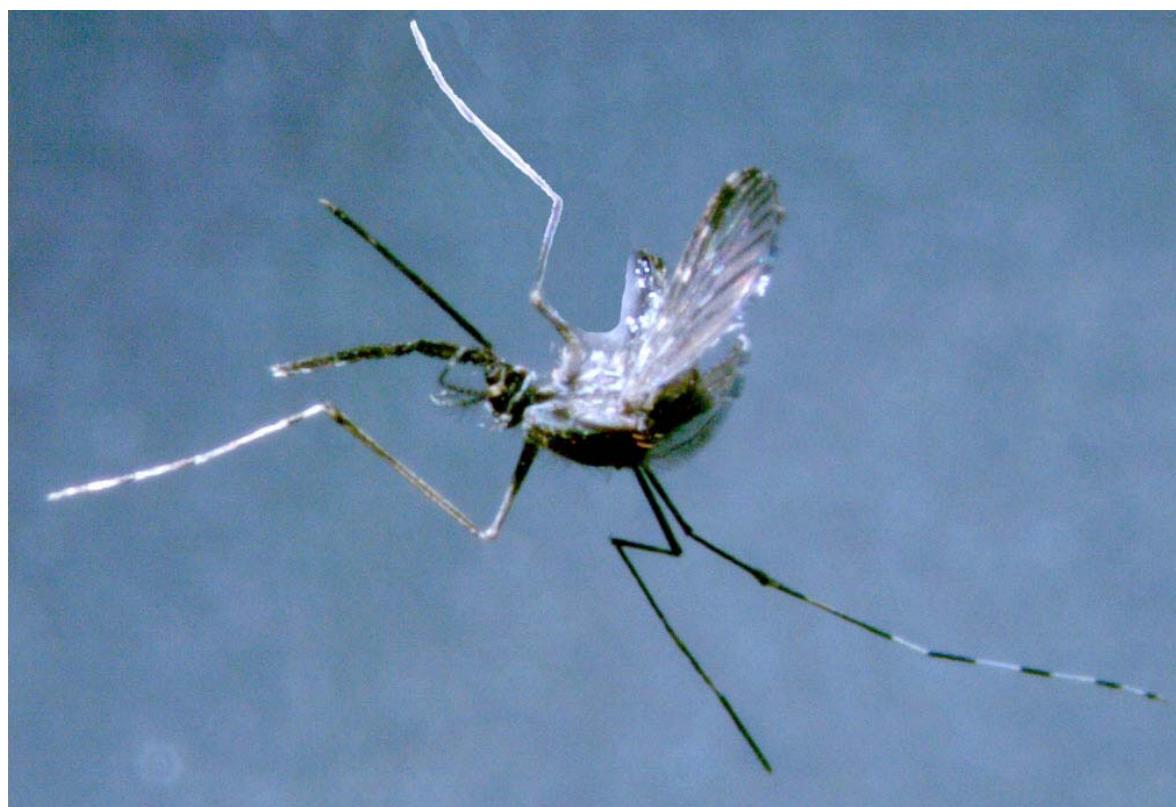
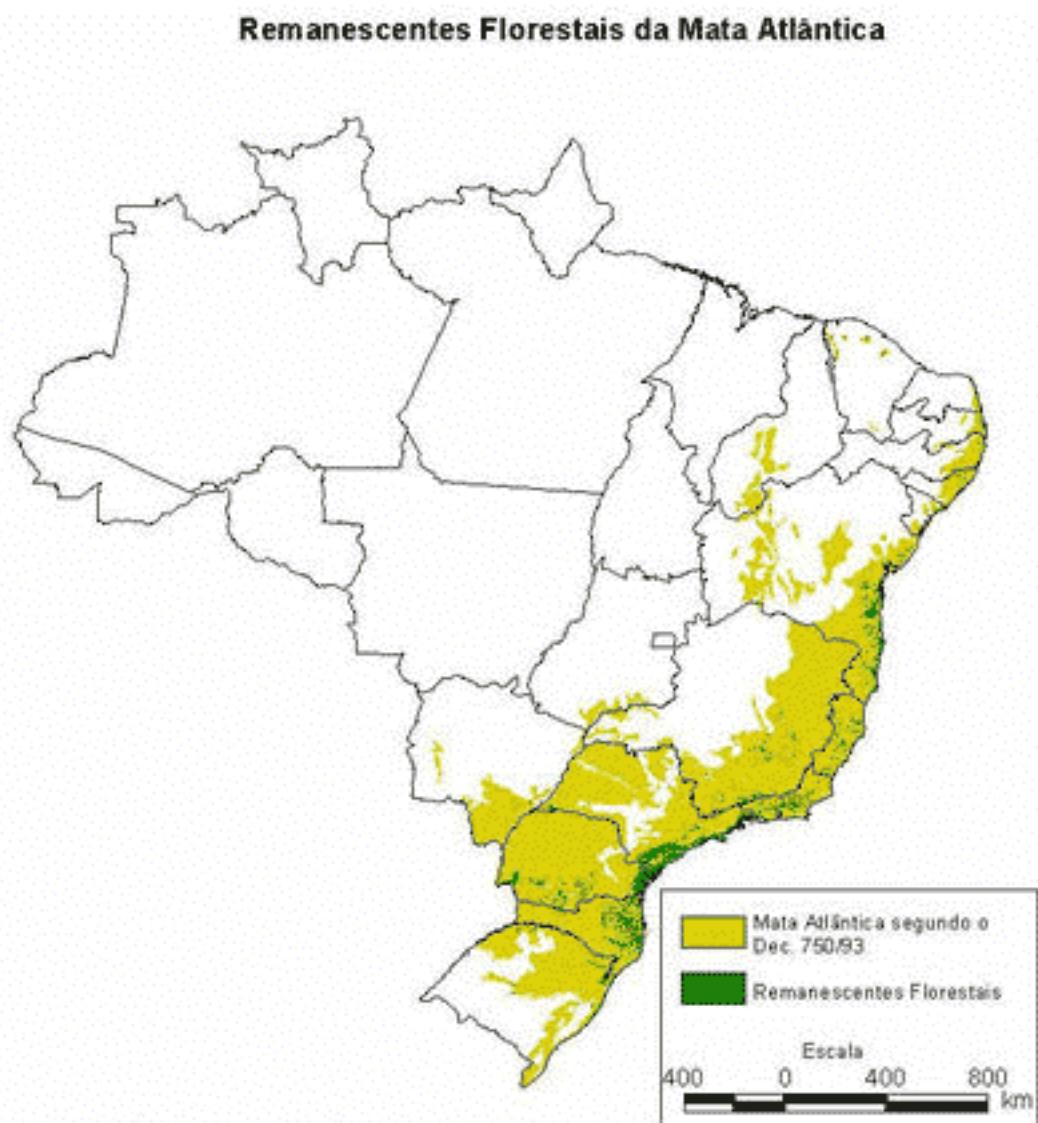


Figura 4. Distribuição da Mata Atlântica



Fonte: FUNDAÇÃO SOS MATA ATLÂNTICA / INPE / ISA.
"Atlas da Evolução dos Remanescentes Florestais e Ecossistemas
Associados no Domínio da Mata Atlântica", 1998.

C.I./FUND. BIODIVERSITAS/SOC. NORDESTINA DE ECOLOGIA
Dados organizados para o Workshop "Prioridades para conservação
da biodiversidade da Mata Atlântica no Nordeste", 1993.

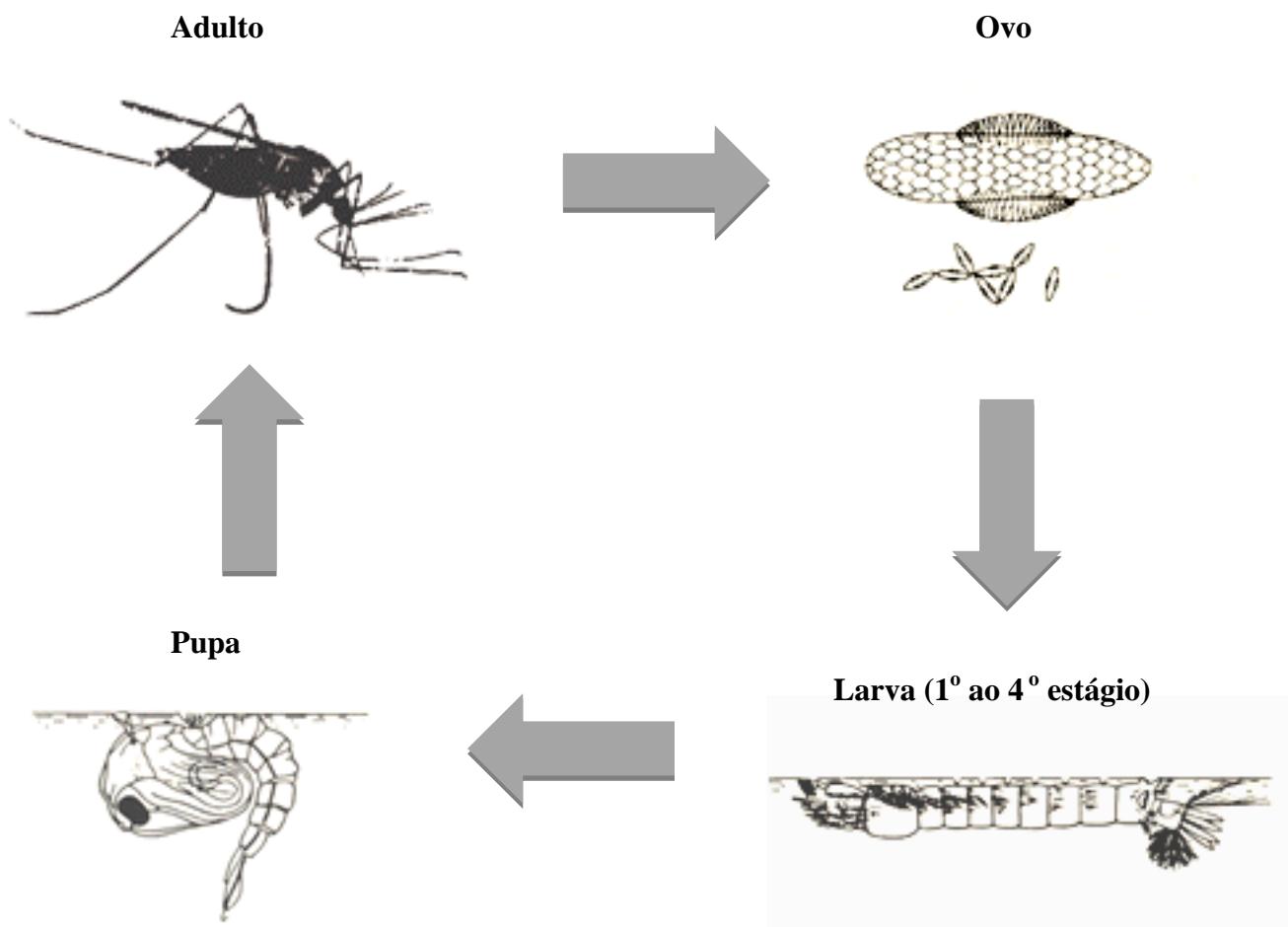
Figura 5. Local de coleta de fêmeas de *Anopheles cruzii*, UCAD (Unidade de Conservação Desterro), Florianópolis, Santa Catarina (SC) ($27^{\circ}31'S$ / $48^{\circ}30'W$).



As fêmeas são altamente antropofílicas e picam preferencialmente durante a noite (Veloso *et al.*, 1956; Corrêa *et al.*, 1961; Aragão, 1963), podendo picar mais de um hospedeiro para completar a maturação de seus ovos, o que é epidemiologicamente relevante para a transmissão da malária (Wilkerson & Peyton, 1991; Natal & Barata, 1998; Dalla Bona & Navarro-Silva, 2006).

Durante seu ciclo de vida, *Anopheles cruzii* passa por quatro estágios: ovo, larva, pupa, e adulto. Em condições de laboratório, Wilkerson & Peyton (1991) verificaram que a duração da fase de ovo ao adulto é de aproximadamente 35 dias e Chahad-Ehlers *et al.* (2007) observaram que cerca de 31 dias após a deposição do ovo, as pupas transformam-se em mosquitos adultos. Estes últimos autores observaram também que após 4 dias da alimentação sanguínea ocorre a oviposição e os ovos demoram um pouco mais 4 dias para maturar. As larvas passam por quatro estágios, sofrendo, posteriormente, metamorfose em pupas e a duração do primeiro ao quarto estágio larval é de aproximadamente 24 dias. As pupas continuam a desenvolver-se em mosquitos adultos, o que leva cerca de 3 dias (Chahad-Ehlers *et al.*, 2007). Um esquema completo do ciclo de vida de *An. cruzii* está mostrado na Figura 6.

Figura 6. Ciclo de vida de *Anopheles cruzii*. A longevidade da alimentação sanguínea até a emergência do adulto dura 35.22 ± 1.54 dias (observação feita em laboratório sob natural fotoperíodo a temperatura de $25.1 \pm 0.6 ^\circ\text{C}$ e 57 – 81% de humidade relativa) (Chahad-Ehlers *et al.*, 2007).



Entre 1930 e 1960, *An. cruzii* juntamente com *An. bellator* e *An. homunculus*, foram considerados os principais vetores da malária uma vez endêmica no sul do Brasil (Rachou, 1958). Métodos de controle reduziram consideravelmente e até interromperam a transmissão da doença em algumas áreas, mas a erradicação da doença não foi alcançada e *An. cruzii* é ainda responsável por vários casos de malária, principalmente oligossitomática, no sul e sudeste do Brasil.

A Região Amazônica é uma área altamente endêmica para malária. A detecção de casos importados da doença, proveniente dessa região, é freqüente em diferentes estados brasileiros (MS – Ministério da Saúde). Apesar da ocorrência de casos autóctones, reportados num estudo realizado em Santa Catarina, a maioria dos casos encontrados neste estado foi importada da Região Amazônica (Machado *et al.*, 2003). Em São Paulo e Rio de Janeiro, onde *An. cruzii* é considerado um dos principais vetores da doença, assim como na Bahia, onde *Anopheles (Nyssorynchus)* spp. são considerados os principais vetores (SESAB; Davis & Kumm, 1932), vários casos importados e autóctones de malária são reportados todos os anos (SINAN). Reforçando a importância epidemiológica de *An. cruzii* como vetor de malária na região sudeste do Brasil, outro estudo realizado no Espírito Santo, onde os casos de malária autóctone estão distribuídos na região serrana próximo aos fragmentos de Mata Atlântica, incluindo a localidade de Santa Teresa, sugeriu que esta espécie é o potencial vetor dos casos de malária autóctone neste Estado (Rezende *et al.*, 2005).

Recentemente, estudos entomológicos e de infecção natural confirmaram que *An. cruzii* é responsável pela transmissão da malária neste Estado já que a detecção de *Plasmodium vivax* no tórax desta espécie, por meio de PCR, reforça esse argumento (Rezende *et al.*, 2009).

Além disso, *An. cruzii* é o único vetor natural de malária simiana que se conhece, transmitindo os plasmódios de macacos que também são infectantes para o homem (Deane *et al.*, 1970; 1971). Em locais onde ocorrem proporções parecidas entre a densidade desse vetor nas copas e junto ao solo, pode ocorrer infecção humana por plasmódios simianos (Deane, 1984; Ueno *et al.*, 2007).

O uso de medidas de controle e o estudo epidemiológico são necessários para evitar a expansão ou a introdução da malária em áreas onde os vetores são encontrados em abundância e os hospedeiros humanos susceptíveis estão presentes. Então, o acesso ao *status* epidemiológico de tais localidades assim como o conhecimento sobre a biologia, o comportamento e as características genéticas das espécies vetoras são potencialmente relevantes para prevenir a ocorrência de surtos de malária ou a conduzir estratégias de controle, especialmente em áreas que foram endêmicas no passado.

Complexo de espécies

Apesar da importância epidemiológica, os dados disponíveis a respeito de *Anopheles cruzii* são insuficientes, provavelmente devido a dificuldade de manutenção das espécies sob condições de laboratório e porque em muitas áreas, o número de mosquitos capturados é pequeno (Carvalho-Pinto, informação pessoal). Assim, pouco é conhecido a respeito da variabilidade genética de populações de *An. cruzii* e o seu *status taxônomico* é ainda confuso.

Zavortink (1973) observou diferenças morfológicas entre as larvas de *An. cruzii* das populações provenientes dos Estados do Rio de Janeiro e de Santa Catarina, sugerindo que *An. cruzii* poderia representar mais de uma única espécie.

Ramirez & Dessen (2000a, 2000b) estudando o padrão de bandeamento em cromossomos politênicos de diferentes populações dos estados de São Paulo e Santa Catarina, observaram a existência de três formas cromossômicas e sugeriram a existência de pelo menos três espécies crípticas, nomeadas provisoriamente por *Anopheles cruzii A, B e C*. As populações que apresentam duas formas alternativas de cromossomo X em simpatria são: Cananéia e Guaratuba, no Estado de São Paulo, onde as formas de cromossomo X denominadas A e B aparecem juntas; Boracéia e Juquitiba, também no Estado de São Paulo, onde as formas A e C aparecem juntas. Já na Aldeia dos Índios,

que fica situada em Peruíbe, Estado de São Paulo, apenas a forma A está presente e em São Francisco do Sul, Santa Catarina, apenas a forma B. Nas populações com dois tipos de X em simpatria, a proporção de um dos tipos é sempre baixa (Ramirez & Dessem, 2000a,b).

Onde as formas A/B e A/C coexistem, heterozigotos entre os grupos não foram detectados na natureza. A falta de heterozigotos para estas formas cromossômicas em populações naturais, onde elas coexistem, é uma evidência de fluxo gênico muito limitado (ou inexistente) entre estes grupos (Ramirez & Dessen, 2000a, 2000b).

Outro estudo utilizando a análise de isoenzimas e do perfil de restrição do DNA mitocondrial sugere a ocorrência de apenas dois grupos geneticamente isolados, um formado pela população da Bahia e o outro pelas populações do sul e sudeste do Brasil (RJ, SP e SC) (Carvalho-Pinto, 2001; Carvalho-Pinto & Lourenço-de-Oliveira, 2004). Reforçando o estudo anterior e baseado nas seqüências de ITS2 (região intergênica de genes do DNA ribossomal), Malafronte *et al.* (2007) não conseguem afirmar a existência de mais de uma espécie críptica nas populações de *An. cruzii* coletadas no sul e sudeste do Brasil.

Esclarecer a estrutura genética populacional desses complexos de espécies, como parece ser o caso de *An. cruzii*, é um pré-requisito para determinar quais membros do complexo são verdadeiros vetores da malária, e

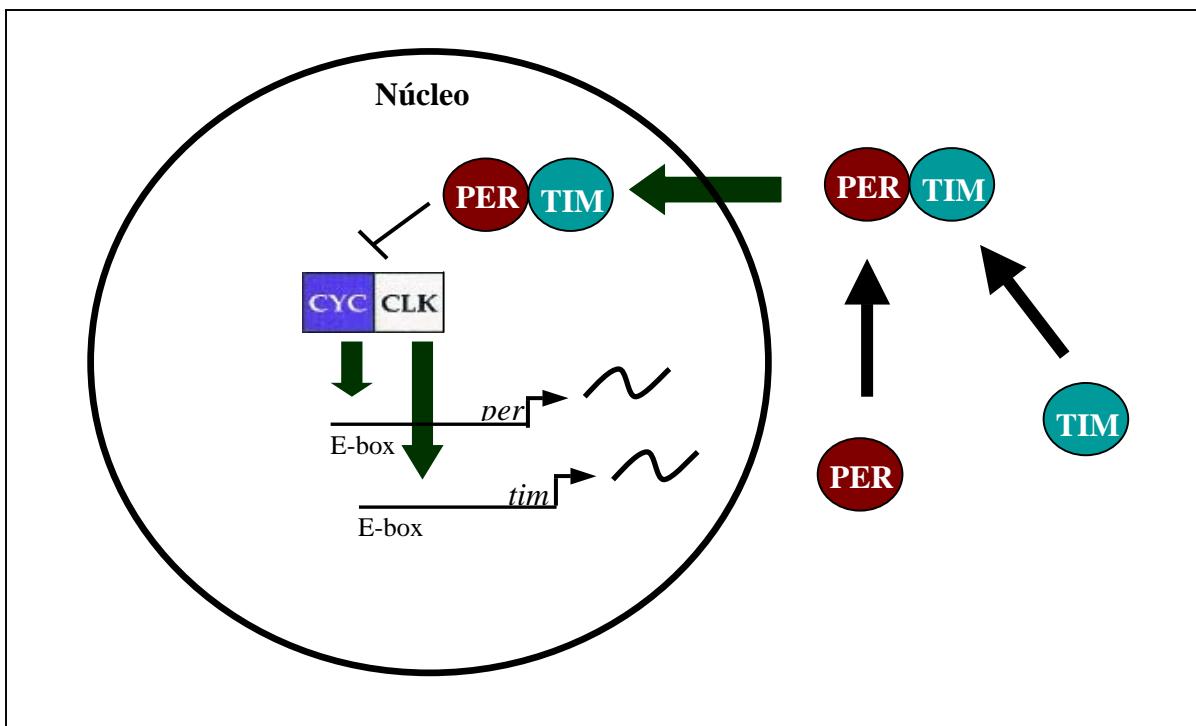
desvendar as diferenças ecológicas e etológicas que são tão importantes na transmissão da doença.

O conhecimento da existência dessas espécies permite a execução de estratégias apropriadas de controle vetorial àquelas que realmente tem responsabilidade na transmissão da doença (Deane, 1992). O sucesso no combate à malária tem sido complementado com o controle do mosquito vetor. Hoje em dia, esta estratégia é muitas vezes falha, devido a várias razões, que vão desde o desenvolvimento de populações resistentes a inseticida, a falta de conhecimento básico sobre a biologia desses vetores.

Análise molecular

Ritmos circadianos já foram estudados em vetores de malária pertencentes ao subgênero *Kerteszia* (Pittendrigh, 1950) e mais recentemente em *An. cruzii* (Chahad-Ehlers *et al.*, 2007). Genes que controlam os ritmos circadianos podem contribuir bastante para o nosso conhecimento desses vetores. Os padrões de atividade e alimentação apresentados por insetos hematófagos, tão importantes na transmissão de doenças, são certamente controlados por relógios biológicos internos, que estão por sua vez sob controle genético (Stanewsky, 2003; Hardin, 2005). Além disso, genes do relógio biológico também estão envolvidos nos ritmos de atividade sexual, potencialmente importante no isolamento reprodutivo entre espécies próximas (Sakai & Ishida, 2001; Tauber *et al.*, 2003; Hardin, 2005). Os quatro principais genes do relógio biológico de *Drosophila* são *period*, *timeless*, *Clock* e *cycle*. Os dois últimos codificam fatores de transcrição que juntos ativam a expressão cíclica de *period* e *timeless*. Estes por sua vez codificam proteínas que interagem e reprimem o heterodímero CLOCK-CYCLE fechando assim uma alça de autoreregulação negativa (Figura 7) que é muito importante no controle dos ritmos circadianos.

Figura 7. Esquema da regulação dos genes do relógio biológico.



Modificado de Scully & Kay 2000

Os genes *period* (*per*) e *timeless* (*tim*) são ativados pelos fatores de transcrição *dClock* (*dClk*) e *cycle* (*cyc*). Estes genes são traduzidos formando as proteínas PER e TIM, que precisam formar um heterodímero para entrar no núcleo. A região E-box é um sítio de ligação dos fatores de transcrição dCLK e CYC, e estes dimerizados se ligam aos E-box nas regiões promotoras de *per* e *tim* ativando suas transcrições. TIM se liga a PER, e a formação deste dímero é necessária para a estabilização do último. A entrada deste dímero no núcleo é necessária para que PER exerça sua função inibitória. PER se liga a dCLK:CYC e os inibe, formando com isso um “feedback” negativo. Quando PER não está ligado a TIM, ele se torna uma proteína instável quando fosforilada por uma quinase chamada DOUBLETIME (DBT) e com isso sinalizado para degradação. No início do dia, TIM é marcada para degradação pela proteína CRY que é ativada pela luz (Scully & Kay, 2000). Este mecanismo de autoregulação negativa é responsável pela variação cíclica de um grande número de genes de “output” do relógio que controlam os ritmos no comportamento e fisiologia.

O gene *cpr* que codifica a enzima “NADH–citocromo P450 redutase” em *Anopheles gambiae* e em *Drosophila melanogaster*, além de estar envolvido na resistência a inseticida, também tem um possível papel tanto no comportamento sexual quanto no olfato, o que também pode ser potencialmente importante no isolamento reprodutivo entre espécies próximas. Em *An. gambiae* este gene está localizado no cromossomo X (Hovemann et al., 1997; Wen and Scott, 2001; Kasai and Tomita, 2003; Nikou et al., 2003), onde estudos anteriores mostraram a existência de inversões cromossômicas sugerindo três diferentes espécies dentro do táxon *An. cruzii* (Ramirez & Dessem, 2000a,b).

No presente estudo, analisamos a diferenciação genética entre diferentes populações brasileiras de *An. cruzii* (Florianópolis – SC; Cananéia e Juquitiba – SP; Itatiaia – RJ; Santa Teresa – ES, Itaparica – BA) utilizando análises moleculares. Foram selecionados e analisados genes que estão em diferentes braços cromossômicos de *An. gambiae*. Entre eles estão genes que fazem parte do controle do relógio circadiano (*timeless*, *Clock* e *cycle*), um gene controlado pelo relógio biológico e localizado no cromossomo X em *An. gambiae* (*cpr*) e também genes constitutivos que codificam proteínas ribossomais utilizados para efeito de comparação com os demais (*rp49*, *rpS2* e *rpS29*). Desta forma, estaremos comparando o grau de diferenciação genética entre populações de *An. cruzii* utilizando diferentes marcadores moleculares provavelmente posicionados em diferentes regiões do genoma, com o objetivo de entendermos o quão estruturadas estão estas populações e esclarecer se estes mosquitos constituem uma única espécie ou se formam um complexo de espécies críticas.

O presente estudo esta dividido em três partes. A primeira parte aborda a variação molecular e o grau de diferenciação genética no gene *timeless* em seis populações de *Anopheles cruzii*. As populações provenientes de Florianópolis - SC, Cananéia e Juquitiba - SP e Itatiaia - RJ foram analisadas durante o mestrado. Durante o doutorado, as populações de Santa Teresa - ES e de Itaparica - BA também foram incluídas neste estudo.

A segunda parte deste estudo, aborda uma análise *multilocus* utilizando seis genes nucleares, sendo que três estão envolvidos no controle dos ritmos circadianos (*timeless*, *Clock* e *cycle*) e os demais são genes constitutivos que codificam proteínas ribossomais (*rp49*, *rpS2* e *rpS29*) nas populações de *An. cruzii* provenientes de Florianópolis - SC e Itaparica - BA. O objetivo desta análise é determinar se a diferenciação genética entre estas duas populações é afetada por muitos *loci* ou se esta apenas concentrada nos genes do relógio biológico. Além disso, todos os genes juntos foram utilizados para estimar o tempo de divergência entre Florianópolis e Itaparica.

A última parte aborda a variabilidade intraespecífica e a divergência genética entre cinco populações de *An. cruzii* provenientes de Florianópolis – SC, Cananéia e Juquitiba – SP, Itatiaia - RJ e Santa Teresa – ES utilizando o gene *cpr*.

OBJETIVO GERAL

Analisar a diferenciação genética entre diferentes populações de *Anopheles cruzii* utilizando uma análise *multilocus* e confirmar se esta é de fato um complexo de espécies crípticas.

OBJETIVOS ESPECÍFICOS

- Isolar em *Anopheles cruzii* fragmentos homólogos aos genes *Clock*, *cycle*, *cpr*, *Rp49*, *RpS2* e *RpS29* de *Drosophila melanogaster*;
- Investigar a variação molecular e o grau de diferenciação genética no gene *timeless* em seis populações de *Anopheles cruzii* provenientes de Florianópolis – SC, Cananéia e Juquitiba – SP, Itatiaia – RJ, Santa Teresa – ES e de Itaparica – BA;
- Realizar uma análise *multilocus* utilizando seis genes nucleares (*timeless*, *Clock*, *cycle*, *Rp49*, *RpS2* e *RpS29*) nas espécies do complexo *An. cruzii* provenientes de Florianópolis – SC e Itaparica – BA para estimar o fluxo gênico e o tempo de divergência entre elas;
- Analisar a variabilidade intraespecífica e a divergência genética entre cinco populações de *An. cruzii* provenientes de Florianópolis – SC, Cananéia e Juquitiba – SP, Itatiaia – RJ e Santa Teresa – ES utilizando o gene *cpr*.

CAPÍTULO 1

Análise da divergência molecular entre populações de *Anopheles (Kerteszia) cruzii* do Brasil utilizando o gene *timeless*: evidências adicionais para um complexo de espécies.

Esse artigo científico foi publicado em 2009. Neste estudo, a variação molecular e o grau de diferenciação genética em seis populações de *Anopheles cruzii* foram analisados utilizando um fragmento do gene *timeless*. Das seis populações utilizadas neste estudo, quatro foram analisadas durante o mestrado.

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Research

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Assessing the molecular divergence between *Anopheles (Kerteszia) cruzii* populations from Brazil using the *timeless* gene: further evidence of a species complex

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Abstract

Background: *Anopheles (Kerteszia) cruzii* was the most important vector of human malaria in southern Brazil between 1930–1960. Nowadays it is still considered an important *Plasmodium* spp. vector in southern and south-eastern Brazil, incriminated for oligosymptomatic malaria. Previous studies based on the analysis of X chromosome banding patterns and inversion frequencies in *An. cruzii* populations from these areas have suggested the occurrence of three sibling species. In contrast, two genetically distinct groups among *An. cruzii* populations from south/south-east and north-east Brazil have been revealed by isoenzyme analysis. Therefore, *An. cruzii* remains unclear.

Methods: In this study, a partial sequence of the *timeless* gene (~400 bp), a locus involved in the control of circadian rhythms, was used as a molecular marker to assess the genetic differentiation between *An. cruzii* populations from six geographically distinct areas of Brazil.

Results: The *timeless* gene revealed that *An. cruzii* from Itaparica Island, Bahia State (north-east Brazil), constitutes a highly differentiated group compared with the other five populations from south and south-east Brazil. In addition, significant genetic differences were also observed among some of the latter populations.

Conclusion: Analysis of the genetic differentiation in the *timeless* gene among *An. cruzii* populations from different areas of Brazil indicated that this malaria vector is a complex of at least two cryptic species. The data also suggest that further work might support the occurrence of other siblings within this complex in Brazil.

Background

Anopheles cruzii is one of the few mosquito species belonging to the subgenus *Kerteszia*. Immature stages of this species are found associated with water trapped in the

interfoliar space of plants from the Bromeliaceae family, which are abundant in the Brazilian Atlantic forest [1-3]. Accordingly, the distribution of these bromeliad-breeding mosquitoes is restricted to the Atlantic forest, which

stretches from the coast of Rio Grande do Sul State (southern Brazil) to Sergipe State (north-eastern Brazil) [4,5].

The adults are found in a variety of habitats, from sea level in coastal areas to the mountains. Females are strongly anthropophilic and preferably bite during the evening [2,6,7], perhaps biting more than one host to complete egg maturation, which is epidemiologically relevant for malaria transmission [8-10].

Between 1930 and 1960, *An. cruzii* together with *Anopheles bellator* and *Anopheles homunculus*, which also belong to *Kerteszia*, were considered the main vectors of malaria when the disease was endemic in southern Brazil. Vector control measures have significantly reduced or even interrupted malaria transmission in some areas, but eradication of the pathogen was not achieved and *An. cruzii* is still responsible for several oligosymptomatic malaria cases in southern and south-eastern Brazil.

The Amazon region is highly endemic for human malaria, caused by *Plasmodium vivax* and *Plasmodium falciparum*, and imported cases are frequently reported in different states due to emigration from this region [11,12]. However, several autochthonous cases were reported in a study in Santa Catarina State, southern Brazil [12]. In the states of São Paulo and Rio de Janeiro, as well as in the state of Bahia, where *An. cruzii* and *Anopheles (Nyssorhynchus)* spp. are considered the main vectors of the disease, respectively [3,7,13,14], several imported and autochthonous cases of malaria are reported every year in the Atlantic forest region [15]. Reinforcing the epidemiological importance of *An. cruzii* as a malaria vector in south-east Brazil, another recent study in Espírito Santo State, including the locality of Santa Teresa, suggested that this species is the potential vector of recent autochthonous cases of malaria in this state [16].

Anopheles cruzii is also a natural vector of simian malaria in Rio de Janeiro and São Paulo States [17]. Studies on seasonal and vertical distribution of *An. cruzii* in coastal São Paulo State demonstrated high vertical mobility from ground level to tree tops, with significantly more activity in the uppermost branch layer of the forest [18]. This behaviour could be responsible for human infection by simian *Plasmodium* species [19,20].

Epidemiological surveillance and the use of control measures are required to avoid the expansion or introduction of malaria in areas where vector species are abundant and susceptible humans are present. Thus, assessment of the epidemiological status of such localities as well as knowledge concerning the biology, behaviour and the genetic characteristics of the vector species are relevant to prevent

the occurrence of outbreaks or to lead control strategies, especially in formerly endemic areas.

Despite its epidemiological importance, there are only a few population genetic studies of *An. cruzii* [18,21], and its taxonomic status is unclear. *Anopheles cruzii* is polymorphic for chromosome rearrangements. Differences in inversions frequencies, and X chromosome banding patterns from south-eastern and southern Brazil, have suggested the existence of three sibling species [21-24]. On the other hand, isoenzymes indicated two genetically isolated groups, one from Bahia State (north-eastern Brazil), and the other from south-eastern and southern Brazil (Rio de Janeiro, São Paulo and Santa Catarina States) [25]. Finally, in a recent study based on sequence analysis of the second Internal Transcribed Spacer of the nuclear ribosomal DNA (ITS2), the authors found no conclusive evidence for sibling species among samples of *An. cruzii* from south-eastern and southern Brazilian localities [26].

The activity and feeding rhythms of insect vectors are very important to disease transmission. These patterns are controlled by endogenous circadian clocks, which are under genetic control [27]. Furthermore, clock genes are also involved in the control of mating rhythms that are potentially important in maintaining sexual isolation between closely related species [28,29].

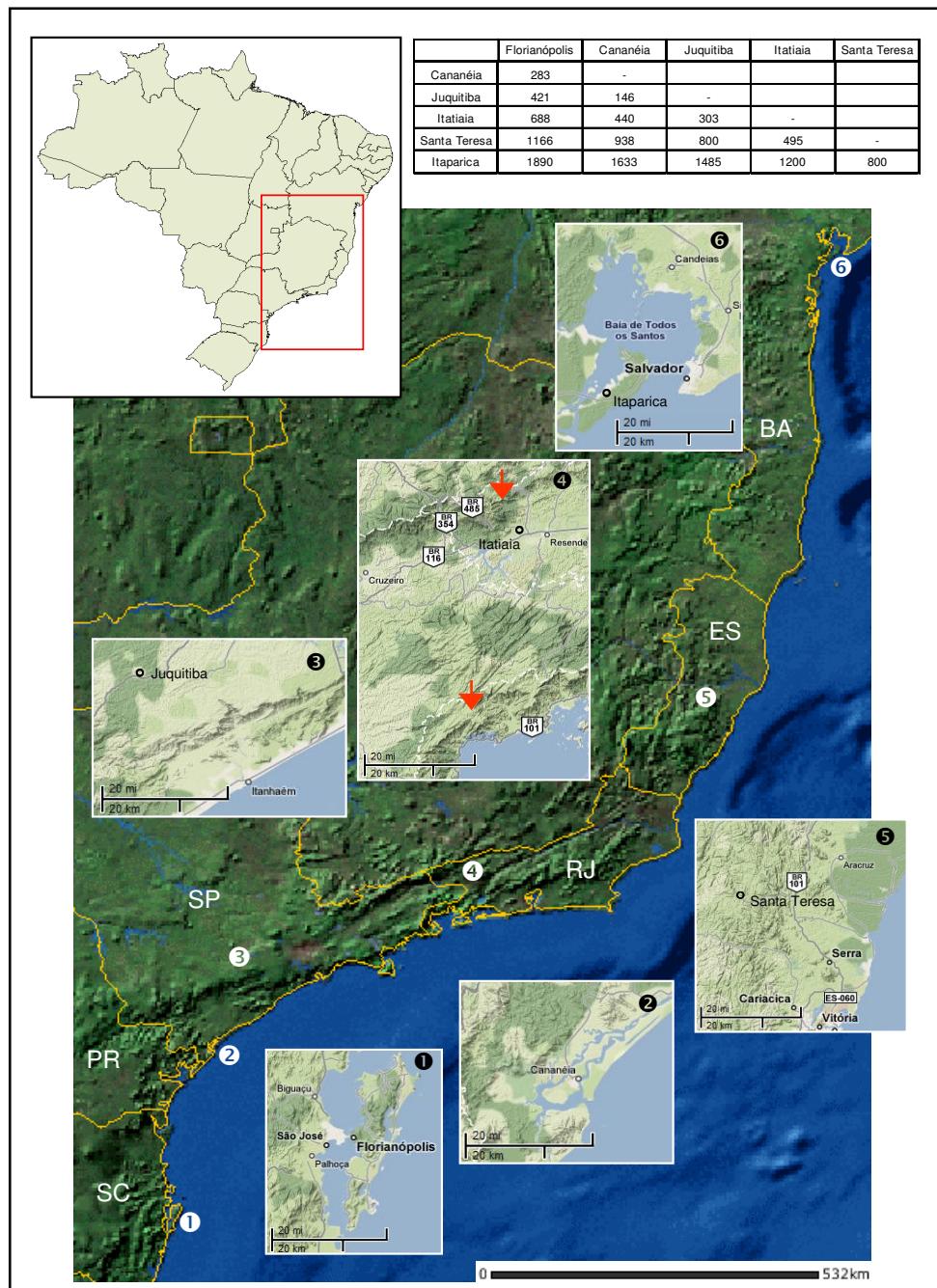
The circadian rhythms of malaria vectors belonging to the subgenus *Kerteszia* were formerly studied by Pittendrigh [30] and, recently, these rhythms were also studied in *An. cruzii* [31]. The *timeless* gene is involved in the control of activity rhythms in *Drosophila* [27], and controls differences in mating rhythms between closely related *Drosophila* species [28].

In the present study, a fragment of ~400 bp of the *An. cruzii* *timeless* gene was used as a molecular marker to assess intraspecific variability and genetic divergence among six populations of *An. cruzii* captured in different locations within the geographic distribution range of this species in Brazil.

Methods

Mosquitoes

All mosquitoes used in this study were females captured at the following localities along the Brazilian Atlantic forest: Florianópolis, Santa Catarina State (SC) ($27^{\circ}31'S/48^{\circ}30'W$), Cananéia and Juquitiba, São Paulo State (SP) ($25^{\circ}01'S/47^{\circ}55'W$ and $23^{\circ}57'S/47^{\circ}03'W$), Itatiaia, Rio de Janeiro State (RJ) ($22^{\circ}27'S/44^{\circ}36'W$), Santa Teresa, Espírito Santo State (ES) ($19^{\circ}56'S/40^{\circ}35'W$) and Itaparica Island (Jaguaribe), Bahia State (BA) ($13^{\circ}05'S/38^{\circ}48'W$) (Figure 1). All mosquitoes were primarily identified on the basis of their morphology according to Con-

**Figure 1**

Anopheles cruzii populations. Localities where the six Brazilian *An. cruzii* populations were collected. Values in table are approximated distances between localities in km. The red arrows on box 4 show the two mountain chains around Itatiaia. The upper arrow shows the Serra da Mantiqueira and the lower shows Serra do Mar mountain chains (Source: IBGE and Google Maps).

soli and Lourenço-de-Oliveira [5]. A total of 67 individuals (12 from Florianópolis, 12 from Cananéia, 11 from Juquitiba, 12 from Itatiaia, 6 from Santa Teresa and 14 from Itaparica, Bahia) were used for molecular assays.

Isolation of the *An. cruzii* timeless gene sequence

To design specific primers for the *An. cruzii* *timeless* gene sequence, genomic DNA was extracted from 10 females according to Jowett [32]. Initially, a pair of degenerated primers based on conserved regions of the TIMELESS proteins from *Drosophila melanogaster* and *Anopheles gambiae* named here 5'timdeg03 and 3'timdeg03 was used (Table 1; see also Figure 2) [33]. PCR was carried out with an Eppendorf Mastercycler® thermocycler using the following conditions: 15 cycles at 94°C for 60 s, 50°C (decreasing 1°C/cycle) for 90 s and 72°C for 60 s, following 20 cycles of 94°C for 60 s, 50°C for 90 s and 72°C for 60 s. The products obtained were then purified and cloned in either Zero Blunt TOPO PCR cloning kit (Invitrogen) or pMOS Blue vector blunt-ended cloning kit (Amersham Biosciences). Sequencing of positive clones was carried out in an ABI Prism 377 or ABI Prism 3730 DNA sequencer at the Oswaldo Cruz Institute using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The identity of the cloned fragments was determined by BlastX analysis using the GenBank [34]. To enlarge the *timeless* gene fragment in *An. cruzii*, a specific forward primer (5'darltim02a) based on a fragment of the *Anopheles darlingi* *timeless* gene (Gentile & Peixoto, unpublished) was used in combination with the specific reverse primer previously designed for *An. cruzii* (3'cruziitim03) in a PCR that amplified a fragment of ~450 bp. This 450 bp fragment from the *An. cruzii* genome was then purified, cloned and sequenced as above. After checking the sequence identity, two new specific forward primers named 5'cruziitim02 and 5'acbatim02a (Table 1 and Figure 2) were designed and in combination with the reverse primer 3'cruziitim03 allowed the amplification of a ~400 bp fragment of the *An. cruzii* *timeless* gene.

Interpopulational analysis of the *An. cruzii* timeless gene

Females were processed individually and genomic DNA was extracted as above [32]. PCR amplification was car-

Table 1: Sequence of primers used to amplify the *timeless* gene fragments

Primers Name	Sequence of primers at 5' → 3'
5'timdeg03	AARGARTTYACNGTNGAYTT (forward)
3'timdeg03	GTNACNARCCARAARAARTG (reverse)
3'cruziitim03	GACGTATCGATCTGCACTT (reverse)
5'cruziitim02	CGCTTCAATGCCGCAAATA (forward)
5'acbatim02a	GCCGCAAATAAGCACCG (forward)

Degenerate and specific primers used to amplify the *timeless* gene fragments in all *Anopheles cruzii* populations.

ried out for 35 cycles at 94°C for 30 s, 62°C for 60 s and 72°C for 90 s using the proofreading *Pfu* DNA polymerase (Biotoools) and primers 5'acbatim02a or 5'cruziitim02 and 3'cruziitim03 (Table 1). Negative controls (no DNA added) were included in all amplification reactions and pre- and post-PCR procedures did not share equipment or reagents. After cloning the fragments obtained as above, at least eight clones of each mosquito were sequenced and two consensus sequences representing both alleles were generated. When only one haplotype was observed among the eight sequences the mosquito was considered a homozygote. The probability that a heterozygote will be mistakenly classified as a homozygote with this procedure is less than 1%. Five mosquitoes were classified as homozygotes in Itatiaia, none in Florianópolis and one in each of the other four populations. The sequences obtained in homozygote mosquitoes were duplicated prior to analysis. However, the population genetics analysis was also carried out without duplicating the homozygote sequences and the results were very similar.

DNA sequence analysis

The *timeless* gene fragments were aligned using the GCG package (Wisconsin Package Version 10.2, Genetics Computer Group) and ClustalX software [35]. Analyses of the polymorphism and differentiation between populations were performed using DNAsP4.0 [36] and $P_{RO}S_{EQ}$ programs [37]. F_{ST} was calculated as described by Hudson *et al* [38] and significance was evaluated by 1,000 random permutations. Phylogenetic analysis was carried out using MEGA 4.0 [39] using the default parameters.

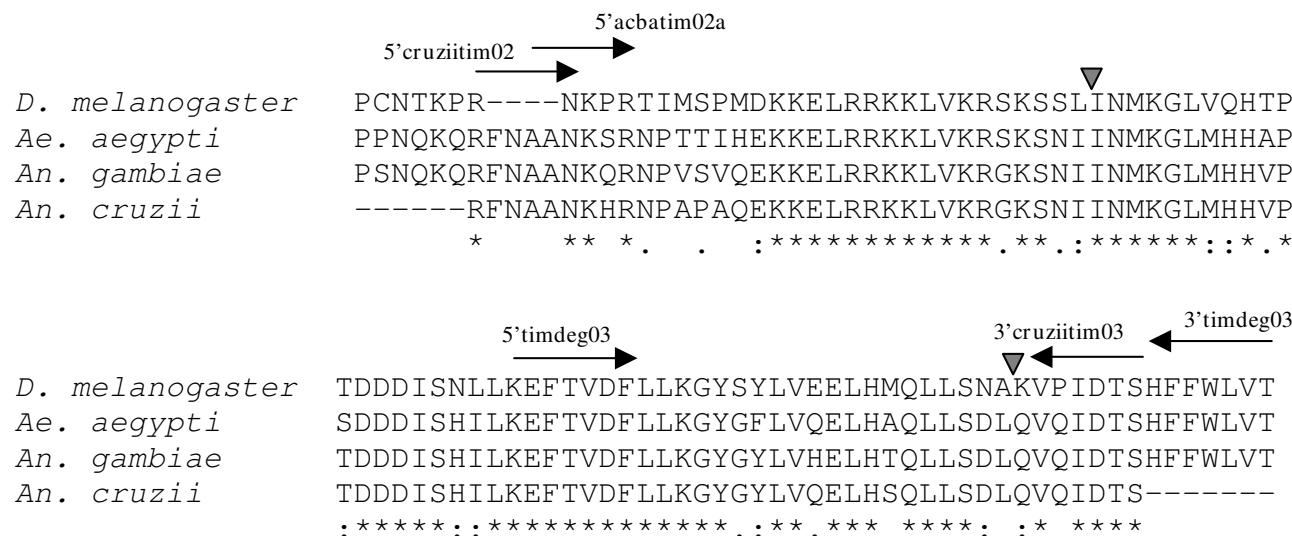
Results

Isolation of *An. cruzii* timeless gene fragment

Different PCR schemes were tested to amplify a fragment of the *An. cruzii* *timeless* gene (see Methods). Figure 2 shows an alignment of the predicted amino acid sequence encoded by this fragment obtained from *An. cruzii* compared to the TIMELESS protein of other insect species (*D. melanogaster*, *Aedes aegypti* and *An. gambiae*). A fairly high degree of inter-specific similarity is observed, but the putative protein encoded by 5' end of this fragment is variable, presenting some amino acid changes among the species compared. Figure 2 also shows the approximate positions of the two introns that occur in this region of the gene, as well as the location of the primers used to amplify the fragment from *An. cruzii* used for the population genetics analysis described below.

Molecular variation and divergence among *An. cruzii* populations

The geographic distribution of the six Brazilian populations of *An. cruzii* used in this study is shown at Figure 1. Initially, using the primers 5'cruziitim02 and 3'cruziitim03 (see Figure 2), a ~420 bp fragment of the

**Figure 2**

Timeless protein multiple alignment and primer positions. The putative fragment of *An. cruzii* TIMELESS deduced protein is aligned with *D. melanogaster*, *An. gambiae* and *Ae. aegypti* homologues. Arrows point to the approximated positions of the primers used in this study. The inverted triangles represent the positions of the two introns.

timeless gene was amplified in all *An. cruzii* populations analyzed, with the exception of samples from Bahia State (Itaparica Island), which revealed a ~400 bp amplification product, indicating the existence of length variation among the studied populations. The sample from Bahia, however, displayed lower amplification in some cases using these primers, and so a new internal forward primer named 5'acbatim02a (Table 1) was designed based on the initial sequences obtained. Using this new primer in conjunction with 3'cruziit03, a ~410 bp fragment of *time-*

less gene was obtained for all *An. cruzii* populations from south and south-east Brazil and a ~390 bp from Bahia.

A total of 24 sequences from Florianópolis, 24 from Cananéia, 22 from Juquitiba, 24 from Itatiaia, 12 from Santa Teresa and 28 from Itaparica (Bahia State) populations were obtained. The sequences were submitted to GenBank (accession numbers: FJ408732 – FJ408865). A full alignment of all sequences is shown in Additional file 1. Most of the base substitutions were silent or occurred

Table 2: Polymorphisms of all *An. cruzii* populations

Population	n	S	θ	π	D_T	D_{FL}	F_{FL}
Florianópolis	24	57 (17)	0.04258 (0.02322)	0.03018 (0.01483)	-1.00660 (-1.24295)	-0.62541 (-1.24456)	-0.87450 (-1.45349)
Cananéia	24	46 (12)	0.03334 (0.01665)	0.02677 (0.01021)	-0.64691 (-1.30282)	-0.47115 (-0.96989)	-0.61709 (-1.24756)
Juquitiba	22	52 (20)	0.03522 (0.02652)	0.03086 (0.02217)	-0.48955 (-0.51723)	-0.47485 (-0.34701)	-0.56076 (-0.46415)
Itatiaia	24	26 (12)	0.01864 (0.01665)	0.01829 (0.01825)	-0.00645 (0.40503)	-0.32168 (0.25917)	-0.25815 (0.35329)
Santa Teresa	12	35 (15)	0.03042 (0.02558)	0.02518 (0.02248)	-0.65598 (-0.41589)	-0.86793 (-0.58337)	-0.92570 (-0.61405)
Bahia	28	24 (9)	0.01661 (0.01099)	0.01035 (0.00571)	-1.31797 (-1.49603)	-0.83982 (-0.91433)	-1.16519 (-1.27249)

n, number of DNA sequences of each population; S, number of polymorphic (segregating) sites; θ , nucleotide diversity based on the total number of mutations (Θ); π , nucleotide diversity based on the average number of pair-wise differences; D_T , Tajima's [40]D; D_{FL} , Fu & Li's [41]D and F_{FL} , Fu & Li's [41]F, based on the total number of mutations. In no cases were Tajima's D-values or Fu & Li's D and F-values significant ($P > 0.10$ in all cases). The values in parentheses were calculated using only coding regions of the *timeless* gene fragment.

within the two introns, which show a number of indels. A few non-synonymous changes were also observed, causing seven amino acid differences among the sequences.

Table 2 shows the number of DNA sequences of each *An. cruzii* population studied (*n*) and the number of polymorphic sites (S). The values in parentheses were calculated using only coding regions of the *timeless* gene fragment. Based on the sequences, two measures of nucleotide diversity were calculated for each population: π , based on the average number of pair-wise differences and θ , based on the total number of mutations (Table 2). The population from Bahia was the least polymorphic, showing the lowest values of θ and π , as well as the smaller number of polymorphic sites (S). Table 2 also shows the results of Tajima [40] and Fu & Li [41] tests of natural selection, based on the total number of mutations of each population. In all cases, Tajima's *D* or Fu & Li's *D* and *F* statistics were non-significant ($P > 0.10$) indicating no deviations from neutrality.

Table 3 shows the pair-wise estimates of population differentiation (F_{ST}) between all *An. cruzii* populations. In all cases the F_{ST} values were significant, except for the comparison between Juquitiba and Santa Teresa when the coding regions of the *timeless* gene fragment were used. Very high F_{ST} values were found between Bahia State and the others (0.8353 – 0.8761). The average number of nucleotide substitutions per site (*Dxy*) and the number of net nucleotide substitutions per site between populations (*Da*) are shown in Table 3. The distribution of the four

mutually exclusive categories of segregating sites observed in each comparison, i.e. the number of polymorphisms exclusive for each population (S_1 and S_2), the number of shared polymorphisms (S_s) and the number of fixed differences (S_f) between populations are also included in Table 3. These polymorphic and fixed sites include some of the non-synonymous changes observed (see Table 4 for a detailed description).

The values using only coding regions (shown in parentheses in Table 3) show some differences compared with those obtained with the whole sequence. Yet even using the more conserved coding regions, the values of differentiation between the population from Bahia and all others revealed a high number of fixed differences and only a few shared polymorphisms. Among the southern and south-eastern populations, there were shared polymorphisms and no fixed differences, suggesting they belong to the same or to very closely related species.

Divergence time between *An. cruzii* populations

The estimate of the time of divergence between *An. cruzii* populations from Bahia and the others were calculated using the *Da* value based on the third codon positions. This estimate assumed that substitutions rates observed between *An. cruzii* from Bahia State and the other populations originally from southern regions of Brazil are similar to the estimated rates in the same fragment of the *timeless* gene between closely related *Drosophila persimilis* and *Drosophila pseudoobscura*, species that diverged around 0.85 millions of years ago (MYA) (FlyBase Accession Numbers

Table 3: Genetic differentiation between all *An. cruzii* populations

Populations	F_{ST}	P-value	<i>Dxy</i>	<i>Da</i>	S_s	S_f	S_1	S_2
1. Florianópolis × Cananéia	0.0548 (0.0622)	0.002 (0.003)	0.0308 (0.0136)	0.0017 (0.0008)	30 (7)	0 (0)	28 (11)	17 (6)
2. Juquitiba × Santa Teresa	0.0693 (0.0487)	0.040 (0.156)	0.0290 (0.0236)	0.0020 (0.0011)	21 (10)	0 (0)	26 (10)	11 (6)
3. Florianópolis × Juquitiba	0.0875 (0.1384)	0.000 (0.000)	0.0333 (0.0216)	0.0029 (0.0030)	21 (8)	0 (0)	37 (10)	26 (12)
4. Cananéia × Juquitiba	0.1077 (0.1849)	0.002 (0.000)	0.0322 (0.0201)	0.0035 (0.0037)	20 (5)	0 (0)	27 (8)	27 (15)
5. Florianópolis × Itatiaia	0.1450 (0.2078)	0.000 (0.000)	0.0293 (0.0213)	0.0042 (0.0044)	16 (7)	0 (0)	42 (11)	11 (6)
6. Florianópolis × Santa Teresa	0.1582 (0.2652)	0.000 (0.000)	0.0325 (0.0256)	0.0051 (0.0068)	14 (8)	0 (0)	44 (10)	18 (8)
7. Itatiaia × Santa Teresa	0.1837 (0.2414)	0.000 (0.000)	0.0265 (0.0273)	0.0049 (0.0066)	10 (6)	0 (0)	17 (7)	22 (10)
8. Juquitiba × Itatiaia	0.2030 (0.2078)	0.000 (0.000)	0.0310 (0.0258)	0.0063 (0.0054)	10 (6)	0 (0)	37 (14)	17 (7)
9. Cananéia × Santa Teresa	0.2154 (0.3152)	0.000 (0.000)	0.0328 (0.0243)	0.0071 (0.0076)	11 (4)	0 (0)	36 (9)	21 (12)
10. Cananéia × Itatiaia	0.2251 (0.2720)	0.000 (0.000)	0.0302 (0.0201)	0.0068 (0.0055)	8 (3)	0 (0)	39 (10)	19 (10)
11. Florianópolis × Bahia	0.8353 (0.8345)	0.000 (0.000)	0.1197 (0.0625)	0.1000 (0.0522)	6 (4)	27 (7)	52 (14)	17 (5)
12. Juquitiba × Bahia	0.8403 (0.7874)	0.000 (0.000)	0.1212 (0.0656)	0.1019 (0.0516)	2 (1)	30 (8)	45 (19)	21 (8)
13. Cananéia × Bahia	0.8506 (0.8703)	0.000 (0.000)	0.1211 (0.0626)	0.1030 (0.0545)	1 (0)	29 (8)	46 (13)	22 (9)
14. Santa Teresa × Bahia	0.8624 (0.7926)	0.000 (0.000)	0.1187 (0.0685)	0.1024 (0.0543)	3 (2)	32 (9)	29 (14)	20 (7)
15. Itatiaia × Bahia	0.8761 (0.8020)	0.000 (0.000)	0.1130 (0.0617)	0.0990 (0.0495)	3 (2)	30 (8)	24 (11)	20 (7)
16. *An. cruzii × Bahia	0.8370 (0.7935)	0.000 (0.000)	0.1187 (0.037)	0.0993 (0.0505)	8 (5)	25 (6)	107 (39)	15 (4)

F_{ST} , pair-wise estimates of population differentiation. P-value, significance of F_{ST} values (evaluated by 1,000 random permutations). *Dxy*, average number of nucleotide substitutions per site between populations [49]; *Da*, number of net nucleotide substitutions per site between populations [49]. S_1 , number of polymorphic sites exclusive to the first population shown in the first column. S_2 , number of polymorphic sites exclusive to the second population shown in the first column. S_s , number of shared polymorphisms between the two populations. S_f , number of fixed differences between the two populations. The values in parentheses were calculated using only coding regions of the *timeless* gene fragment. *An. cruzii: all populations from south and south-east Brazil together without Bahia population.

Table 4: Non-synonymous changes on the *timeless* gene fragment

Polymorphic Sites:			
Site Position:	Individuals:	Codon:	Amino acid:
05 (first codon base)	Individuals from all populations analysed Can03a	CCC	Proline
06 (second codon base)	Individuals from all populations analysed Juq66a; Juq66b; Can06b; Can12b	TCC	Serine
08 (first codon base)	Individuals from south and south-east populations All individuals from Bahia population and Flo37a; Can02b	CCC	Proline
18 (second codon base)	All individuals from south and south-east populations and Bahia19a; Bahia33a; Bahia20b Individuals from Bahia population	CTC	Leucine
		GCG	Alanine
		ACG	Threonine
		CAG	Glutamine
		CTG	Leucine

Fixed Differences:			
Site Position:	Individuals:	Codon:	Amino acid:
11 (first codon base)	All individuals from Florianópolis, Cananéia, Juquitiba, Itatiaia and Santa Teresa populations All individuals from Bahia population	CCG	Proline
188 (first codon base)	All individuals from Florianópolis, Cananéia, Juquitiba, Itatiaia and Santa Teresa populations All individuals from Bahia population	TCG	Serine
275 (first codon base)	All individuals from Florianópolis, Cananéia, Juquitiba, Itatiaia and Santa Teresa populations All individuals from Bahia population	ACG	Threonine
		TCG	Serine
		TCC	Serine
		ACC	Threonine

List of non-synonymous changes on the studied *timeless* gene fragment between *An. cruzii* populations. Flo: Florianópolis population; Can: Cananéia population; Juq: Juquitiba population; Ita: Itatiaia population; San: Santa Teresa population; Bahia: Bahia population.

FBtr0185090 and FBtr0282161, respectively) [42]. The divergence observed for the *timeless* gene between these two *Drosophila* species based on the third codon positions is 0.03030. Based on the *Da* value (0.05426), the estimated time of divergence between *An. cruzii* populations from south and south-east Brazil and that from Bahia State, is approximately 1.5 MYA.

Genealogy of the *An. cruzii* *timeless* sequences

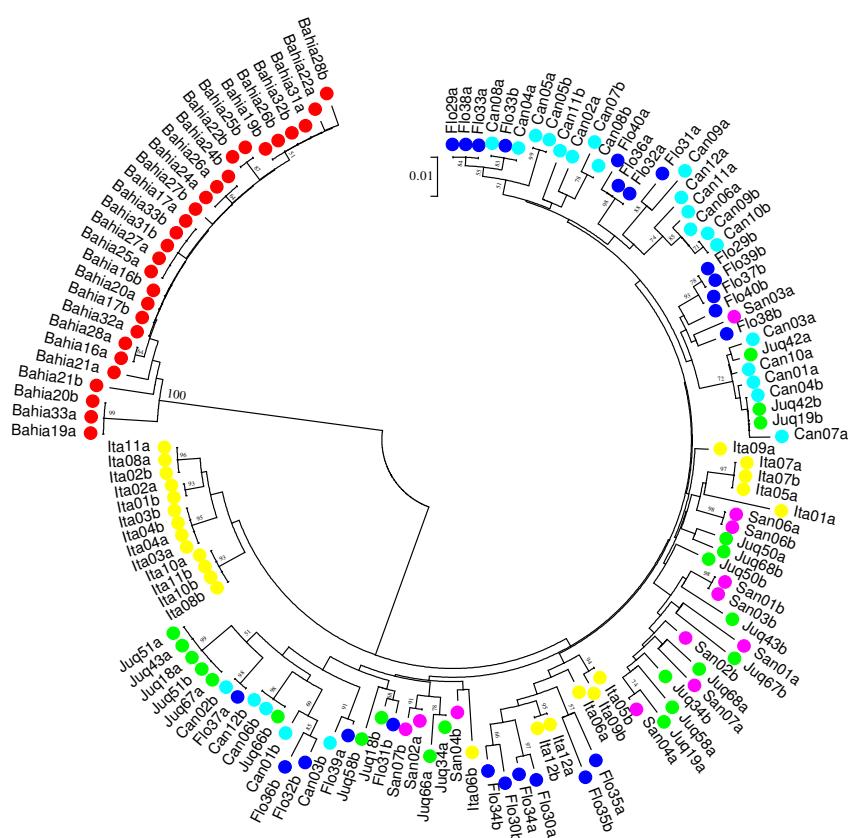
Figure 3 shows a Neighbour-joining tree of the sequences from all *An. cruzii* populations using the Kimura 2-parameter distance and the *timeless* gene sequences. The resulting tree showed no clear separation between the sequences of the populations from Florianópolis, Cananéia, Juquitiba, Itatiaia and Santa Teresa, but some differentiation was evident since the sequences do not appear at random in the tree, especially in the case of Itatiaia. The *An. cruzii* sequences from the Bahia population, however, were clearly separated on an isolated branch.

Discussion

Zavortink [4] pointed out morphological differences in the larval stage of populations of *An. cruzii* from Rio de Janeiro and Santa Catarina States, suggesting that *An. cruzii* could represent more than a single species. A moderately high F_{ST} value between Florianópolis (Santa Catarina State) and Itatiaia (Rio de Janeiro State) populations was reported here. In addition, comparison of Itatiaia with the other populations (excluding Bahia) revealed

even higher F_{ST} values, perhaps suggesting that this population is indeed in a process of differentiation and incipient speciation. Moreover, sequences from Itatiaia showed some clustering in the Neighbour-joining tree (Figure 3). Itatiaia was also the least polymorphic population of south and south-east Brazil and showed the highest number of homozygotes suggesting some inbreeding. It is possible that this reflects a smaller effective size and the relative isolation of this population, since its location in a valley between two mountain chains (Serra do Mar and Serra da Mantiqueira – Figure 1) might reduce gene flow with other populations.

In a recent review, Ayala and Coluzzi [43] argue that many siblings are outcomes of recent speciation processes associated with paracentric inversions, mostly involving the X chromosome. Ramirez and Dessen [23,24], studying the X chromosome banding patterns and inversion frequencies of distinct populations of *An. cruzii* from south and south-east Brazil, showed that there are three X chromosomal forms (A, B and C), suggesting a process of incipient speciation acting on *An. cruzii* populations. Among the localities analysed in this study, only Juquitiba and Cananéia were also investigated by Ramirez and Dessen [23,24]. They observed that in Juquitiba the majority of mosquitoes had form A and the remainder had form C, while in Cananéia form B predominated with the remainder having form A [23,24]. Although the differentiation in the *timeless* gene between these two populations is not

**Figure 3**

Neighbour-joining tree. Neighbour-joining tree using *timeless* nucleotide sequences of the *Anopheles cruzii* populations obtained with Kimura 2-parameters distance. Numbers on the nodes represent the percentage bootstrap values based on 1,000 replications. Flo: Florianópolis population; Can: Cananéia; Juq: Juquitiba; Ita: Itatiaia; San: Santa Teresa; Bahia: Itaparica Island population.

high, the F_{ST} value is significant and does not contradict the results of the chromosomal analysis. The relatively low differentiation in *timeless* among most populations from south and south-east Brazil might reflect introgression at this locus. It would be interesting to analyse the same populations with an X-linked molecular marker to see whether a higher level of differentiation is found.

Recently, Malafronte *et al* [26] compared sequences of ITS2 (Internal Spacer Region 2) from several *An. cruzii* populations from south and south-east Brazil. Although, they found some differences between sequences from different localities, including Juquitiba and Cananéia, they considered premature to conclude based on their results that there are distinct sibling species in the areas they investigated. Similar results were observed by Calado *et al* [44] using PCR-RAPD and PCR-RFLP of the ITS2 region.

Very strong evidence was presented here that confirms the existence of a different species in Bahia State, a finding that supports a previous isoenzyme study [25]. The extremely high F_{ST} values detected between this population and the other five populations studied, as well as the higher number of fixed differences observed, show that Bahia represents a different species. This population also presented lower levels of variability than those from south and south-east Brazil, possibly indicating a smaller population size or past founder effects. However, although the isoenzyme heterozygosity reported for Bahia is lower than Cananéia it is similar to that observed in Florianópolis [25].

A very rough estimate suggests that the divergence between the Bahia population and the more southern populations of *An. cruzii* possibly occurred around 1.5

MYA, during the Pleistocene. Climate changes during this period such as an intense precipitation decrease and more arid conditions fragmented the Brazilian Atlantic forest [45] creating refugia that played an important role in the differentiation among populations of a number of forest species, such as marmosets [46], tree frogs and many others [47]. Forest fragmentation has also been proposed to explain differentiation among populations of the Atlantic forest mosquito *Sabethes albiprivus* [48]. Since *An. cruzii* is also a forest-obligate species, it is possible that the Bahia and southern populations of this species complex suffered fragmentation due a constriction of the forest. Although Tajima's *D* and Fu & Li's *D* and *F* statistics were non-significant, they were negative in most cases and that is consistent with population expansion following the forest recovery after the Pleistocene. Analysis of a number of other molecular markers will allow more precise estimates of the divergence time between the Bahia population and those of south and south-east Brazil. It may also help in determining whether further *An. cruzii* siblings exist in the latter area.

Finally, although malaria cases are reported annually in Bahia State, the main vector implicated in *Plasmodium* spp. transmission in this area is *An. darlingi* and not *An. cruzii*, the most important vector in the southern states. This suggests that the differentiation observed within the *An. cruzii* complex might also explain aspects of the vectorial capacity of these mosquitoes, however further studies are needed to confirm or reject this hypothesis.

Conclusion

Analysis of the molecular polymorphism and genetic differentiation of the *timeless* gene among Brazilian populations of *An. cruzii* indicates that this malaria vector is a complex of at least two cryptic species, one occurring in the north-east (Bahia State) and another in south and south-east Brazil. In addition, the data also suggest that populations of the latter region might also constitute different incipient species and that further work might support the occurrence of other siblings within this complex in Brazil.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LDPR participated in data generation and analysis, and drafted the manuscript. She also helped capture mosquitoes in Florianópolis. CJCP carried out the capture and morphological identification of mosquitoes collected in Florianópolis and Itaparica. CG participated in the cloning of *An. cruzii* *timeless* gene fragments. ECG participated in the DNA sequencing and helped to write the manuscript. AAP is the principal investigator, participated in its

design and coordination, and helped to write the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Alignment of the DNA sequences of An. cruzii. Alignment of the DNA sequences from the timeless gene fragment from all populations of *An. cruzii* analysed. The translated amino acid sequence is shown above the alignment and the introns are presented in the darkened regions. Dots represent the identity of the first nucleotide sequence and asterisks represent the identity of all sequences. The non-synonymous changes found among the sequences are highlighted in yellow boxes. Flo: individuals from Florianópolis; Can: Cananéia; Juq: Juquitiba; Ita: Itatiaia; San: Santa Teresa; Bahia: individuals from Itaparica Island, Bahia State. Click here for file [<http://www.biomedcentral.com/content/supplementary/1475-2875-8-60-S1.doc>]

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RESUMO CAPÍTULO 1

- A análise da diferenciação genética do gene *timeless* entre as populações de *An. cruzii* confirma que este vetor forma um complexo de pelo menos duas espécies crípticas, uma proveniente de Itaparica (Bahia) e a outra do sul/sudeste do Brasil.
- Os dados também sugerem que as populações do sul/sudeste podem constituir diferentes espécies do complexo, principalmente no caso de Itatiaia.

CAPÍTULO 2

**Estimativa do tempo de divergência entre duas espécies do complexo
Anopheles (Kerteszia) cruzii usando uma abordagem *multilocus*. (artigo em
preparação)**

Neste artigo está descrita a análise *multilocus* de seis diferentes *loci* (*timeless*, *Clock*, *cycle*, *Rp49*, *RpS2* e *RpS29*) realizada entre duas populações do complexo *Anopheles cruzii* provenientes de Florianópolis – SC e Itaparica – BA com o objetivo de estimar o fluxo gênico e o tempo de divergência entre elas utilizando genes nucleares.

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Estimate of divergence time between two sibling species of *Anopheles (Kerteszia) cruzii* complex using a multilocus approach

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Abstract

Background

Anopheles cruzii is the primary human *Plasmodium* vector in south and south-east Brazil. The distribution of this mosquito follows the coast of Brazilian Atlantic forest. Previous studies indicated that *An. cruzii* is a complex of cryptic species.

Methods

A multilocus approach using six loci, three circadian clock genes and three encoding ribosomal proteins, was implemented to investigate in more detail the genetic differentiation between the *An. cruzii* populations from Santa Catarina (southern Brazil) and Bahia States (north-eastern Brazil) that represent two sibling species.

Results

Analysis of the genetic differentiation in six different gene fragments revealed very high F_{ST} values and fixed differences between the two *An. cruzii* siblings in all loci, irrespective of their function. The data was fit into an Isolation-with-Migration model using the IM program. The results reveal no migration in either direction and allowed a rough estimate of the divergence time between the two siblings.

Conclusions

Population genetics analysis of *An. cruzii* samples from two Brazilian localities using a multilocus approach confirmed that they represent two different sibling species in this complex. The results suggested that two species have not exchange migrants since their

separation and that they possibly diverged around 2.4 million of years ago, a period of intense climatic changes.

Background

Anopheles cruzii (Diptera: Culicidae) is the primary vector of human and simian malaria parasites in southern and south-eastern Brazil (Deane *et al.*, 1970; Rachou *et al.*, 1958).

Earlier studies that evaluated *X* chromosome inversion frequencies (Ramirez & Dessen, 2000a, 2000b) and isoenzyme profiles (Carvalho-Pinto & Lourenço- de-Oliveira, 2004) suggest that *Anopheles cruzii* is a species complex. A recent analysis of genetic differentiation using the *timeless* gene among *An. cruzii* populations from south, south-east and north-east Brazil indicated that the population from Itaparica, Bahia State (north-east Brazil) is a different species (Rona *et al.*, 2009), corroborating previous studies.

In the current study, a multilocus analysis using six different nuclear gene fragments was performed comparing two populations of *An. cruzii* (Florianópolis and Itaparica), representing respectively the south-east and north-east siblings. Three of the fragments used are orthologues of *Drosophila melanogaster* genes involved in the control of circadian rhythms: *timeless* (*tim*), *Clock* (*Clk*) and *cycle* (*cyc*); and three code for Ribosomal proteins: *Rp49* (Ribosomal protein 49, known also as RpL32 - Ribosomal protein L32), *RpS2* (Ribosomal protein S2) and *RpS29* (Ribosomal protein S29).

The aim of the study was to determine if there is still gene flow between the two siblings and to estimate their divergence time. The study also aimed at verifying whether the differentiation in circadian genes putatively involved in the control of mating rhythms,

such as the *timeless*, *Clock* and *cycle* (Hardin, 2005; Sakai & Ishida, 2001), which are potentially important in maintaining temporal reproductive isolation between closely related species, is higher than the divergence in constitutive loci, such as the ribosomal protein genes *rp49*, *rpS29* and *rpS2*. Finally we also aimed at estimating the divergence time between the two species.

Methods

Molecular analysis

The mosquitoes used in this study were females captured in Florianópolis, Santa Catarina State (SC) ($27^{\circ}31'S$ / $48^{\circ}30'W$) and Itaparica Island (Jaguaripe), Bahia State (BA) ($13^{\circ}05'S$ / $38^{\circ}48'W$). They were identified on the basis of their morphology according to Consoli and Lourenço-de-Oliveira (1994). For the molecular analysis, 12 individuals from Florianópolis and 12 to 14 from Itaparica were used for each gene.

The sequences of the *timeless* gene from Florianópolis and Itaparica populations were those previously published by our group (Rona *et al.*, 2009) (Accession numbers: FJ408732 – FJ408865). The sequences of the other genes were obtained as described below using the primers listed in Table 1.

To design specific primers for the *cycle* gene, degenerate primers (5'C_YC_Dg_El x 3'C_YC_Dg_El) previously designed in our lab (Meirelles *et al.* 2006) to isolate this gene in other insect species were used with *An. cruzii* genomic DNA extracted according to Jowett (Jowett, 1998). The same procedure was applied to isolate a fragment of the *Clock*

gene from *An. cruzii* using the degenerate primers 5'CLKdeg3 x 3'CLKdeg10, available in our lab and kindly provided by João Gesto.

After checking the sequence identity, new specific primers were designed (5'cruziiclock x 3'cruziiclock and 5'cruziicycle x 3'cruziicycle).

To isolate the *rpS2* and *rpS29* genes in *An. cruzii*, semi-degenerate primers were designed (5'cruziiRP_S2 x 3'cruziiRP_S2 and 5'cruziiRP_S29b x 3'cruziiRP_S29, respectively) based on conserved nucleotide regions of these genes in *Anopheles gambiae* and *Aedes aegypti*.

Finally, the specific primers previously designed in our lab to isolate the *rp49* gene in both *An. aquasalis* and *Ae. aegypti* (5'aquaRP1 x 3'aeaquaRP1b, Gentile *et al.*, 2005) were also used in this study.

All PCR reactions using degenerate primers were carried out in an Eppendorf Mastercycler® thermocycler under the following conditions: 15 cycles at 94°C for 60 s, 50°C (decreasing 1°C /cycle) for 90 s and 72°C for 60 s, followed by 20 cycles of 94°C for 60 s, 50°C for 90 s and 72°C for 60 s. PCR products were then purified and cloned using Zero Blunt TOPO PCR cloning kit (Invitrogen).

Sequencing of positive clones was carried out in an ABI Prism 3730 DNA sequencer at the Oswaldo Cruz Institute using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The identity of the cloned fragments was determined by BlastX analysis using GenBank (**GenBank database** [<http://www.ncbi.nlm.nih.gov/BLAST/>]).

The specific and the semi-degenerate primers were used in PCR reactions using the proofreading *Pfu* DNA polymerase (Biotoools) to amplify the fragments of each loci from individual mosquitoes from the two *An. cruzii s.l.* siblings.

PCR fragments were cloned using either Zero Blunt TOPO PCR cloning kit (Invitrogen) or pMOS *Blue* vector blunt-ended cloning kit (Amersham Biosciences) and at least eight clones were sequenced for each mosquito.

Sequences were edited and in most cases consensus sequences representing the two alleles were generated. In a number of individuals only one haplotype was observed among the eight sequences and in these cases mosquitoes were classified as homozygotes. The probability of incorrectly classifying a heterozygote as a homozygote individual with this procedure is less than 1%. The sequences from homozygote mosquitoes were duplicated prior to analysis. However, when carried out without duplicating homozygote sequences, the analysis rendered similar results. Sequences were submitted to GenBank (Accession numbers: XXXXX-XXXXX).

DNA sequence analysis

The sequences were aligned using ClustalX software (Thompson *et al.*, 1997) and population genetics analysis was carried out using DNAsP4.0 (Rozas *et al.*, 2003) and PROSEQ v 2.91 (Filatov & Charlesworth, 1999) softwares.

The Modeltest version 3.7 (Posada and Crandall, 1998) was used with a model block implemented in PAUP 4.0d105 (Swofford, 1993) to find the most suitable model for each gene evolution. Models selected by the Bayesian Information Criterion (BIC) were

favored and used in the phylogenetic analysis, carried out using MEGA 4.0 (Tamura *et al.*, 2007).

The Isolation-with-Migration program (*IM*, Hey, 2005; Hey & Nielsen, 2004), an implementation of the MCMC (Markov Chain Monte Carlo) method for the analysis of population divergence with genetic data, was carried out for the simultaneous estimation of six demographic parameters from multilocus data: effective population size for an ancestral and two descendent populations (θ_A , θ_1 , and θ_2 , respectively), divergence time (t) and migration parameters in both directions (m_1 and m_2). Initial runs in the MCMC mode were performed to find the upper limits for the priors of each demographic parameter and to have a rough idea of what the marginal distributions look like. The convergence was assessed through multiple long runs (four independent MCMC runs with different seed numbers were carried out with at least 30,000,000 recorded steps after a burn-in of 100,000 steps) and by monitoring the ESS values, the update acceptance rates and the trend lines.

The Infinite Sites model (Kimura, 1969) was chosen as the mutation model in the IM simulations because the species are closely related and all genes are nuclear.

Since IM requires sequences with no evidence of recombination (four-gamete violations), the optimal recombination-filtered block was extracted from each gene alignment using the *IMgc* program, which also removes haplotypes that represent likely recombinant sequences (Hammer lab, *IMgc*; Woerner *et al.*, 2007). Table 2 shows the position of the non-recombinating (NR) blocks used in this study as well as the putative recombinant sequences that were removed.

Results

Polymorphism and divergence between Florianópolis and Itaparica populations

Table 3 shows the minimum number of recombination events for each gene (RM) and the length of the whole fragment and for the NR block of each gene (values in parentheses). The larger differences in length between the whole fragment and the NR block were observed for *timeless* and *cycle* and this was due to the higher number of recombination events identified in these two genes ($RM = 14$ and 5 respectively). The alignments of the whole sequences of each gene are presented in Additional files 1 to 6. All loci include at least one intron of variable size, except the *cycle* gene fragment, which was composed entirely of an exon. Except for *timeless* gene, all base substitutions were silent or occurred within introns. The few non-synonymous changes found in the *timeless* gene are described in Rona *et al.* (2009).

Table 3 also shows the number of polymorphic sites (S) for each *An cruzii* population and two measures of nucleotide diversity: π , based on the average number of pairwise differences and θ , based on the total number of mutations (values for the NR blocks in parentheses). In general, the population from Itaparica was less polymorphic than Florianópolis, having showed the lowest θ and π values, as well as fewer polymorphic sites (S). The results of the Tajima (Tajima, 1989) test of neutrality are also presented in Table 3 and no significant deviations were observed after Bonferroni correction.

Table 4 shows the pairwise estimates of population differentiation between the two *An. cruzii* siblings. Very high F_{ST} values (ranging from ~ 0.6 to 0.9) were found between Florianópolis and Itaparica populations using both the whole fragment as well as the NR

blocks for all loci. Table 4 also shows the average number of nucleotide substitutions per site (D_{xy}), the number of net nucleotide substitutions per site between populations (D_a) and the distribution of the four mutually exclusive categories of segregating sites observed in each comparison: the number of exclusive polymorphisms for each population (S_1 and S_2), the number of shared polymorphisms (S_s) and the number of fixed differences (S_f). The *timeless* and the *cycle* loci were the only ones that shared polymorphisms between Florianópolis and Itaparica, albeit they were few (7 and 1 for the whole fragment, respectively). All loci presented a large number of fixed differences between the two populations. For the *Clock* and *rpS29* genes, the number of fixed differences was higher when using the NR block. This slightly increased the population differentiation estimates (F_{ST}) between Itaparica and Florianópolis using these genes (Table 4).

Estimation of Demographic Population Parameters

The IM program was used to simultaneously estimate six demographic parameters (θ_1 , θ_2 , θ_4 , t , m_1 , m_2) from two populations through an “Isolation with Migration” model using multiple loci (Hey & Nilsen, 2004). The method assumes no recombination within the loci that are being studied. Accordingly, only the NR blocks were used and some recombining sequences were removed before IM analysis (Table 2).

Figure 1 shows the posterior probability distributions for each of the six demographic parameters estimated using IM and Table 5 summarizes the features from the marginal histograms for each of the parameters in all MCMC runs. Among four independent runs, the simulations between the two populations showed good convergence and consistency resulting in complete posterior distributions.

The estimates of θ suggest that the ancestral population effective population size is smaller than the current Florianópolis and Itaparica populations indicating that both populations may have had a history of growth from their starting point (Figure 1).

The migration rates in both directions for all combined loci were also estimated by the IM software (m_1 and m_2). No indication of migration was found in either direction in the multiple simulations.

The divergence time parameter was estimated for all combined loci in four different IM runs. This parameter cannot be directly converted to numbers in years since the mutation rates in *Anopheles cruzii* species are unknown. Therefore, an estimate of the divergence time between *Anopheles cruzii* species was performed using the average of *Drosophila* synonymous and nonsynonymous substitution rates for several nuclear genes [(0.0156 and 0.00191 per site per million of years respectively (Li, 1997)]. Using this approach, an estimate of the divergence time between Florianópolis and Itaparica populations would be approximately 2.4 Mya (range from 1.1 to 3.6 Mya, based on the average of HPD90Lo and HPD90Hi values).

Another manner of estimating the divergence time between these two *Anopheles* species is to use the same *Drosophila* synonymous substitution rate mentioned above and the average *Da* values from the six loci (Table 4). Based on these values, an estimate of the divergence time was performed for Florianópolis and Itaparica populations, which were approximately 1.91 ± 0.76 Mya and 1.93 ± 0.65 Mya for the whole sequence and NR blocks, respectively.

Genealogy analysis

Gene trees of the sequences from all loci for both whole sequences and NR blocks were estimated using the Neighbor-Joining method (NJ) (Figure 2 and Additional file 7, respectively). The most suitable model selected using Modeltest 3.7 (Posada and Crandall, 1998) was Kimura 2-parameters (Kimura, 1980) for all loci excepted for the *Clock* gene where the JC (Jukes and Cantor, 1969) model was chosen. All trees were performed with 1,000 bootstrap replicates. The resulting NJ trees clearly grouped the sequences from the two siblings in different clusters with high bootstrap values.

Discussion

The results presented here confirm the high level of differentiation between the Itaparica and Florianópolis siblings (Carvalho-Pinto & Lourenço-de-Oliveira, 2004; Rona *et al.*, 2009).

Less differentiation was expected in the three genes that code for the highly conserved ribosomal proteins (*rp49*, *rpS29* and *rpS2*) than in loci possibly involved in the control of mating rhythms (*timeless*, *Clock* and *cycle*), which might be good candidate genes for speciation in insects since they are potentially important in maintaining temporal reproductive isolation between closely related species (Hardin, 2005; Sakai & Ishida, 2001). In fact, Rona *et al.* (2009) showed very high differentiation between Itaparica and the more southern Brazilian populations, including Florianópolis, using the *timeless* gene as a molecular marker.

However, very high F_{ST} values were detected in all loci between these two populations

and they were even higher for *rp49*, *rpS29* and *rpS2* (0.8854, 0.8865 and 0.8502, respectively for the whole fragment) than for *timeless*, *Clock* and *cycle* (0.8150, 0.7088 and 0.5806, respectively for the whole fragment). Mazzoni *et al.* (2008) found similar results in a multilocus analysis between two sand fly vectors of leishmaniasis, where fixed differences were only observed in ribosomal protein genes. A higher number of fixed differences were found for these loci in *An. cruzii* and a possible explanation for these findings is codon usage bias since only synonymous changes were found for these three ribosomal genes. In fact, the degree of codon usage variation across 12 *Drosophila* genomes was observed to be greater for ribosomal protein genes than for other genes (Heger & Ponting, 2007).

No indication of migration was found in either direction in the multiple IM simulations, which was consistent with the very high differentiation values for all loci. Itaparica also presented lower levels of variability than those from Florianópolis, possibly indicating a smaller population size. This is confirmed by IM results, which also indicated a smaller effective population size for Itaparica. The estimated difference in population size between these populations seems coherent, since the Southern *An. cruzi* sibling found in Florianópolis is distributed throughout most of the South and Southeast Brazilian Atlantic Forest (from Santa Catarina to Espírito Santo State) and the Northeast sibling found in Itaparica seems to occur only in a more restricted region (Rona *et al.*, 2009).

The multilocus results corroborate previous data (Carvalho-Pinto & Lourenço-de-Oliveira, 2004; Rona *et al.*, 2009) indicating that these populations represent two

different species in the *An. cruzii* complex. This was also confirmed by NJ trees, which show that Florianópolis and Itaparica are clearly separated in two isolated groups.

The estimated divergence time of approximately 2.4 Mya based on the IM results, corresponds to the end of the Pliocene and beginning of the Pleistocene. Significant climate changes, including the onset of heavy Northern Hemisphere glaciation, ~2.75 million years ago, occurred at the end of the Pliocene (Ravelo *et al.*, 2004). A very important consequence of this cooling was an extensive increase in aridification, which lead to fragmentation of forests, including the Brazilian Atlantic forest (Vasconcelos *et al.*, 1992; Ravelo *et al.*, 2004). Interestingly, Carnaval *et al.* (2009) discussed the hypothesis of refugia for neotropical species occurring in the Atlantic forest. Itaparica is located in an area proposed to be a large central refugium in the Brazilian Atlantic Forest and another refugium is proposed in the South and Southeast Brazil. Climate changes have been proposed to explain differentiation among many groups such as fruit flies (Tamura *et al.*, 2004), insect vectors (Conn & Mirabello, 2007) as well as many forest-obligate species (Marroig *et al.*, 2004; Grazziotin *et al.*, 2006; Carnaval *et al.*, 2009; Pedro *et al.*, 2008). Since *An. cruzii* is endemic to the Atlantic Forest, it is seems likely that it underwent differentiation due to the forest fragmentation, which separated a single prevalent ancestral species in two or more isolated groups.

Conclusions

The results of the multilocus analysis corroborate previous data indicating that Florianópolis and Itaparica populations of *An. cruzii* represent two different species in the complex and suggest that they have not exchanged migrants since their separation around 2.4 Mya.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LDPR participated in data generation and analysis, and drafted the manuscript. She also helped capture mosquitoes in Florianópolis. CJCP carried out the capture and morphological identification of mosquitoes. CJM helped to analyze the IM data. AAP is the principal investigator, participated in its design and coordination, and helped to write the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Posterior probability distributions of demographic parameters

Posterior probability distributions for each of the six demographic parameters estimated using IM: effective population size for an ancestral and two descendent populations (θ), divergence time between Florianópolis and Itaparica, and migration rates in both directions. Four IM simulations (a, b, c and d) using different seed numbers were plotted for each parameter estimate (see also Table 5). All curves are shown including the range of the priors.

Figure 2. Neighbour-joining trees of all loci.

Neighbour-joining trees using the six genes nucleotide sequences of the *Anopheles cruzii* populations obtained with Jukes and Cantor distance for *Clock* gene and Kimura 2-parameters distance for the others. Numbers on the nodes represent the percentage bootstrap values based on 1,000 replications. Flo: Florianópolis population; Bah: Itaparica population.

Tables

Table 1. Sequence of primers used to amplify the gene fragments

Degenerate and specific primers used to amplify the six gene fragments in *Anopheles cruzii* populations.

locus	Primers name	Sequence of primers
<i>clock</i>	5'CLKdeg3	5'-SNGGNTAYGAYTAYTAYCA-3'
	3'CLKdeg10	5'-TCNGTYTGNARCCADATCCA-3'
	5'cruziiclock	5'-TTGACGATCTGGAAAAGGTG-3'
	3'cruziiclock	5'-CTTGGTCAGGAAGCGATA GT-3'
<i>cycle</i>	5'CYCdeg1	5'-ARMGNMGNMNGAYAARATGAA-3'
	3'CYCdeg1	5'-ACYTTNCCDATRTCYTTNGGRTG-3'
	5'cruziicycle	5'-CACCTACATCACCGAACTG-3'
	3'cruziicycle	5'-GACTCGGAAACGTACAGGATA-3'
<i>rp49</i>	5'aquaRP1	5'-GTGAAGAACGGACGAAGAAGTT-3'
	3'aeaquaRP1b	5'-TCATCAGCACCTCCAGCTC-3'
<i>rpS2</i>	5'cruziRP_S2	5'-GGCTACTGGGGTAACAAGA-3'
	3'cruziRP_S2	5'-CAGRACGGAACCGCACTT-3'
<i>rpS29</i>	5'cruziRP_S29b	5'-TCGCATCCSCGTAAATA-3'
	3'cruziRP_S29	5'-TTCCKGAAGCCAATATCCT-3'

Table 2. NR blocks and sequences excluded from the IM analysis.

Edition of sequences prior to IM analysis using the *IMgc* program and based on alignment presented in additional files 1 to 6. NR blocks, fragment positions of the non-recombinating blocks used in the analyses; Removed sequences, the putative recombinant sequences removed before the IM analysis.

locus	NR blocks	Removed sequences
<i>timeless</i>	124 – 381	Flo31a, Flo31b, Flo32b, Flo35a, Flo35b, Flo36b, Flo37a, Flo39a, Flo40a
<i>clock</i>	1 – 154	Bah02b, Bah03a, Bah03b, Flo08a, Flo12a, Flo16b
<i>cycle</i>	36 – 131	Flo06a, Flo18a, Flo18b
<i>rp49</i>	47 – 269	Flo06a, Flo06b, Flo09b
<i>rpS2</i>	1 – 266	Flo09b
<i>rpS29</i>	36 – 274	Bah31b, Flo07b, Flo09b, Flo12a

Table 3. Polymorphisms of *An. cruzii* populations from Florianópolis and Itaparica.

Locus	Population	RM	Length(bp)	n	S	θ	π	D_T
<i>timeless</i>	Florianópolis	14	413 (258)	24 (15)	59 (16)	0.04314 (0.02141)	0.03081 (0.02080)	-0.98320 (-0.02183)
	Itaparica			28 (28)	24 (14)	0.01661 (0.01602)	0.01035 (0.00994)	-1.31797 (-1.25698)
<i>Clock</i>	Florianópolis	03	159 (154)	24 (21)	10 (09)	0.01750 (0.01688)	0.01774 (0.01703)	0.11242 (0.09477)
	Itaparica			24 (21)	08 (06)	0.01592 (0.01282)	0.02253 (0.01789)	1.41474 (1.30746)
<i>cycle</i>	Florianópolis	05	218 (96)	24 (21)	21 (12)	0.02787 (0.03929)	0.02802 (0.02946)	0.13573 (-0.77817)
	Itaparica			24 (24)	03 (03)	0.00370 (0.00845)	0.00304 (0.00691)	-0.43933 (-0.43933)
<i>rp49</i>	Florianópolis	01	269 (223)	24 (21)	10 (09)	0.01134 (0.01299)	0.00849 (0.00876)	-0.82070 (-1.09222)
	Itaparica			24 (24)	09 (09)	0.00915 (0.01109)	0.00678 (0.00820)	-0.82379 (-0.82379)
<i>rpS2</i>	Florianópolis	01	270 (266)	24 (23)	17 (17)	0.01879 (0.01931)	0.01723 (0.01719)	-0.22879 (-0.33019)
	Itaparica			24 (24)	08 (07)	0.00807 (0.00716)	0.00863 (0.00679)	0.25353 (-0.13512)
<i>rpS29</i>	Florianópolis	02	274 (238)	24 (21)	13 (07)	0.01375 (0.00836)	0.00907 (0.00774)	-1.14756 (-0.21099)
	Itaparica			24 (23)	20 (13)	0.02101 (0.01499)	0.01002 (0.00877)	-1.87423 (-1.42007)

RM, the minimum number of recombination events; n, number of DNA sequences of each population; S, number of polymorphic (segregating) sites; θ , nucleotide diversity based on the total number of mutation (*Eta*); π , nucleotide diversity based on the average number of pair-wise differences; D_T , Tajima's D (Tajima, 1989) based on *Eta*. No significant deviations from neutrality were observed after Bonferroni correction. Numbers in parentheses are related to the non-recombining block (NR) for each locus.

Table 4. Genetic differentiation between Florianópolis and Itaparica.

Locus	F_{ST}	P (F_{ST})	Dxy	Da	S_s	S_f	S_I	S_2
<i>timeless</i>	0.8150 (0.8144)	0.0000 (0.0000)	0.1154 (0.0877)	0.0941 (0.0714)	07 (01)	25 (15)	56 (17)	16 (12)
<i>Clock</i>	0.7088 (0.7500)	0.0000 (0.0000)	0.0593 (0.0579)	0.0420 (0.0434)	00 (00)	03 (04)	07 (06)	08 (06)
<i>cycle</i>	0.5806 (0.5852)	0.0000 (0.0000)	0.0371 (0.0441)	0.0215 (0.0258)	01 (00)	02 (02)	21 (13)	02 (03)
<i>Rp49</i>	0.8854 (0.8903)	0.0000 (0.0000)	0.0606 (0.0695)	0.0536 (0.0619)	00 (00)	12 (12)	10 (09)	09 (09)
<i>rpS2</i>	0.8502 (0.8598)	0.0000 (0.0000)	0.0843 (0.0836)	0.0717 (0.0718)	00 (00)	16 (16)	18 (18)	08 (07)
<i>rpS29</i>	0.8865 (0.9276)	0.0000 (0.0000)	0.0843 (0.0950)	0.0747 (0.0881)	00 (00)	11 (19)	14 (05)	19 (11)

F_{ST} , pair-wise estimates of population differentiation. P-value, significance of F_{ST} values (evaluated by 1,000 random permutations). Dxy, average number of nucleotide substitutions per site between populations (Nei & Kumar, 2000); Da, number of net nucleotide substitutions per site between populations (Nei & Kumar, 2000). S_I , number of polymorphic sites exclusive to the first population shown in the first column. S_2 , number of polymorphic sites exclusive to the second population shown in the first column. S_s , number of shared polymorphisms between the two populations. S_f , number of fixed differences between the two populations. Numbers in parentheses are related to the non-recombining block (NR) for each locus.

Table 5. Summarized features of the marginal histograms for each parameter.

		Minbin	Maxbin	HiPt	HiSmth	Mean	95Lo	95Hi	HPD90Lo	HPD90Hi
θ_1	a	0.6770	5.5448	2.0120	2.0057	2.0624	1.3886	3.0007	1.4200	2.7425
	b	0.6644	5.7211	1.9805	1.9931	2.0624	1.3886	3.0007	1.4200	2.7425
	c	0.7210	5.6518	1.9868	1.9805	2.0624	1.3886	3.0007	1.4200	2.7425
	d	0.7210	6.2312	1.9994	1.9931	2.0624	1.3886	3.0007	1.4200	2.7425
θ_2	a	1.0659	7.2149	2.7815	2.7475	2.8410	1.9662	4.0555	2.0086	3.7242
	b	0.9385	7.6141	2.7815	2.7730	2.8410	1.9662	4.0555	2.0086	3.7242
	c	1.0064	7.9114	2.7815	2.7730	2.8410	1.9662	4.0555	2.0086	3.7242
	d	0.9810	7.5801	2.7900	2.7475	2.8410	1.9662	4.0555	2.0086	3.7242
θ_A	a	0.0099	19.8074	0.2081	0.1883	3.9734	0.1883	16.4583	0.0099	11.3652
	b	0.0099	19.8074	0.0495	0.1288	3.9139	0.1684	16.4781	0.0099	11.3454
	c	0.0099	19.8074	0.0099	0.0099	3.9932	0.1684	16.5178	0.0099	11.4049
	d	0.0099	19.8074	0.0099	0.0099	3.9932	0.1883	16.5178	0.0099	11.4247
t	a	1.1300	19.9900	5.6700	5.6500	5.7300	2.8900	12.9100	2.7300	8.4900
	b	1.2300	19.9900	5.6700	5.6900	5.7700	2.9500	13.3900	2.7500	8.5900
	c	1.0900	19.9900	5.7900	5.7700	5.7500	2.8900	13.5500	2.6900	8.5700
	d	1.2300	19.9900	5.6700	5.7100	5.7300	2.8900	13.4300	2.6700	8.5300
m_1	a	0.0001	0.0999	0.0003	0.0003	0.0201	0.0008	0.0854	0.0001	0.0617
	b	0.0001	0.0999	0.0001	0.0001	0.0203	0.0008	0.0858	0.0001	0.0622
	c	0.0001	0.0999	0.0001	0.0001	0.0202	0.0008	0.0857	0.0001	0.0621
	d	0.0001	0.0999	0.0004	0.0003	0.0202	0.0008	0.0857	0.0001	0.0620
m_2	a	0.0001	0.0999	0.0002	0.0002	0.0154	0.0006	0.0764	0.0001	0.0506
	b	0.0001	0.0999	0.0001	0.0001	0.0155	0.0006	0.0765	0.0001	0.0507
	c	0.0001	0.0999	0.0001	0.0001	0.0155	0.0006	0.0767	0.0001	0.0508
	d	0.0001	0.0999	0.0001	0.0001	0.0154	0.0006	0.0765	0.0001	0.0507

Values of the six parameters that span the prior distribution are presented for each of the four runs with different seed numbers (a, b, c and d). Population size parameter for Itaparica, Florianópolis and ancestral populations (θ_1 , θ_2 , θ_A); Time of population splitting parameter (t); Migration rate estimate from Florianópolis to Itaparica population (m_1) and from Itaparica to Florianópolis population (m_2); Minbin and Maxbin, the midpoint values of the lowest and the highest bin, respectively; HiPt, the value of the bin

with the highest count; HiSmth, the value of the bin with the highest count, after the counts have been smoothed by taking a running average of 9 points centered on each bin; 95Lo and 95Hi, the estimated points to which 2.5% of the total area lies to the left and to the right, respectively; HPD90Lo and HPD90Hi, the lower and upper bounds of the estimated 90% highest posterior density (HPD) interval, respectively.

Additional files

Additional file 1. Alignment of the *timeless* sequences from Florianópolis and Itaparica populations

Alignment of the DNA sequences from the *timeless* gene fragment from Florianópolis and Itaparica populations. The translated amino acid sequence is shown above the alignment and the intron is highlighted in grey. Dots represent identity and dashed represent gaps. The asterisks in the bottom line represent identity of all sequences. Flo: individuals from Florianópolis and Bah: individuals from Itaparica.

Additional file 2. Alignment of the *Clock* sequences from Florianópolis and Itaparica populations

Alignment of the DNA sequences from the *clock* gene fragment from Florianópolis and Itaparica populations. The translated amino acid sequence is shown above the alignment and the intron is highlighted in grey. Dots represent identity and dashed represent gaps. The asterisks in the bottom line represent identity of all sequences. Flo: individuals from Florianópolis and Bah: individuals from Itaparica.

Additional file 3. Alignment of the *cycle* sequences from Florianópolis and Itaparica populations

Alignment of the DNA sequences from the *cycle* gene fragment from Florianópolis and Itaparica populations. The translated amino acid sequence is shown above the alignment. Dots represent identity and dashed represent gaps. The asterisks in the bottom line represent identity of all sequences. Flo: individuals from Florianópolis and Bah: individuals from Itaparica.

Additional file 4. Alignment of the *rp49* sequences from Florianópolis and Itaparica populations

Alignment of the DNA sequences from the *rp49* gene fragment from Florianópolis and Itaparica populations. The translated amino acid sequence is shown above the alignment and the intron is highlighted in grey. Dots represent identity and dashed represent gaps. The asterisks in the bottom line represent identity of all sequences. Flo: individuals from Florianópolis and Bah: individuals from Itaparica.

Additional file 5. Alignment of the *rpS2* sequences from Florianópolis and Itaparica populations

Alignment of the DNA sequences from the *rpS2* gene fragment from Florianópolis and Itaparica populations. The translated amino acid sequence is shown above the alignment and the intron is highlighted in grey. Dots represent identity and dashed represent gaps. The asterisks in the bottom line represent identity of all sequences. Flo: individuals from Florianópolis and Bah: individuals from Itaparica.

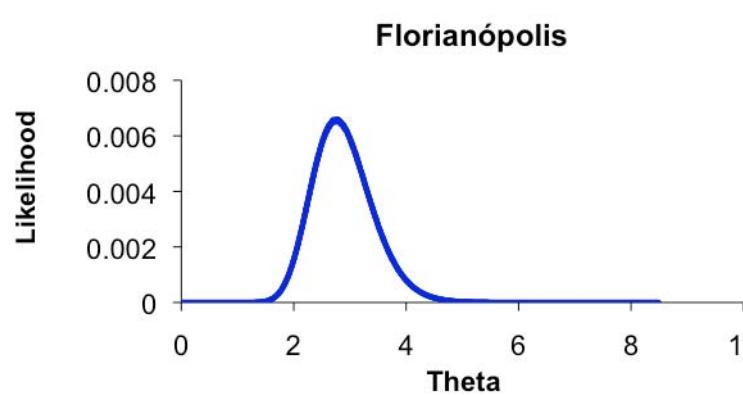
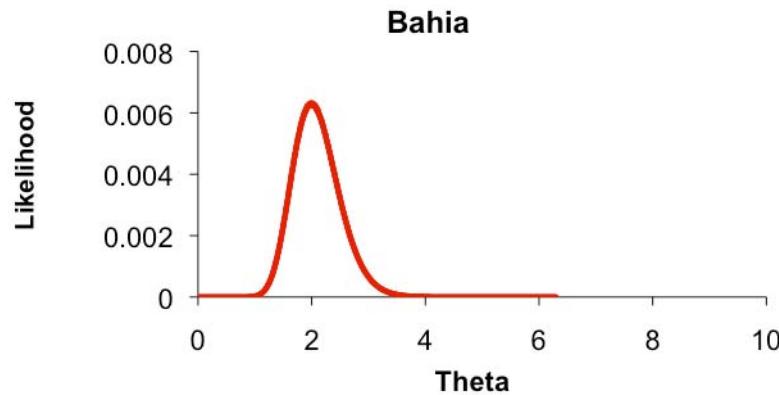
Additional file 6. Alignment of the *rpS29* sequences from Florianópolis and Itaparica populations

Alignment of the DNA sequences from the *rpS29* gene fragment from Florianópolis and Itaparica populations. The translated amino acid sequence is shown above the alignment and the intron is highlighted in grey. Dots represent identity and dashed represent gaps. The asterisks in the bottom line represent identity of all sequences. Flo: individuals from Florianópolis and Bah: individuals from Itaparica.

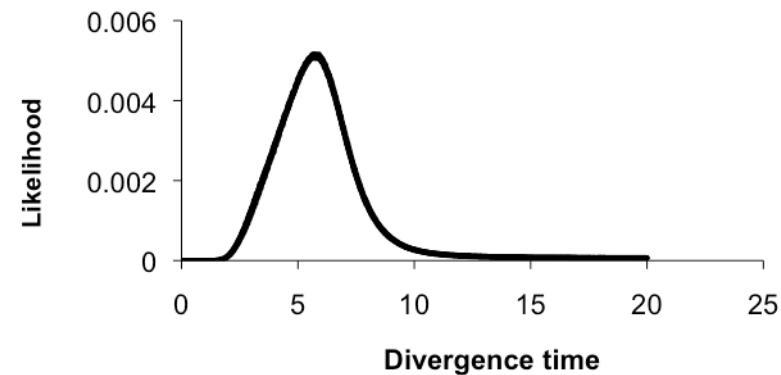
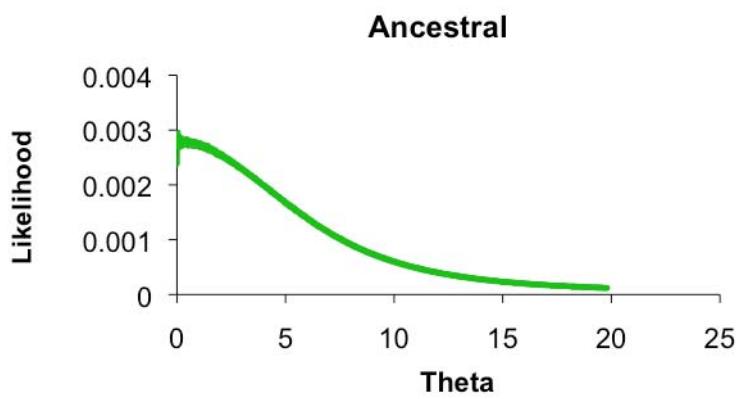
Additional file 7. Neighbour-joining trees of NR blocks of all loci.

The trees were estimated using the neighbor-joining method with 1,000 bootstrap replicates. The Jukes and Cantor distance was used for the *Clock* gene and Kimura 2-parameters distance for the others.

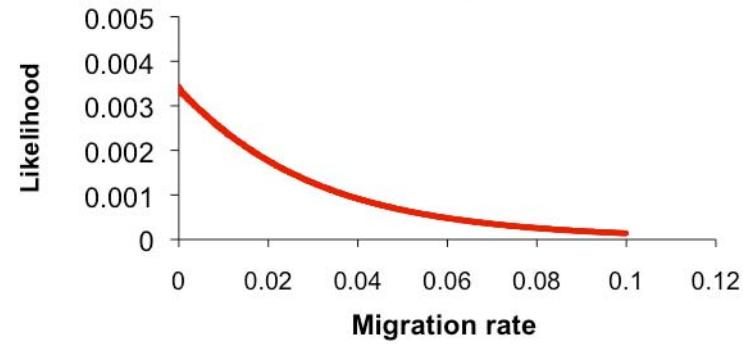
Figure 1



68



from Florianópolis to Bahia



from Bahia to Florianópolis

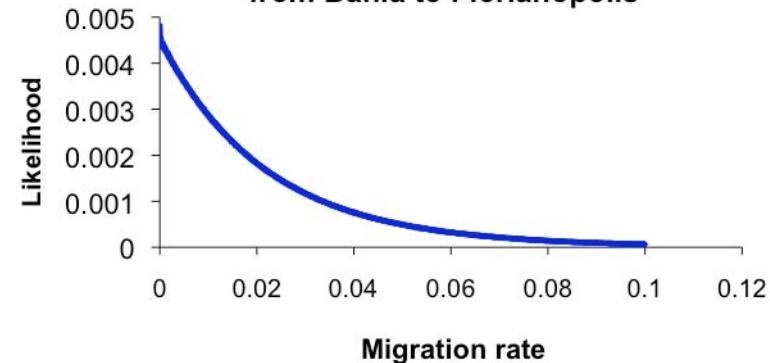
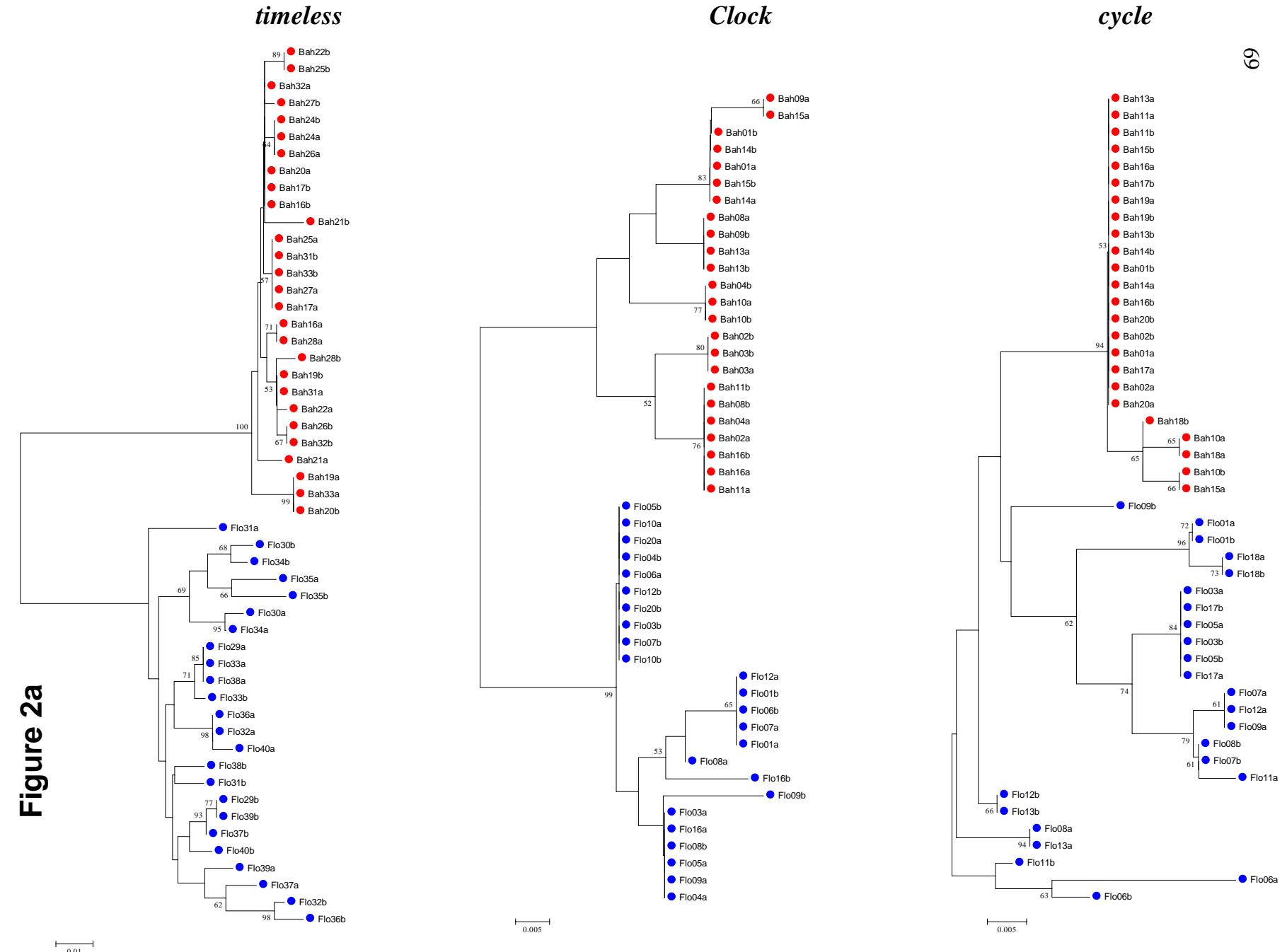
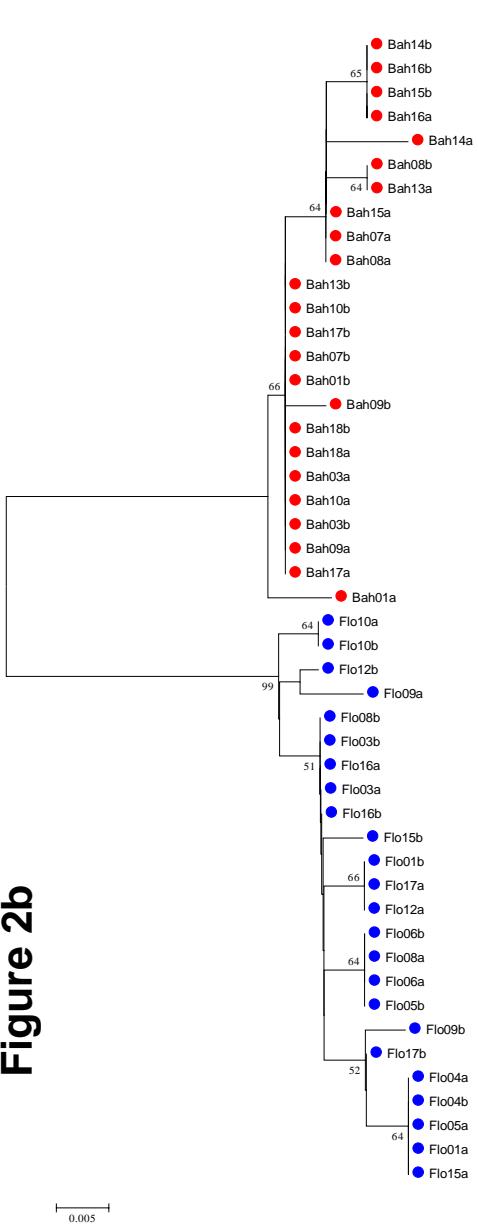
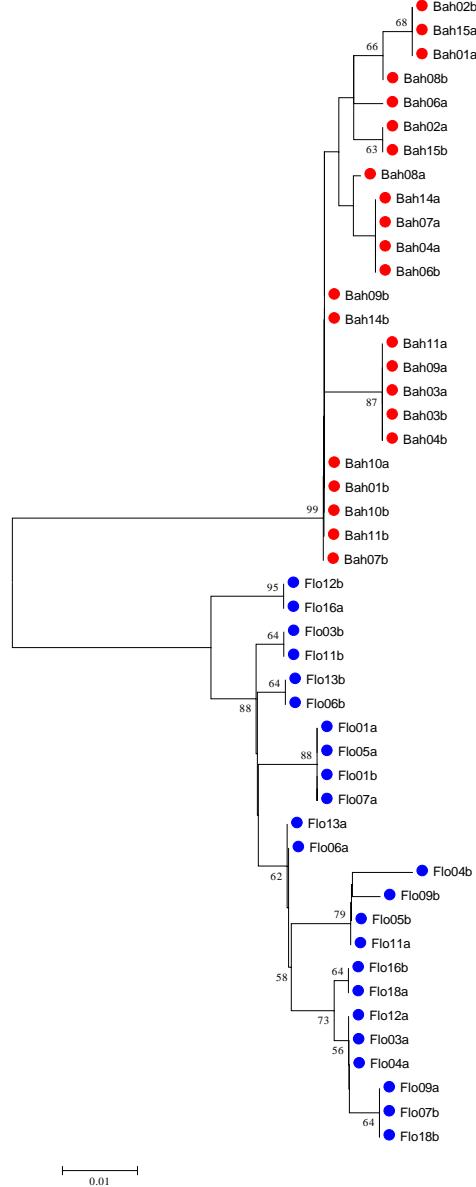


Figure 2a

Rp49



RpS2



RpS29

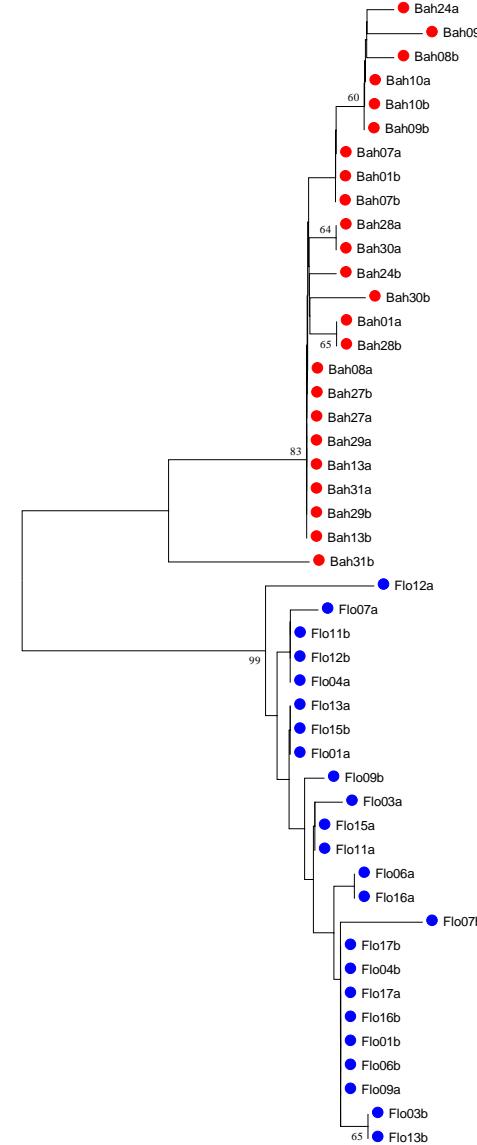


Figure 2b

Additional file 2

V A C H E A L	M Q K G E G T S C
Bah16a	GTGGCTTGTATGAAGCAT G AAGCGCTACAATACCTCTACTCTGTAATCTCCGCCGGCTGCTCAATGATTAAAT-CCGTTTCTTTCTTCGGTTGG-----
Bah16b	-.-.C.....G...T..-.-.C.....C.....
Bah01aC.....G...T..-.-.C.....C.....
Bah01bC.....G...T..-.-.C.....C.....
Bah02aA.....A.....A.....A.....
Bah02bA.....A.....A.....A.....
Bah03aA.....A.....A.....A.....
Bah03bA.....A.....A.....A.....
Bah04aA.....A.....A.....A.....
Bah04bG...T..-.-.C.....A.....C.....
Bah08aG...T..-.-.C.....A.....C.....
Bah08bG...T..-.-.C.....A.....C.....
Bah09aC.....G...T..-.-.C.....A.....C.....
Bah09bC.....G...T..-.-.C.....A.....C.....
Bah10aG...T..-.-.C.....A.....C.....
Bah10bG...T..-.-.C.....A.....C.....
Bah11aG...T..-.-.C.....A.....C.....
Bah11bG...T..-.-.C.....A.....C.....
Bah13aG...T..-.-.C.....A.....C.....
Bah13bG...T..-.-.C.....A.....C.....
Bah14aC.....G...T..-.-.C.....A.....C.....
Bah14bC.....G...T..-.-.C.....A.....C.....
Bah15aC.....G...T..-.-.C.....A.....C.....
Bah15bC.....G...T..-.-.C.....A.....C.....
Flo01aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo01bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo03aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo03bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo04aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo04bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo05aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo05bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo06aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo06bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo07aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo07bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo08aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo08bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo09aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo09bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo10aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo10bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo12aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo12bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo16aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo16bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo20aT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.
Flo20bT.....G...T..C..T.A.G-..G.....A.....TACGCTTCTCTCCCGCTGCT.

Additional file 4

	I R H Q S D R Y D K L A	P A W R R F P K G I D N R V R R R F K G Q Y L M P N I G Y G S N K R T R H M L P C G F K L V H N V R	TATCCGCCACCACTCGGATCGCTATGACAAGCTTGCA	AAAGGGTTT-C-CGTTAAGGTTGGGGTCGGCGAAGTTGTTGACCGTTGCTTCGATTCCTCATTCCTCGC	CCCTGCATGCCGTGGCCGAAGGTAATCGACACCGGGTGTCTGCGTTCAAGGGACAGTACCTGATGCCAACATCGGTACCGCTGAAACAGCGCACAGSCATATTGCTGGATTCAAGAAGTTCTGCTGACAACTGCGC
Bah10a
Bah10b
Bah01a
Bah03a
Bah03b
Bah07a
Bah07b
Bah08a
Bah08b
Bah09a
Bah13a
Bah13b
Bah14a
Bah14b
Bah15a
Bah15b
Bah16a
Bah16b
Bah17a
Bah17b
Bah18a
Bah18b
Flo01a	...T	CGC.G.A..GG.C..A..
Flo01b	...T	CGC.G.A..G..A..A..
Flo03a	...T	CGC.G.A..G..C..A..
Flo03b	...T	CGC.G.A..G..C..A..
Flo04a	...T	CGC.G.A..GG.C..A..A..
Flo04b	...T	CGC.G.A..GG.C..A..A..
Flo05a	...T	CGC.G.A..GG.C..A..A..
Flo05b	...T	CGC.G.A..G..C..A..
Flo06a	...T	CGC.G.A..G..C..A..
Flo06b	...T	CGC.G.A..G..C..A..
Flo08a	...T	CGC.G.A..G..C..A..
Flo08b	...T	CGC.G.A..G..C..A..
Flo09a	...T	CGC.G.A..G..CC..A..
Flo09b	...T	CGC.G.A..G..CC..A..
Flo10a	...T	CGC.G.A..GG.C..A..A..
Flo10b	...T	CGC.G.A..G..C..A..
Flo12a	...T	CGT.G.A..G..C..A..
Flo12b	...T	CGT.G.A..G..C..A..
Flo15a	...T	CGC.G.A..GG.C..A..A..
Flo15b	...T	CGC.G.A..G..C..A..
Flo16a	...T	CGC.G.A..G..C..A..
Flo16b	...T	CGC.G.A..G..C..A..
Flo17a	...T	CGC.G.A..G..AC..A..
Flo17b	...T	CGC.G.A..GG.C..A..A..

	G	K	P	H	T	V	P	C	K	V	S	G
Bah01a	TCGTTAACACATACCGTACCGTCCAACTGAGCATGATGTTGGCTTGTGCTTCAAGACTAAGCGAACCGATTGGCTGGCTTCCAAATGATGAGAAAATTCAAAACCTTATGGGTTTCA CGATAGGTGCTGATGGAAACCCTCCGATACCACTCGACTTACAGTTATGATGGCTTGATGGAAACCCTCCGATTCGGAACCGTGAATTAGCTCGAATTCTGTAATGGGTTATGTTATTATTCTGGTTCAGTGCGC											
Bah02a		A									T	C..
Bah02b											GG	C..
Bah02c											GG	C..
Bah04											GG	C..
Bah04b											GG	C..
Bah06a											GG	C..
Bah06b											GG	C..
Bah07a											GG	C..
Bah07b											GG	C..
Bah08a											GG	C..
Bah08b											GG	C..
Bah09a											GG	C..
Bah09b											GG	C..
Bah10a											GG	C..
Bah10b											GG	C..
Bah11a											GG	C..
Bah11b											GG	C..
Bah14a											GG	C..
Bah14b											GG	C..
Bah15a											GG	C..
Bah15b		A..									GG	C..
Flo01a		C..	A---		GA			T..		A..	G..	G.T..
Flo01b		C..	A---		GA			T..		A..	G..	G.T..
Flo03a		C..	---		GA		A..	T..		A..	G..	G.T..
Flo03b		C..	C..	---	GA			T..A..		A..	G..	G.T..
Flo04a		C..	C..	---	GA		A..	T..		A..	G..	G.T..
Flo04b		C..	C..	---	GA		A..	T..		A..	G..	G.T..
Flo05a		C..	C..	A---	GA			T..		A..	G..	G.T..
Flo05b		C..	C..	---	GA		A..	T..		A..	G..	G.T..
Flo06a		C..	C..	---	GA		A..	T..		A..	G..	G.T..
Flo06b		C..	C..	---	GA			T..		A..	G..	G.T..
Flo07a		C..	C..	A---	GA			T..		A..	G..	G.T..
Flo07b		C..	C..	---	GA		T..	A..T..		A..	G..	G.T..
Flo09a		C..	C..	---	GA		T..	A..T..		A..	G..	G.T..
Flo09b		C..	C..	---	GA		T..	A..T..	A..	A..	G..	G.T..
Flo11a		C..	C..	---	GA		A..	T..	A..	A..	G..	G.T..
Flo11b		C..	C..	---	GA			T..A..		A..	G..	G.T..
Flo12a		C..	C..	---	GA		A..	T..		A..	G..	G.T..
Flo12b		C..	C..	---	GA		A..	A..T..		A..	G..	G.T..
Flo13a		C..	C..	---	GA		A..	T..		A..	G..	G.T..
Flo13b		C..	C..	---	GA			A..		A..	G..	G.T..
Flo16a		C..	C..	---	GA			T..		A..	G..	G.T..
Flo16b		C..	C..	---	GA			A..T..		A..	G..	G.T..
Flo18a		C..	C..	---	GA			A..		A..	G..	G.T..
Flo18b		C..	C..	---	GA			A..T..		A..	G..	G.T..

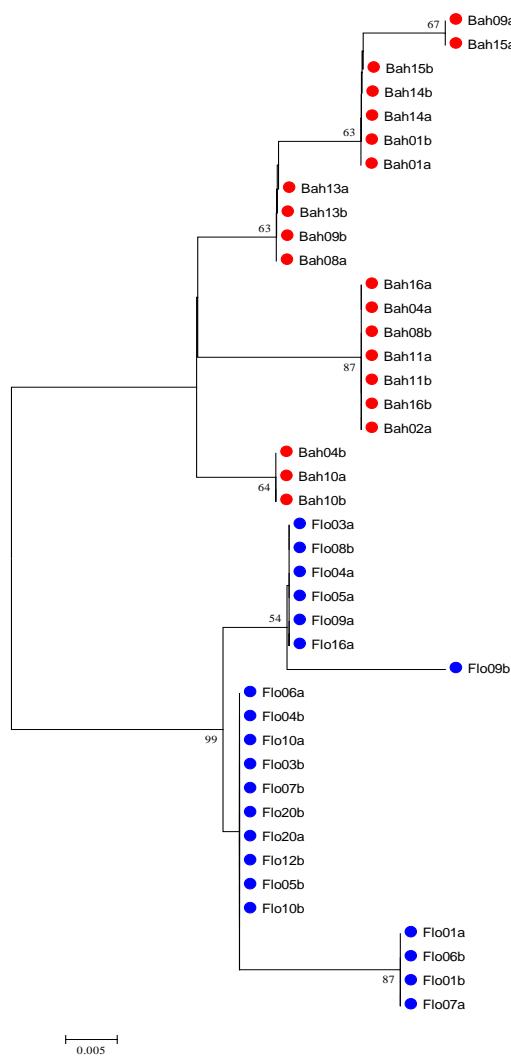
Additional file 6

Additional file 7a

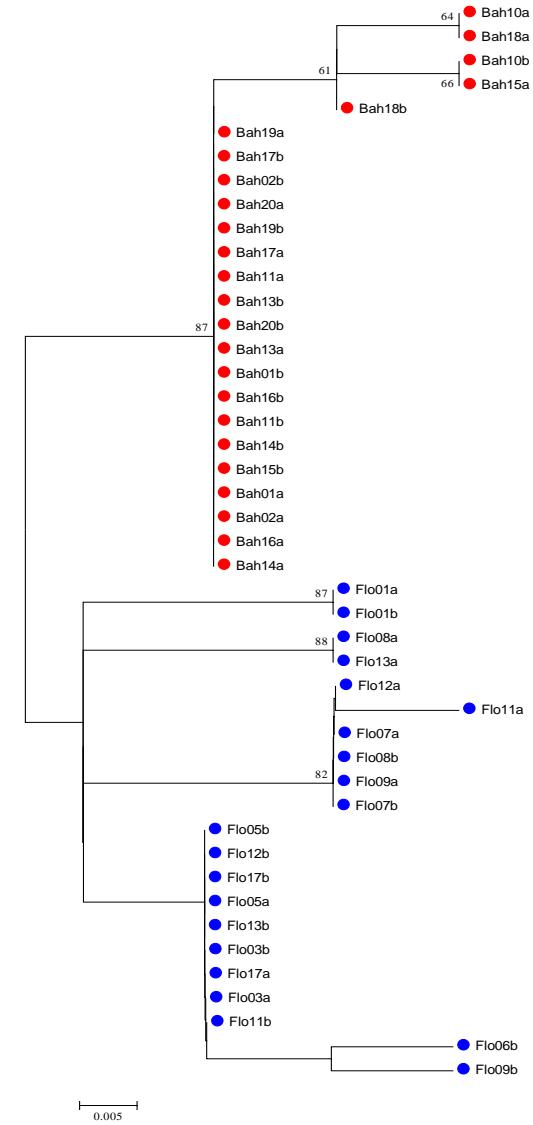
timeless



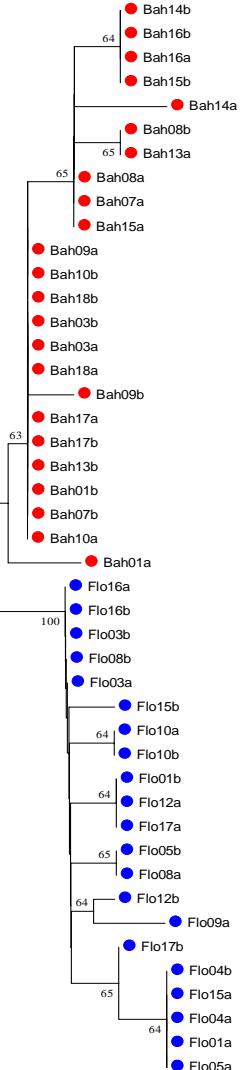
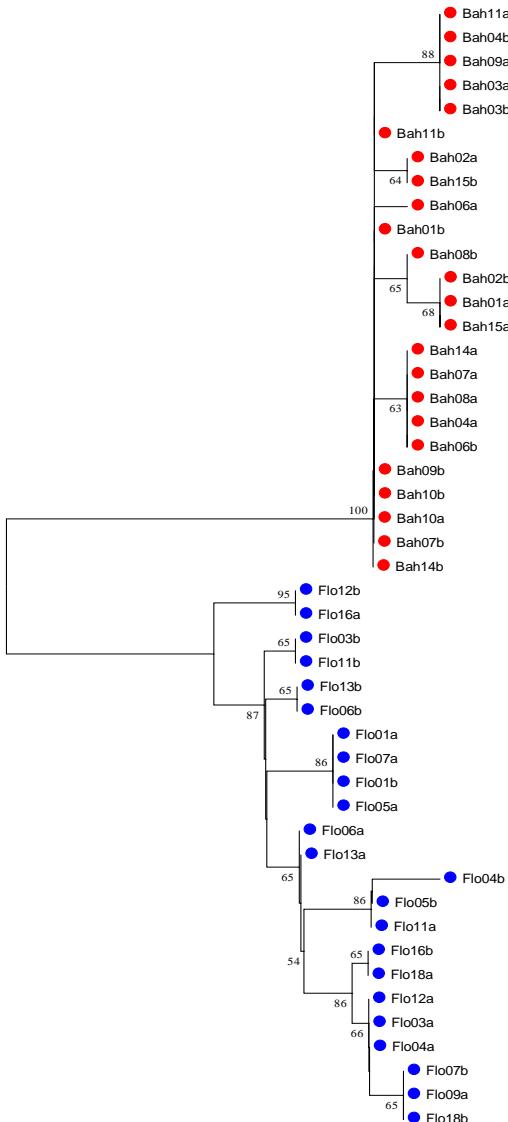
Clock



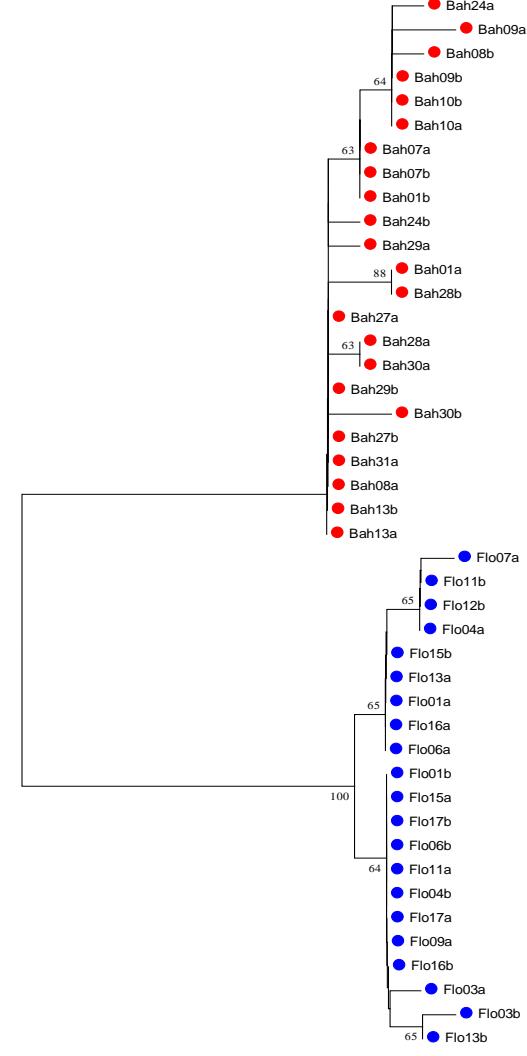
cycle



Additional file 7b

rp49*rpS2*

0.01

rpS29

0.01

RESUMO CAPÍTULO 2

- Os resultados desta análise *multilocus* corroboram com estudos anteriores confirmado que as populações de Florianópolis e Itaparica representam duas espécies diferentes dentro do complexo *An. cruzii* e sugerem que elas não trocam migrantes desde sua separação que ocorreu há aproximadamente 2.4 milhões de anos.

CAPÍTULO 3

Evidência molecular para a ocorrência de novas espécies crípticas do complexo *Anopheles (Kerteszia) cruzii* no sudeste do Brasil. (artigo em preparação)

Neste estudo, um fragmento do gene *cpr* foi seqüenciado em cinco populações de *Anopheles cruzii* provenientes do sul/sudeste do Brasil e análises da variabilidade intraespecífica e da diferenciação genética foram realizadas com o objetivo de verificar a divergência entre as populações.

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Molecular evidence for the occurrence of new sibling species within the *Anopheles (Kerteszia) cruzii* complex in south-east Brazil

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Abstract

Background

Anopheles cruzii (Diptera: Culicidae) has long been known as the primary vector of human and simian malaria parasites in southern and south-eastern Brazil. Previous studies have provided evidence that *An. cruzii* is a species complex but the status of the different populations and the number of sibling species remains unclear. A recent analysis of the genetic differentiation of the *timeless* gene among *An. cruzii* populations from south and south-east Brazil suggested that the population from Itatiaia, Rio de Janeiro State (south-east Brazil), is in a process of incipient differentiation.

Methods

A ~180 bp fragment of *cpr*, a gene encoding the NADPH–cytochrome P450 reductase, an enzyme involved in metabolic insecticide resistance and odorant clearance in insects, was used in this study as a molecular marker to analyze the divergence between five *An. cruzii* populations from south and south-east Brazil.

Results

Analysis of the genetic differentiation in the *cpr* gene revealed very high F_{ST} values and fixed differences between Itatiaia and the other four populations studied (Florianópolis, Cananéia, Juquitiba and Santa Teresa). In addition, the data also provided preliminary evidence for the occurrence of two sympatric siblings in Itatiaia. Finally, significant genetic differences were also observed among some of the other four populations.

Conclusions

Population genetics analysis of *An. cruzii* samples from different localities using a fragment of the *cpr* gene strongly suggests that the Itatiaia sample represents one, or perhaps two, new sibling species in this complex.

Background

Anopheles cruzii has long been known as the primary vector of human and simian malaria parasites in southern and south-eastern Brazil (Deane *et al.*, 1970; Rachou *et al.*, 1958). This species, which belongs to the subgenus *Kerteszia*, is found from the coast of Rio Grande do Sul State in southern Brazil to Sergipe State in north-eastern Brazil (Zavortink, 1973; Consoli & Lourenço-de-Oliveira, 1994), all along the Brazilian Atlantic forest. This forest provides an excellent environment for *An. cruzii*, since it is an ecosystem abundant in bromeliads, the larval habitat for this anopheline (Pittendrigh, 1949; Veloso *et al.*, 1956; Rachou, 1958).

The possibility that *An. cruzii* could represent more than a single species was first supported by morphological differences observed among populations from the states of Santa Catarina and Rio de Janeiro (Zavortink, 1973). Later it was revealed that *Anopheles cruzii* is polymorphic for chromosome rearrangements (Ramirez & Dessen, 1994; Ramirez *et al.*, 1994). Differences in inversion frequencies and X chromosome banding patterns from populations in south-eastern and southern Brazil have suggested a process of incipient speciation (Ramirez & Dessen, 2000a, 2000b). Malafronte *et al.* (2007) compared sequences of ITS2 (Internal Spacer Region 2) from several *An. cruzii* populations from south and south-east Brazil and found differences between sequences from different localities, although they considered premature to conclude based on their results that there are distinct sibling species in the areas investigated. Similar results were observed by Calado *et al.* (2006), using PCR-RAPD and PCR-RFLP of the ITS2 region.

Finally, isoenzyme analysis indicated two genetically isolated groups, one from Bahia State (north-eastern Brazil), and the other from south-eastern and southern Brazil (Rio de Janeiro, São Paulo and Santa Catarina States) (Carvalho-Pinto & Lourenço-de-Oliveira, 2004). Supporting the isoenzyme results, analysis of the molecular polymorphism and genetic differentiation of the *timeless* gene among Brazilian populations of *An. cruzii* also indicated two cryptic species, one occurring in the north-east (Bahia State) and another in south and south-east Brazil (Espírito Santo, Rio de Janeiro, São Paulo and Santa Catarina States). In addition, the *timeless* gene sequences also suggested that populations from the south and south-east regions might also constitute different incipient species within this complex in Brazil (Rona *et al.*, 2009). Since previous X chromosome analyses suggested the existence of sibling species in these Brazilian regions (Ramirez & Dessem 2000a, 2000b), it would be interesting to analyze the same populations with an X-linked molecular marker to see whether a higher level of differentiation is found.

The gene encoding the NADPH-cytochrome P450 reductase (CPR) has been cloned from several insect species (Koener *et al.*, 1993; Hovemann *et al.*, 1997; Nikou *et al.*, 2003). In *Anopheles gambiae*, this protein is encoded by a single copy gene located on the X chromosome (Holt *et al.*, 2002; Nikou *et al.*, 2003). Previous studies have shown that the cytochrome P450 gene family, which is involved in metabolic insecticide resistance, requires CPR to function (Henderson *et al.*, 2003; Smith *et al.*, 1994). Additionally, knockdown of CPR expression increases *An. gambiae* sensitivity to the insecticide permethrin (Lycett *et al.*, 2006). Another putative function associated with CPR in insects is odorant clearance, since the *cpr* gene is highly expressed in the antennae of the fruit fly *Drosophila melanogaster* and more specifically at the base of olfactory sensilla in the

moth *Mamestra brassicae* (Hovemann *et al.*, 1997; Maibeche-Coisne *et al.*, 2005).

Olfactory cues are important environmental stimuli affecting mosquito behavior, playing significant roles in the location of food sources, mates and oviposition sites (Takken and Knols, 1999). Therefore, genes involved in the regulation of antennal response to pheromonal odors can be potentially important in maintaining sexual isolation between closely related species, and in this case, the CPR would be an interesting molecular marker for population studies of the *An. cruzii* complex.

In this study, we carried out an analysis of intraspecific variability and genetic divergence among five Brazilian populations of *An. cruzii* using a fragment of the *cpr* gene.

Methods

The mosquitoes used in this study were females captured at different localities along the Brazilian Atlantic forest and identified on the basis of their morphology according to Consoli and Lourenço-de-Oliveira, 1994. A total of 56 individuals were used for the molecular analysis: 14 from Florianópolis, Santa Catarina State (SC) ($27^{\circ}31'S$ / $48^{\circ}30'W$), 12 from Cananéia, São Paulo State (SP) ($25^{\circ}01'S$ / $47^{\circ}55'W$), 12 from Juquitiba, São Paulo State (SP) ($23^{\circ}57'S$ / $47^{\circ}03'W$), 11 from Itatiaia, Rio de Janeiro State (RJ) ($22^{\circ}27'S$ / $44^{\circ}36'W$) and 7 from Santa Teresa, Espírito Santo State (ES) ($19^{\circ}56'S$ / $40^{\circ}35'W$) (Figure 1).

For the isolation of a fragment of the *An. cruzii cpr* gene, initially a pair of degenerated primers based on conserved regions of the CPR proteins from *Drosophila melanogaster*, *Drosophila pseudoobscura*, *Musca domestica* and *Anopheles gambiae* named here

5'Cpr01deg and 3'Cpr01deg was designed (Table 1 and Figure 2) and used in PCR with *An. cruzii* genomic DNA extracted according to Jowett (Jowett, 1998). PCR was carried out with an Eppendorf Mastercycler® thermocycler using the following conditions: 15 cycles at 94°C for 60 s, 50°C (decreasing 1°C/cycle) for 90 s and 72°C for 60 s, followed by 20 cycles of 94°C for 60 s, 50°C for 90 s and 72°C for 60 s. PCR products were then purified and cloned using Zero Blunt TOPO PCR cloning kit (Invitrogen).

Sequencing of positive clones was carried out in an ABI Prism 3730 DNA sequencer at the Oswaldo Cruz Institute using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The identity of the cloned fragments was confirmed by BlastX analysis using the GenBank (**GenBank database** [<http://www.ncbi.nlm.nih.gov/BLAST/>]). Based on these initial sequences, two new specific primers named 5'cpr01ancruzii and 3'cpr01ancruzii (Table 1 and Figure 2) were designed to amplify a ~180 bp fragment of the *An. cruzii* cpr gene from individual mosquitoes of the different localities listed above. This short fragment includes an intron of variable size (see below) and two small segments of the flanking exons (Figure 2). PCR amplification using the specific primers was carried out for 35 cycles at 94°C for 30 s, 50°C for 60 s and 72°C for 90 s using the proofreading *Pfu* DNA polymerase (Biotoools). PCR fragments were cloned using either Zero Blunt TOPO PCR cloning kit (Invitrogen) or pMOS Blue vector blunt-ended cloning kit (Amersham Biosciences) and at least eight clones of each mosquito were sequenced.

Sequences were edited and in most cases consensus sequences representing the two alleles were generated. In a number of individuals only one haplotype was observed among the eight sequences and in these cases the mosquitoes were classified as

homozygotes. The probability of incorrectly classifying a heterozygote as a homozygote individual with this procedure is less than 1%. Eight homozygotes were found in Florianópolis, four in Cananéia, seven in Juquitiba, three in Itatiaia and two in Santa Teresa. The sequences from homozygote mosquitoes were duplicated prior to analysis. However, the analysis was also carried out without duplicating the homozygote sequences with similar results.

The *cpr* sequences were aligned using ClustalX software (Thompson *et al.*, 1997) and the population genetics analysis was carried out using DNAsP4.0 (Rozas *et al.*, 2003), PROSEQ v 2.91 (Filatov & Charlesworth, 1999) and Arlequin 3.0 (Excoffier *et al.*, 2005) softwares. The haplotype network was estimated using TCS1.21 (Clement *et al.*, 2000).

Results

Polymorphism and divergence among *An. cruzii* populations

A total of 112 sequences were obtained (28 from Florianópolis, 24 from Cananéia, 24 from Juquitiba, 22 from Itatiaia and 14 from Santa Teresa). The sequences were submitted to the GenBank (accession numbers: XXXXX - XXXXX). An alignment of the variable sites is shown in Figure 3 (an alignment of the whole sequences is presented in Additional file 1). The small segments of the flanking exons in this *cpr* fragment are totally conserved among the five populations analyzed. Therefore, all base substitutions occurred in the intron which also shows a number of indels, including three polymorphic dinucleotide repetitions (Figure 3 and Additional file 1). Table 2 shows the number of copies of each dinucleotide repeat in all *An. cruzii* populations. In Itatiaia only one repeat

of CG dinucleotide was found, while in the other four populations there are two or three repeats. Similar pattern was observed for the CT repeat which shows four copies in Itatiaia while there are six to nine in the other populations (Table 2).

Table 3 shows the pair-wise estimates of population differentiation between the *An. cruzii* populations. Because this *cpr* fragment contains a number of indels, the F_{ST} values were calculated in two different ways. In the first one ($F_{ST(1)}$) the gaps were treated as single mutations in the analysis performed with the PROSEQ v 2.91 software. In the second one ($F_{ST(2)}$), the Arlequin 3.0 software was used to calculate the differentiation values considering the three types of dinucleotide repeats as microsatellite loci. In all cases the F_{ST} values were significant and the two types of estimates showed similar results in most cases. Very high F_{ST} values (ranging from ~ 0.6 to 0.8) were found between Itatiaia and the other populations. Albeit significant, the pair-wise F_{ST} values in the comparisons among the samples of Florianópolis, Cananéia and Santa Teresa are usually under 0.2, while those between Juquitiba and the other three populations are moderately high.

Table 3 also shows the average number of nucleotide substitutions per site (D_{xy}), the number of net nucleotide substitutions per site between populations (Da) and the distribution of the four mutually exclusive categories of segregating sites observed in each comparison: the number of exclusive polymorphisms for each population ($S1$ and $S2$), the number of shared polymorphisms (Ss) and the number of fixed differences (Sf). As in the case of the F_{ST} , the highest D_{xy} and Da values are those involving the Itatiaia population. In addition, this sample shows few shared polymorphisms and it is the only one presenting fixed differences in comparisons with the other populations.

Genealogy of the *An. cruzii* cpr sequences

A network of genealogical relationships of *An. cruzii* haplotypes was estimated using the method of Templeton *et al.* (1992) available in the TCS program (Figure 4). Gaps were treated as a 5th state. A network was also estimated ignoring the gaps, but in this case much of the divergence among the sequences was lost and the network was not informative. The haplotype network shows that the Itatiaia population is clearly separated in an isolated group. A less clear separation was found among the sequences of the other populations. Even Juquitiba which shows moderately high F_{ST} values in the comparisons with Florianópolis, Cananéia and Santa Teresa, shared haplotypes with these populations (Figure 4).

Divergence between Itatiaia A and Itatiaia B

Inspection of the *cpr* sequences presented in Figure 3 and Additional file 1 suggests that the Itatiaia sample might include two different sets of individuals. Based on the number uninterrupted AG repeats between positions 32 to 49 (Figure 3) the Itatiaia population was divided in two groups: the first one, called henceforth Itatiaia A, has more than three AG repeats (04 to 06 repeats) and the second, called henceforth Itatiaia B, has exactly three AG repeats (Table 2). According to this classification the individuals Ita2, Ita3, Ita4, Ita8, Ita10 and Ita11 belong to Itatiaia A (genotype “4-6/4-6”), the mosquitoes Ita5, Ita6, Ita7 and Ita9 belong to Itatiaia B (genotype “3/3”) and individual Ita12 is the only “hybrid” between the two groups (genotype “3/4-6”). Therefore, the Itatiaia sample is not in Hardy-Weinberg equilibrium ($X^2 = 7.24$; d.f. = 1; P<0.01) suggesting the possibility that two sibling sympatric might exist in this locality. The separation between the two groups is also evident in Figure 4. Besides, the F_{ST} value (considering gaps as

single mutations) between Itatiaia A and B is quite large (0.6678) and highly significant ($P<0.001$) despite the small sample sizes.

Finally, to test the hypothesis that the Itatiaia population might include two different sympatric siblings we reanalyzed the recently published *timeless* data (Rona *et al.*, 2009) from the same sample. As for the *cpr* data, the *timeless* sequences were divided into Itatiaia A (Ita2, Ita3, Ita4, Ita8, Ita10 and Ita11) and Itatiaia B (Ita5, Ita6, Ita7 and Ita9). The *timeless* gene also suggests that the sequences might belong to two different siblings with a F_{ST} value (0.3418) that is highly significant ($P<0.001$) and the occurrence of two fixed differences. There is also a clear separation between the Itatiaia A and B *timeless* sequences in a haplotype network (Additional file 2).

Discussion

The *X* chromosome seems to be enriched in genes that cause reproductive isolation between species in the genus *Drosophila* (Tao *et al.*, 2003). In addition, many siblings including the *Anopheles gambiae* complex are outcomes of recent speciation processes associated with paracentric inversions involving this chromosome (Ayala & Coluzzi, 2005). The *X* chromosome banding patterns and inversion frequencies studies of Brazilian south and south-east *An. cruzii* populations, showed three *X* chromosomal forms (A, B and C), suggesting a process of incipient speciation (Ramirez & Dessen, 2000a, 2000b). The authors observed that the majority of mosquitoes from Juquitiba population had form A, while form B predominated in Cananéia (Ramirez & Dessen, 2000a, 2000b). In the current study, although there are no fixed differences in the *cpr*

gene between Juquitiba and Cananéia, a moderately high F_{ST} value was observed. In *Anopheles gambiae*, *cpr* is located on the X chromosome. Therefore, if this molecular marker has a similar chromosomal location in *An. cruzii*, it might be associated with the chromosomal forms described by Ramirez & Dessen (2000a, 2000b).

Comparisons of the F_{ST} values observed with *cpr* and *timeless* in all pair-wise comparisons involving the five populations analyzed in the current study and in Rona *et al.* (2009) show only a partial consistence but this is expected. Wang-Sattler *et al.* (2007) demonstrated that the phylogenetic relationships in the *An. gambiae* complex could vary widely between different genomic regions, thus indicating the mosaic nature of the genome of these species (Wang-Sattler *et al.*, 2007).

As mentioned above the *cpr* gene in *An. gambiae* is X-linked, while *timeless* is autosomal. Assuming these two markers have similar locations in *An. cruzii*, *cpr* is expected to be under more efficient selection than the *timeless*, since in species with X/Y sex determination, as *An. cruzii*, rare recessive mutations are fully expressed in the heterogametic sex, which could lead to ‘faster-X evolution’ if a large proportion of mutations are fixed by positive selection (Vicoso & Charlesworth, 2006). If positive selection is more efficient on the X chromosome, we expect it to harbour less variability than the autosomes (Betancourt *et al.*, 2004). The X chromosome is indeed less variable than the autosomes in non-African populations of *D. simulans* (Schofl & Schlotterer, 2004). Comparing *timeless* and *cpr*, the first is more polymorphic than the latter, but the latter shows higher differentiation among the southern populations of *An. cruzii*.

Since *An. cruzii* is polymorphic for chromosomal inversions and Ramirez & Dessem (2000a, 2000b) found evidence for sibling species carrying different X

chromosomal forms, another hypothesis that might explain the differences between the two markers is the suppressed-recombination model of speciation proposed by Coluzzi (1982) (Ayala & Coluzzi, 2005). According to this model, first proposed to account for the speciation patterns observed in the *An. gambiae* species complex, in incipient species the genes within fixed inversions will accumulate allelic differences faster than genes in the colinear chromosomes, where gene flow can occur (Coluzzi, 1982).

Analysis of the molecular polymorphism and genetic differentiation of the *timeless* gene among Brazilian populations of *An. cruzii* suggested that the population from Itatiaia (Rio de Janeiro State) is in a process of differentiation and incipient speciation (Rona *et al.*, 2009). High F_{ST} values between Itatiaia (Rio de Janeiro State) and the other populations from south and south-east Brazil was reported here. In addition, comparison of Itatiaia with other populations revealed some fixed differences and only a few shared polymorphisms. Moreover, the haplotype network shows that Itatiaia is clearly separated in an isolated group (Figure 4). These results therefore strongly suggest that this population represents a different species in the *An. cruzii* complex.

Preliminary evidence was also presented here that raised the possibility of the existence of two different sympatric incipient species in Itatiaia. This is based on the analysis of the genetic differentiation of the *cpr* gene and a reanalysis of the recently published *timeless* data (Rona *et al.*, 2009). Although a putative heterozygote was found in *cpr* analysis considering the AG repeats and shared polymorphisms were observed in *timeless*, high F_{ST} values were detected between Itatiaia A and Itatiaia B in these two molecular markers, as well as fixed differences which demonstrate that these two groups might represent different incipient species. Inspection of the Neighbor-joining tree

presented in the *timeless* study (Rona *et al.*, 2009) reveals that the individuals classified here as Itatiaia A are clearly isolated in a separated branch. However, the individuals classified as Itatiaia B are mixed with the other individuals from south and south-east populations. In that study, only one putative heterozygote was found (Ita01) sharing alleles with the two Itatiaia groups. Unfortunately this DNA sample was lost and therefore it was not possible to analyze the *cpr* gene of this individual mosquito. The sample sizes available of the two Itatiaia groups are quite small and further work is clearly needed to determine beyond any doubt that two sympatric incipient sibling species exist in this locality but the results presented here seem to indicate that is the case.

Analysis of a number of other molecular markers will allow more precise estimation of the Itatiaia population differentiation and would provide a more complete representation of the divergence history of this species complex.

Conclusions

Evidence was presented here suggesting the existence of at least one new sibling species within the *Anopheles (Kerteszia) cruzii* complex in Itatiaia, south-east Brazil, a finding that supports a previous *timeless* gene study. In addition, according to *cpr* and *timeless* genes analysis, the Itatiaia sample might be in fact composed by two sympatric incipient species, named here Itatiaia A and Itatiaia B.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LDPR participated in data generation and analysis, and drafted the manuscript. She also helped capture mosquitoes in Florianópolis. CJCP carried out the capture and morphological identification of mosquitoes collected in Florianópolis. AAP is the principal investigator, participated in its design and coordination, and helped to write the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1 - *Anopheles cruzii* populations

Localities where the five Brazilian *An. cruzii* populations were collected (Source: IBGE).

Figure 2 - CPR protein multiple alignment and primer positions

The putative fragment of *An. cruzii* CPR deduced protein is aligned with *D.*

melanogaster, *D. pseudoobscura*, *M. domestica*, *An. gambiae* and *Ae. aegypt*

homologues. Arrows point to the approximated positions of the primers used in this study. The inverted triangle represents the position of the intron.

Figure 3: Alignment of the variable sites in the *cpr* fragment of *An. cruzii*

Alignment of the variable positions in the DNA sequences from the *cpr* gene fragment from all populations of *An. cruzii* analyzed. The sequences of homozygote individuals were grouped and are represented as a/b. Dots represent the identity of the first nucleotide sequence and asterisks represent the identity of all sequences. Flo: individuals from Florianópolis; Can: Cananéia; Juq: Juquitiba; Ita: Itatiaia; San: Santa Teresa.

Figure 4 - Haplotype network of *cpr* sequences

Each color represents one population of *An. cruzii*. Each circle represents a different haplotype with size proportional to its relative frequency. Haplotype numbers are given in Roman and the number of sequences of each haplotype is given in brackets. The small

white circles represent missing intermediates and the lines connecting the haplotypes represent one mutational step between two observed haplotypes. Each individual of Itatiaia population is discriminated next to respective haplotype.

Tables

Table 1 - Sequence of primers used to amplify the *cpr* gene fragments

Primers Name	Sequence of primers at 5' → 3'
5'Cpr01deg	ATGAARGGNATGGTNGCNGA (forward)
3'Cpr01deg	ATCCARTCRTARAAYTCCAT (reverse)
5'cpr01ancruzii	AGTGTAAATATGGTAAGCG (forward)
3'cpr01ancruzii	GATTCTCGATGTCTTCAG (reverse)

Degenerate and specific primers used to amplify the *cpr* gene fragments in all *Anopheles cruzii* populations.

Table 2 - Number of each dinucleotide repeats in *An. cruzii* populations.

		Nº of alleles in each population				
		Florianópolis	Cananéia	Juquitiba	Santa Teresa	Itatiaia
CG	01 repeat	-	-	-	-	22
	02 repeats	28	24	24	08	-
	03 repeats	-	-	-	06	-
AG	02 repeats	-	-	-	07	-
	03 repeats	24	15	21	05	09
	04 repeats	-	01	02	-	01
	05 repeats	-	-	-	-	07
	06 repeats	-	-	-	01	05
	07 repeats	01	05	01	-	-
	08 repeats	02	-	-	-	-
	09 repeats	01	03	-	01	-
	04 repeats	-	-	-	-	22
CT	06 repeats	01	-	-	-	-
	07 repeats	04	13	23	07	-
	08 repeats	21	10	01	07	-
	09 repeats	02	01	-	-	-

Location of each type of dinucleotide repeat (see Additional file 1): CG repeats at positions 22 to 27, uninterrupted AG repeats (excluding flanking repeats with point mutations) at positions 32 to 49 and CT repeats at positions 120 to 137.

Table 3 - Genetic differentiation between *An. cruzii* populations

Populations	$F_{ST(1)}$	P-value	$F_{ST(2)}$	P-value	D_{xy}	Da	S_s	S_f	S_1	S_2
1. Florianópolis x Cananéia	0.0845	0.013	0.1445	0.004	0.0220	0.0029	07	00	03	02
2. Cananéia x Santa Teresa	0.1811	0.000	0.1827	0.007	0.0339	0.0061	04	00	05	07
3. Florianópolis x Santa Teresa	0.1575	0.000	0.3196	0.000	0.0307	0.0075	04	00	06	07
4. Cananéia x Juquitiba	0.2673	0.000	0.2008	0.000	0.0216	0.0064	06	00	03	02
5. Santa Teresa x Juquitiba	0.2661	0.000	0.4267	0.000	0.0296	0.0104	04	00	07	04
6. Florianópolis x Juquitiba	0.3616	0.000	0.5238	0.000	0.0231	0.0126	05	00	05	03
7. Cananéia x Itatiaia	0.6105	0.000	0.6627	0.000	0.0597	0.0362	03	04	06	07
8. Santa Teresa x Itatiaia	0.6557	0.000	0.5980	0.000	0.0630	0.0355	02	03	09	08
9. Florianópolis x Itatiaia	0.6812	0.000	0.7355	0.000	0.0564	0.0375	02	04	08	08
10. Juquitiba x Itatiaia	0.7710	0.000	0.8078	0.000	0.0618	0.0470	02	04	06	08

F_{ST} , pair-wise estimates of population differentiation (see text for more details); P-value, significance of F_{ST} values (evaluated by 1,000 random permutations). D_{xy} , average number of nucleotide substitutions per site between populations (Nei & Kumar, 2000); Da , number of net nucleotide substitutions per site between populations (Nei & Kumar, 2000). S_1 , number of polymorphic sites exclusive to the first population shown in the first column. S_2 , number of polymorphic sites exclusive to the second population shown in the first column. S_s , number of shared polymorphisms between the two populations. S_f , number of fixed differences between the two populations. These values were calculated at P_{RO}S_{EQ} v 2.91 (Filatov & Charlesworth, 1999) using the alignment shown in Additional file 1 and considering the gaps as single mutations.

Additional files

Additional file 1 – Alignment of the *An. cruzii* cpr sequences

Alignment of the DNA sequences from the *cpr* gene fragment from all populations of *An. cruzii* analyzed. The translated amino acid sequence is shown above the alignment and the intron is highlighted in grey. Dots represent identity and dashes represent gaps. The asterisks in the bottom line represent identity of all sequences. Flo: individuals from Florianópolis; Can: Cananéia; Juq: Juquitiba; Ita: Itatiaia; San: Santa Teresa.

Additional file 2 – Haplotype network using *timeless* nucleotide sequences of the Itatiaia population

Each circle represents a different haplotype with size proportional to its relative frequency. Haplotype numbers are given in Roman and the number of sequences of each haplotype is given in brackets. The small white circles represent missing intermediates and the lines connecting the haplotypes represent one mutational step between two observed haplotypes. Each individual of Itatiaia population is discriminated next to respective haplotype.

Figure 1



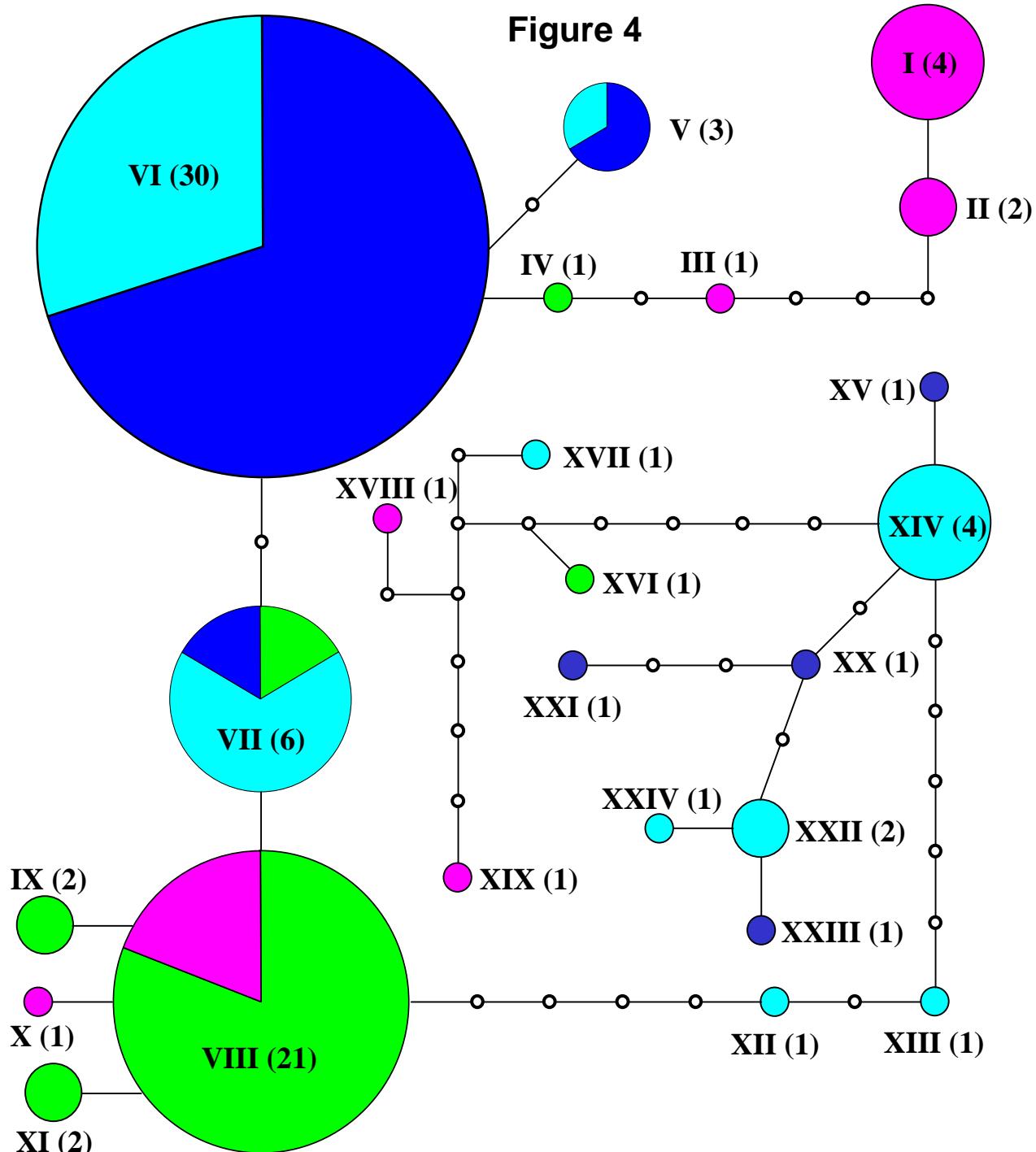
Figure 2

D. melanogaster 5'Cpr01 5'cpr01geral 3'cpr01geral 3'Cpr01
—> LKGMVADPPEECDMEEELLQLKDIDNSLAVFCLATYGEVDPTDNAM
D. pseudoobscura —> LKGMVADPPEECDMEEELLQLKDIDNSLAVFCLATYGEVDPTDNAM
M. domestica MKGMVADPPEECDMEEELLQMKDIPNSLAVFCLATYGEVDPTDNAM
Ae. aegypt MKDMVADPPEECDMEELLSLKDIDKSLAVFCLATYGEVDPTDNCM
An. gambiae MKGMVADPPEECNMEELLMLKDIDKSLAVFCLATYGEVDPTDNCM
An. cruzii MKGMVADPPEECNMEELLQLKDIKSLAVFCLATYGEVDPTDNCM
: * . ***** : ***** : *** : ***** . *

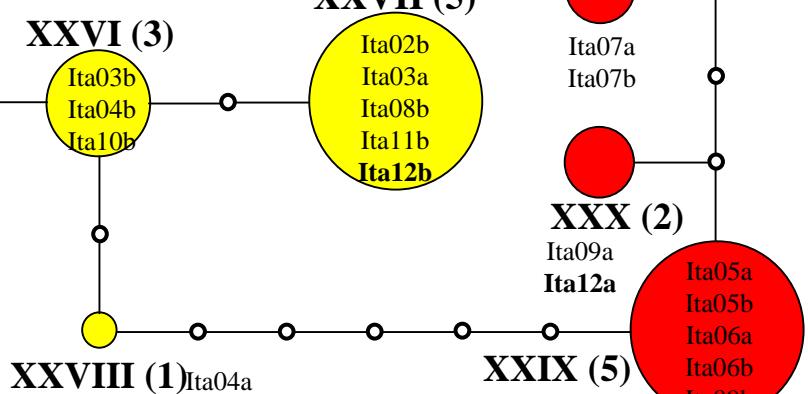
Figure 3

00111111111111
22222333334444444445566778888888899912233333334
14567167890123456789186901013456789016489012345676
Flo04a -CG--GAG-----GCGACGCCGGG---CGCTCTCTCTG
Flo04b -.....
Flo05a -....AGAGAGAGA-C...CGGG.....
Flo05b -.....
Flo06a/b -.....
Flo07a/b -.....
Flo08a -.....
Flo08b -.....
Flo09a/b -.....
Flo10a/b -.....
Flo11a -....AGAGAGAG-A.....C-..CGGG.....
Flo11b -.....
Flo12a -.....
Flo12b -.....
Flo13a/b -.....
Flo15a/b -.....
Flo16a/b -.....
Flo17a/b -.....
Flo18a -....AGAGAGAGAG-A.....C-..CGGG.....
Flo18b -....AGAGAGAGAGA.....C-..CGGG.....
Can01a -.....
Can01b -.....
Can02a -....AGAGAGAG.....C-..CGGG.....
Can02b -....AGAGAGAGAG.....C-..CGGG.....
Can03a/b -.....
Can04a -.....
Can04b -.....C-..CGGG.....
Can05a/b -.....
Can06a/b -....AGAGAGAG.....C-..CGGG.....
Can07a -.....
Can07b -.....
Can08a -.....
Can08b -.....
Can09a/b -.....
Can10a -....AGAGAGAG.....
Can10b -....AGAGAGAGAG.....AC-..CGGG.....
Can11a -.....
Can11b -....AG.....C-..CGGG.....
Can12a -....AGAGAGAGAG.....C-..CGGG.....
Can12b -....AGAGAGAG.....C-..CGGG.....
Jug02a/b -...A.....C.....
Jug11a -....AG.....C.....
Jug11b -....C.....
Jug19a/b -.....
Jug34a/b -.....
Jug35a -....AGAGAGAG.....T.....C.....
Jug35b -.....
Jug42a/b -.....
Jug43a -.....
Jug43b -.....
Jug50a/b -.....
Jug51a/b -.....
Jug58a/b -.....
Jug67a -.....C.....
Jug67b -.....C.....
Jug69a -.....C.....
Jug69b -....AG.....C.....
San01a -.....C.....T.....
San01b -...CG.....A.....
San02a -.....C.....
San02b -.....C.....
San03a -.....C.....
San03b -...CG.....AT.....
San04a -....AGAGAG.....
San04b -....AGAGAGAGAG.....
San06a/b -.....C.....
San07a/b -...CG.....AT.....
San14a -...CG.....AT.....
San14b -...CG.....A.....
Ita02a A.....AGAG.....T.....
Ita02b A.....AGAGAG.....
Ita03a A.....AGAGAG.....
Ita03b A.....AGAG.....
Ita04a A.....AG.....
Ita04b A.....AGAG.....
Ita05a/b A.....
Ita06a/b A.....
Ita07a/b A.....T.T.....A.....A.....
Ita08a A.....AGAG.....T.....
Ita08b A.....AGAGAG.....
Ita09a A.....C.....A.....
Ita09b A.....
Ita10a A.....AGAG.....T.....
Ita10b A.....AGAG.....
Ital1a A.....AGAG.....T.....
Ital1b A.....AGAGAG.....
Ital2a A.....C.....A.....
Ital2b A.....AGAGAG.....

Figure 4



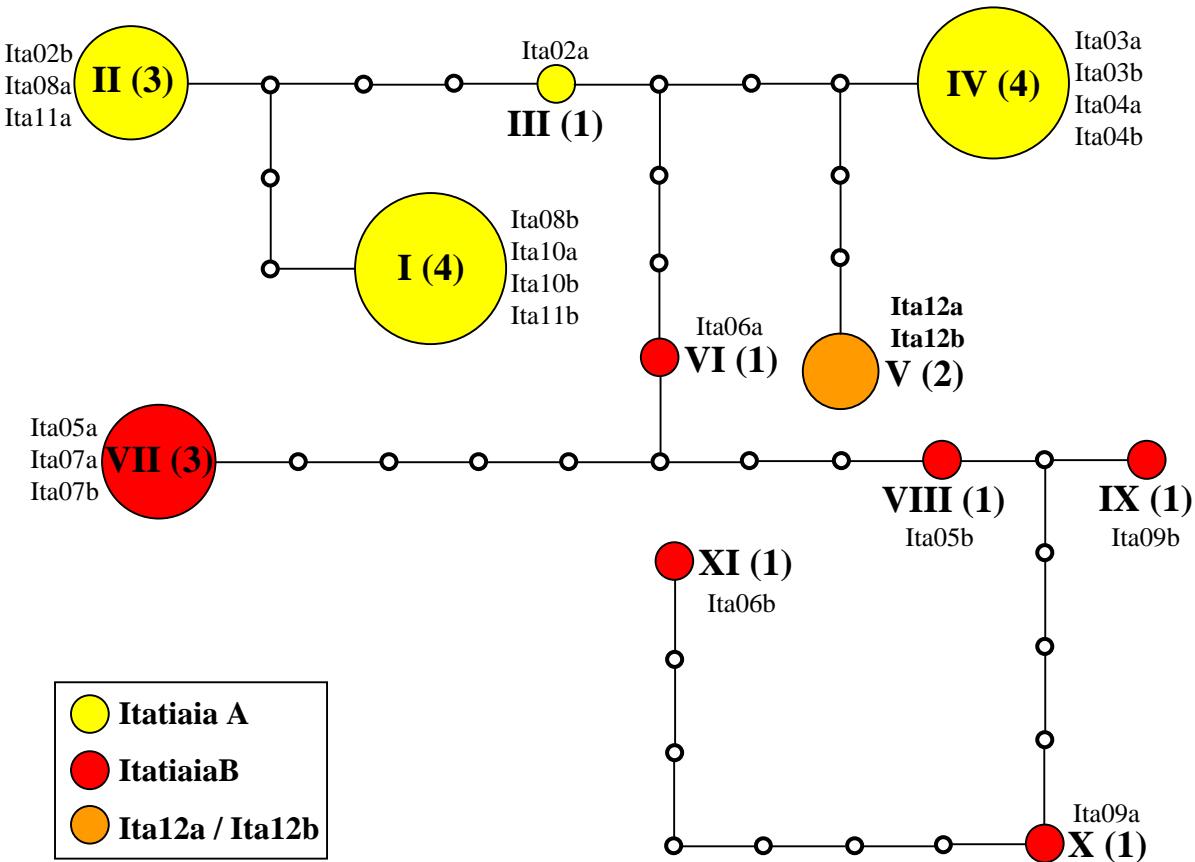
- Florianópolis
- Cananéia
- Juquitiba
- Itatiaia A
- Itatiaia B
- Santa Teresa



Additional file 1

	C N M	E E L L Q L K D I E K
Plo04a	AGTGTAAATAGCAGCGAA-CGGG--AGAGAGAGAG-	-AGTCGCCCTATA CGGTGACCCGGGGGGGGGG--CCAGCATGTTGTAATCCGTTCCACTCTCTCTCTCTGTGACACCGCGAAGACGCTGTGACGCTGAAAGACATCGAAAATC
Plo04b
Plo05a
Plo06a
Plo06b
Plo07a
Plo07b
Plo08a
Plo08b
Plo09a
Plo09b
Plo10a
Plo10b
Plo11a
Plo11b
Plo12a
Plo12b
Plo13a
Plo13b
Plo15a
Plo15b
Plo16a
Plo16b
Plo17a
Plo17b
Plo18a
Plo18b
Can01a
Can01b
Can02a
Can02b
Can03a
Can03b
Can04a
Can04b
Can05a
Can05b
Can06a
Can06b
Can07a
Can07b
Can08a
Can08b
Can09a
Can09b
Can10a
Can10b
Can11a
Can11b
Can12a
Can12b
Jug02a
Jug02b
Jug11a
Jug11b
Jug19a
Jug19b
Jug34a
Jug34b
Jug35a
Jug35b
Jug42a
Jug42b
Jug43a
Jug43b
Jug50a
Jug50b
Jug51a
Jug51b
Jug58a
Jug58b
Jug67a
Jug67b
Jug69a
Jug69b
San01a
San01b
San02a
San02b
San03a
San03b
San04a
San04b
San06a
San06b
San07a
San07b
San14a
San14b
Ita02a
Ita02b
Ita03a
Ita03b
Ita04a
Ita04b
Ita05a
Ita05b
Ita06a
Ita06b
Ita07a
Ita07b
Ita08a
Ita08b
Ita09a
Ita09b
Ita10a
Ita10b
Ita11a
Ita11b
Ita12a
Ita12b

Additional file 2



RESUMO CAPÍTULO 3

- Os resultados desta análise sugerem a existência de pelo menos uma nova espécie do complexo *An. cruzii* em Itatiaia (RJ). Além disso, de acordo com os genes *cpr* e *timeless*, Itatiaia pode ser composta por duas espécies crípticas simpátricas nomeadas provisoriamente de Itatiaia A e Itatiaia B.

APÊNDICE 1

Análise preliminar da divergência entre Itatiaia (RJ) e Florianópolis (SC) utilizando uma abordagem *multilocus*

Nossos resultados com o gene *timeless* mostraram um valor de F_{ST} moderadamente alto entre Itatiaia (RJ) e as outras populações do sul/sudeste do Brasil (Florianópolis, Cananéia, Juquitiba e Santa Teresa), sugerindo uma estruturação incipiente desta população (Capítulo 1). Além disso, os resultados obtidos utilizando o gene *cpr*, mostraram que a população de Itatiaia pertence de fato a um grupo geneticamente isolado das demais populações do sul/sudeste mencionadas acima, apresentando valores de F_{ST} extremamente altos e também diferenças fixas (Capítulo 3). Encontramos também evidência preliminar que Itatiaia possa ser composta por duas espécies simpátricas incipientes (Capítulo 3). A Tabela 1 mostra os resultados dos valores de F_{ST} utilizando os genes *timeless* e *cpr* em que Itatiaia A e Itatiaia B são comparadas com as populações do sul/sudeste. Os menores valores de diferenciação foram obtidos nas comparações com Itatiaia B mostrando que com estes dois marcadores este grupo é menos diferenciado das populações do sul/sudeste do Brasil.

Para investigar com mais detalhes a estruturação genética destas amostras foi realizada uma análise *multilocus* utilizando os outros cinco genes isolados em *An. cruzii* (*Clock*, *cycle*, *Rp49*, *RpS29* e *RpS2*) comparando

Florianópolis (SC) e Itatiaia (RJ), tratando esta última como duas espécies diferentes (Itatiaia A e Itatiaia B).

A Tabela 2 mostra os polimorfismos observados nas populações analisadas. Apesar das seqüências de Florianópolis serem as mesmas utilizadas nos Capítulos 1 e 2, estes mesmos dados de polimorfismo foram colocados nesta tabela para efeito de comparação com Itatiaia A e Itatiaia B e porque o alinhamento entre Itatiaia e Florianópolis é diferente dos outros alinhamentos devido aos indels observados, podendo gerar resultados diferentes. Segundo esta tabela, Itatiaia A foi menos polimórfica que Itatiaia B para quase todos os genes analisados, exceto *RpS2*, mostrando os menores valores de π e de θ assim como menor número de sítios polimórficos.

A Tabela 3 mostra os valores de F_{ST} , as medidas de divergência bruta (D_{XY}) e líquida (D_A), o número de polimorfismos compartilhados (S_s) e de diferenças fixas (S_f) entre as populações de *An. cruzii* e o número de sítios polimórficos exclusivos para cada população (S_1 e S_2). Valores altos e significativos de F_{ST} entre Itatiaia A e Itatiaia B foram observados, de ~0,15 a ~0,5, confirmando a existência em simpatria de dois grupos geneticamente isolados em Itatiaia, apesar do tamanho amostral ser pequeno. No entanto, comparando Florianópolis com Itatiaia A e com Itatiaia B não foi observado um padrão de proximidade como foi evidenciado nas análises com os genes *timeless* e *cpr*, onde alguns genes mostraram maior diferenciação entre Florianópolis e Itatiaia A e outros entre Florianópolis e Itatiaia B. A Figura 1, Apêndice 1, mostra a árvore NJ do gene *timeless*, onde Itatiaia A aparece

claramente separada de Itatiaia B e Florianópolis. Já na árvore feita com o gene *Rp49*, Itatiaia B aparece nitidamente separada dos outros dois grupos (Figura 4, Apêndice 1). Na árvore feita com o gene *RpS2* (Figura 5), as três populações aparecem em grupos bem separados. No entanto, foi encontrado um indivíduo (Ita05), onde um dos alelos desse indivíduo classificado como Itatiaia B está misturado com os alelos de todos os indivíduos classificados como Itatiaia A. Estes três genes, *timeless*, *Rp49* e *RpS2*, que mostraram os mais altos valores de diferenciação entre Itatiaia A e Itatiaia B ($F_{ST} = 0,34$, $0,47$ e $0,44$, respectivamente), revelam árvores que evidenciam visivelmente a separação entre estes dois grupos. Contudo, os outros genes, *Clock*, *cycle* e *RpS29* (Figuras 2, 3 e 6, Apêndice 1), que não possuem uma diferenciação tão alta, apesar de significativa, não mostraram uma separação tão evidente. No entanto, as árvores feitas com estes genes separam Florianópolis das duas espécies incipientes de Itatiaia.

Tabela 1. Medida da diferenciação genética (F_{ST}) entre as populações de *An. cruzii* utilizando os genes *timeless* e *cpr*. $P(F_{ST})$ é a significância dos valores de F_{ST} que foram avaliadas com 1000 permutações. Os valores de F_{ST} do gene *cpr* foram calculados no programa P_{RO}S_{EQ} v 2.91 (Filatov & Charlesworth, 1999) usando o alinhamento mostrado no “Additional file 1” do capítulo 2 e considerando os gaps como mutações únicas (opção: single mutations). Os valores de Itatiaia A x Itatiaia B do capítulo 3 foram repetidos aqui para efeito de comparação.

	F_{ST} <i>timeless</i>	$P(F_{ST})$	F_{ST} <i>cpr</i>	$P(F_{ST})$
Itatiaia A x Florianópolis	0.2834	0.000	0.7993	0.000
Itatiaia A x Cananéia	0.3543	0.000	0.7306	0.000
Itatiaia A x Juquitiba	0.3347	0.000	0.8736	0.000
Itatiaia A x Santa Teresa	0.3501	0.000	0.6871	0.000
Itatiaia B x Florianópolis	0.1404	0.000	0.7321	0.000
Itatiaia B x Cananéia	0.2197	0.000	0.6796	0.000
Itatiaia B x Juquitiba	0.1671	0.000	0.8365	0.000
Itatiaia B x Santa Teresa	0.1180	0.000	0.6212	0.006
Itatiaia A x Itatiaia B	0.3418	0.000	0.6678	0.000

Tabela 2. Polimorfismos observados em Florianópolis, Itatiaia A e Itatiaia B.

Gene	População	<i>n</i>	S	<i>Eta</i>	<i>h</i>	θ	π	D_T
Clock	Florianópolis	24	10	10	06	0.01750	0.01774	0.11242
	Itatiaia A	12	07	07	04	0.01572	0.01283	-0.66471
	Itatiaia B	08	12	15	07	0.04058	0.04003	0.15846
Cycle	Florianópolis	24	21	22	12	0.02787	0.02802	0.13573
	Itatiaia A	12	10	10	06	0.01545	0.01870	0.94964
	Itatiaia B	08	12	12	06	0.02174	0.02359	0.55962
Rp49	Florianópolis	24	10	11	10	0.01126	0.00842	-0.82070
	Itatiaia A	10	05	05	03	0.00662	0.00743	0.52764
	Itatiaia B	08	08	08	06	0.01162	0.01341	0.81775
RPS2	Florianópolis	24	17	18	11	0.01879	0.01723	-0.22879
	Itatiaia A	12	09	09	05	0.01130	0.01163	0.17135
	Itatiaia B	08	04	04	03	0.00582	0.00495	-0.62573
RpS29	Florianópolis	24	13	13	11	0.01375	0.00907	-1.14756
	Itatiaia A	12	12	12	05	0.01479	0.01604	0.42828
	Itatiaia B	08	15	15	06	0.02282	0.01854	-0.85233

n, número de seqüências de DNA de cada amostra; S, número de sítios polimórficos em cada população; *h*, número de haplótipos encontrados em cada população; *Eta*, número total de mutações. θ , parâmetro neutro baseado no número total de mutações (*Eta*); π , número médio de diferenças par-a-par entre as seqüências; D_T , teste *D* de Tajima (1989). Em nenhum caso os valores do teste *D* de Tajima foi significativo ($P > 0.10$). Este teste foi calculado usando o número total de mutações (*Eta*).

Tabela 3. Medida da diferenciação genética (F_{ST}) entre as populações de *An. cruzii*. D_{XY} é a divergência bruta e D_A é a divergência líquida entre populações. A significância dos valores de F_{ST} foram avaliadas com 1000 permutações (em todos os casos $P < 0,001$). Número de polimorfismos compartilhados (S_s) e de diferenças fixas (S_f) entre as populações de *An. cruzii*. S_1 e S_2 são os números de sítios polimórficos exclusivos para cada população. S_1 são os sítios polimórficos exclusivos da primeira população da primeira coluna e S_2 são os sítios polimórficos da segunda população da primeira coluna.

	Populações	F_{ST}	D_{XY}	D_A	S_s	S_f	S_1	S_2
Clock	Itatiaia A x Itatiaia B	0.1783	0.0282	0.0050	03	00	03	08
	Florianópolis x Itatiaia A	0.7525	0.0500	0.0376	00	04	06	06
	Florianópolis x Itatiaia B	0.5542	0.0546	0.0303	00	02	06	11
cycle	Itatiaia A x Itatiaia B	0.1523	0.0249	0.0038	05	00	05	07
	Florianópolis x Itatiaia A	0.3542	0.0362	0.0128	06	00	16	04
	Florianópolis x Itatiaia B	0.2241	0.0333	0.0075	08	00	14	04
Rp49	Itatiaia A x Itatiaia B	0.4784	0.0204	0.0098	00	01	05	08
	Florianópolis x Itatiaia A	0.3038	0.0116	0.0035	02	00	09	03
	Florianópolis x Itatiaia B	0.3740	0.0177	0.0066	00	01	11	08
RpS2	Itatiaia A x Itatiaia B	0.4494	0.0153	0.0069	01	00	08	03
	Florianópolis x Itatiaia A	0.2926	0.0207	0.0060	00	00	18	09
	Florianópolis x Itatiaia B	0.4169	0.0192	0.0080	01	00	17	03
RpS29	Itatiaia A x Itatiaia B	0.1819	0.0216	0.0039	06	00	05	09
	Florianópolis x Itatiaia A	0.7624	0.0545	0.0415	01	05	13	10
	Florianópolis x Itatiaia B	0.7363	0.0536	0.0395	01	04	13	14

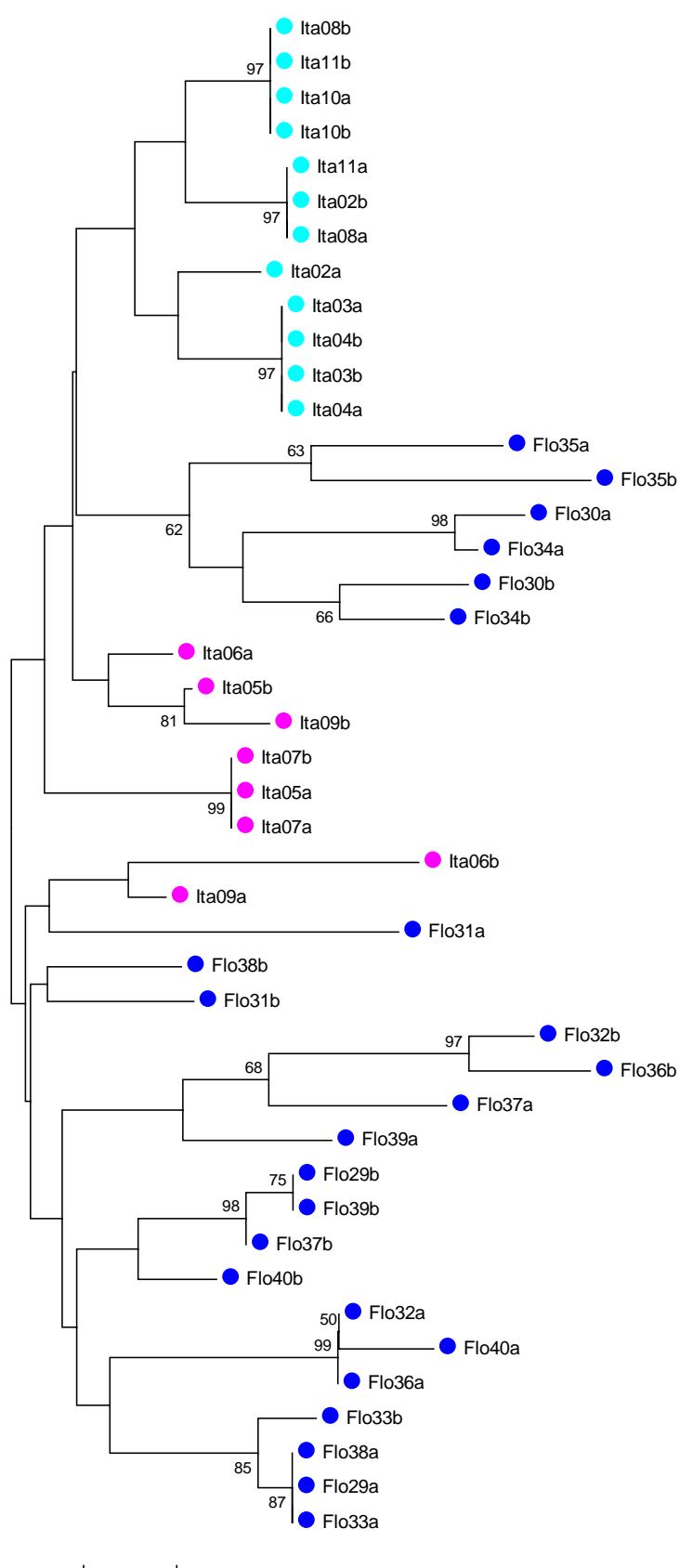


Figura 1. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene *timeless*, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações.

- indivíduos de Florianópolis (SC);
- indivíduos classificados como Itatiaia A;
- indivíduos classificados como Itatiaia B;

Figura 2. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene *Clock*, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações.



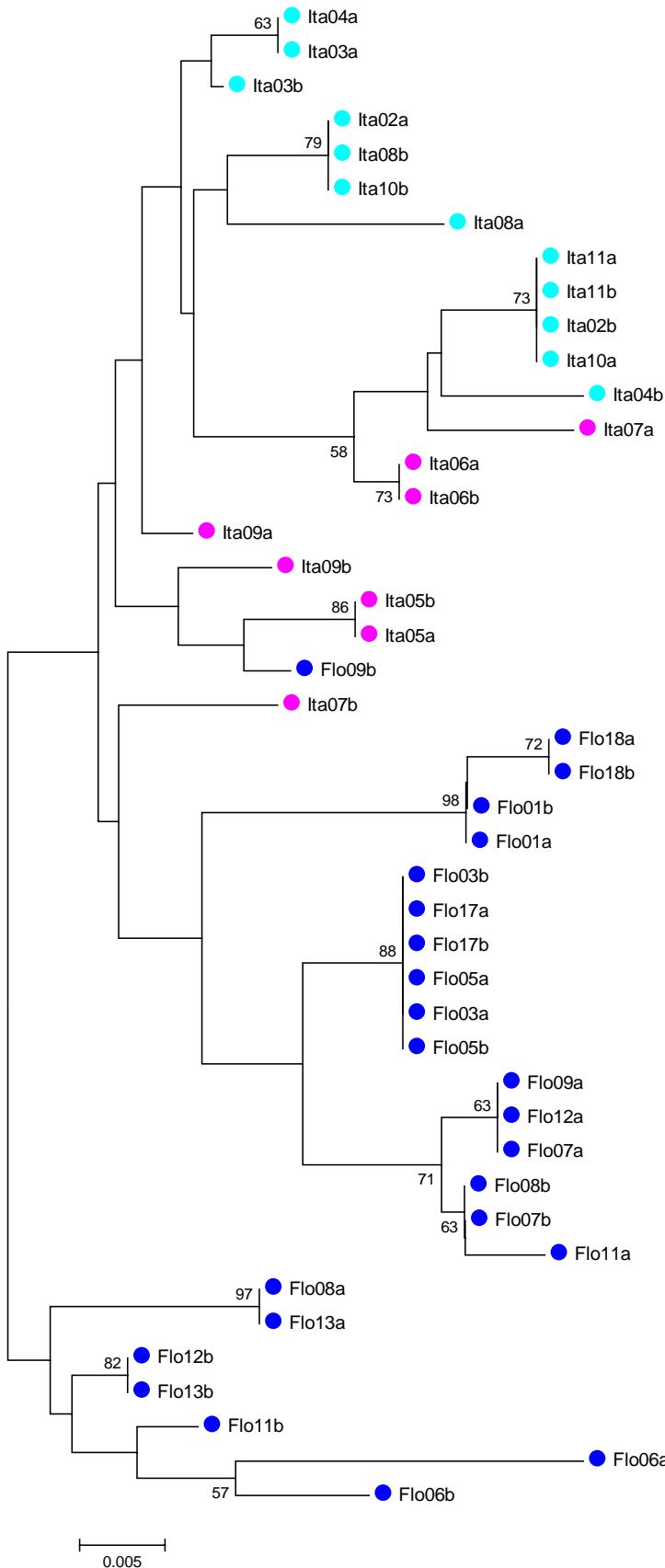


Figura 3. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene *cycle*, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações.

- indivíduos de Florianópolis (SC);
- indivíduos classificados como Itatiaia A;
- indivíduos classificados como Itatiaia B;

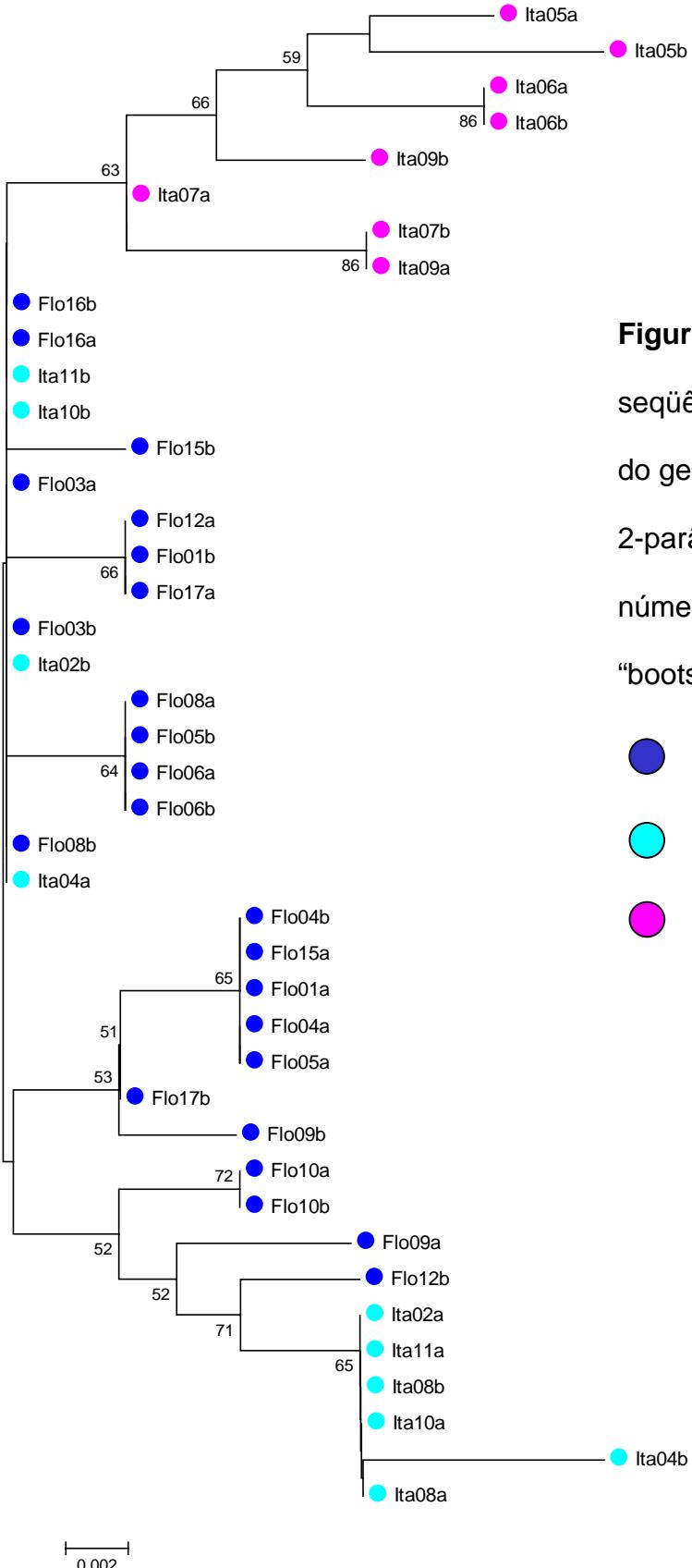


Figura 4. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene *Rp49*, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações.

- indivíduos de Florianópolis (SC);
- indivíduos classificados como Itatiaia A;
- indivíduos classificados como Itatiaia B;

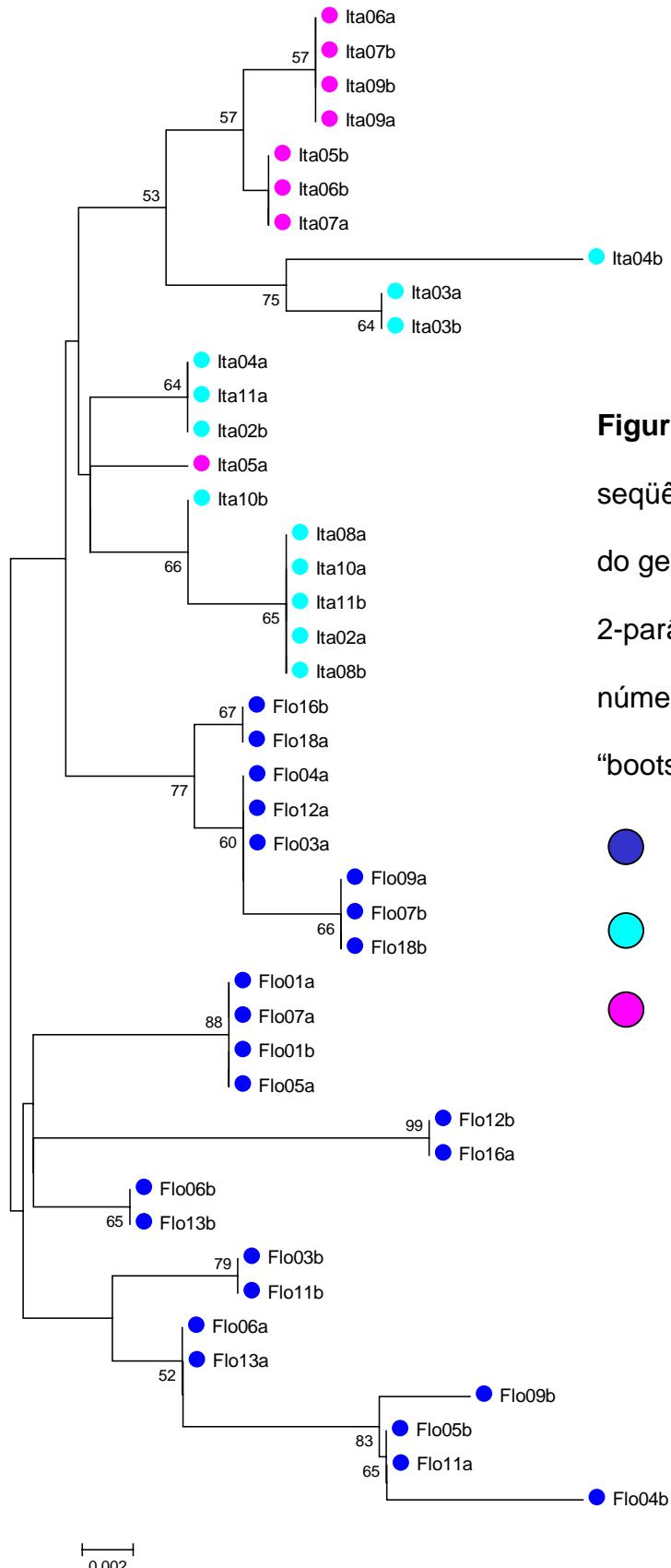


Figura 5. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene *RpS2*, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações.

- indivíduos de Florianópolis (SC);
- indivíduos classificados como Itatiaia A;
- indivíduos classificados como Itatiaia B;

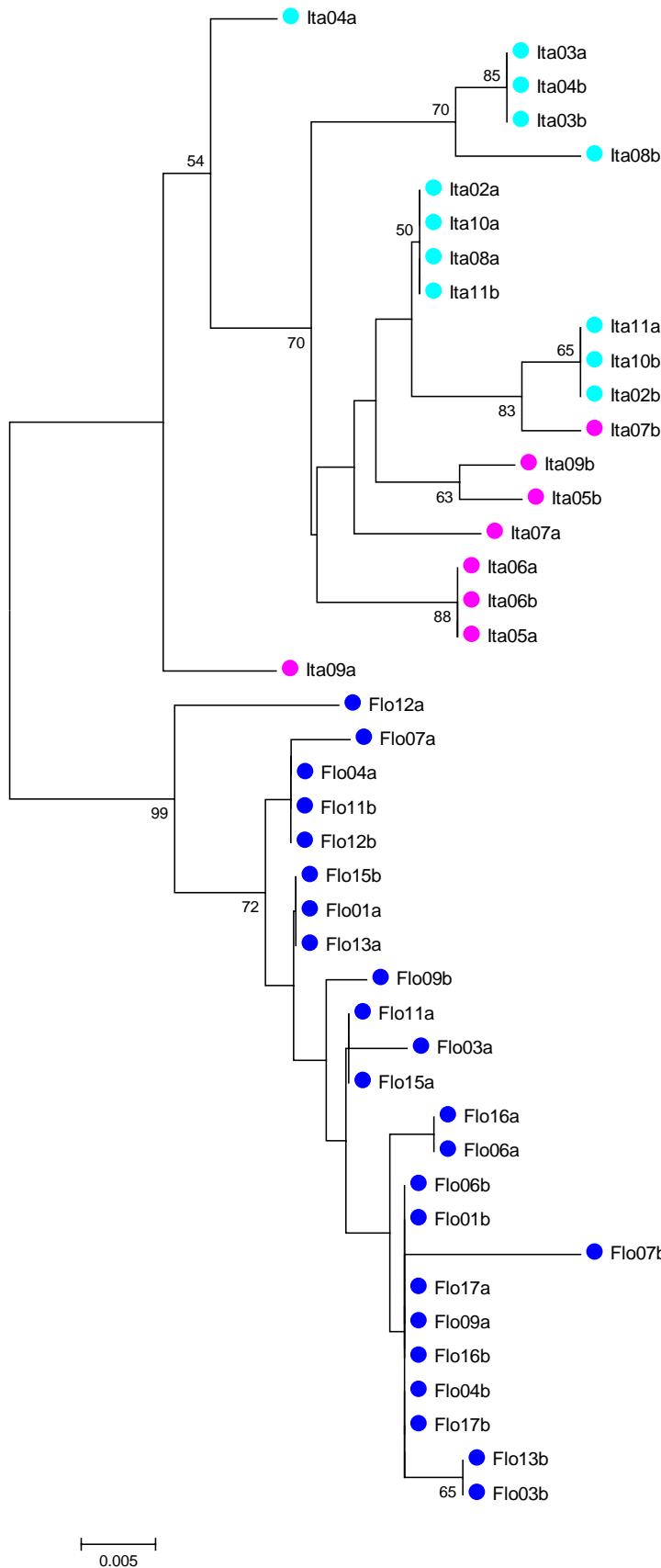


Figura 6. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene *RpS29*, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações.

- indivíduos de Florianópolis (SC);
- indivíduos classificados como Itatiaia A;
- indivíduos classificados como Itatiaia B;

RESUMO APÊNDICE 1

- Os resultados desta análise *multilocus* sugerem fortemente que Itatiaia é constituída por duas espécies incipientes que ocorrem em simpatria nomeadas de Itatiaia A e Itatiaia B, que possuem valores altos e significativos de diferenciação e que também são diferenciadas da população de Florianópolis.

APÊNDICE 2

Evidência para a ocorrência de duas espécies do complexo *Anopheles cruzii* em simpatria na Ilha de Itaparica (BA).

O par de oligos específicos 5'acbatim02a x 3'cruziitim3, desenhados para *An. cruzii* (para mais detalhes, ver Capítulo 1), foi utilizado nas populações do sul/sudeste do Brasil amplificando fragmentos de ~420 pares de base do gene *timeless*. No entanto, na população de Itaparica (BA), fragmentos de tamanhos diferentes das populações do sul/sudeste foram gerados e ainda, dentro desta população onde a maioria dos indivíduos apresentou ~400 pares de base, três indivíduos apresentaram um fragmento ainda menor, de ~360 pares de base do gene *timeless* (Figura 1). Com isso, a maioria dos indivíduos da população de Itaparica, chamada aqui de espécie A, foi utilizada nas análises apresentadas nos Capítulos 1 e 2, enquanto que os três indivíduos que geraram fragmentos de tamanho menor que possivelmente pertencia a outra espécie, que chamamos aqui de Itaparica B, foram separados para uma análise posterior.

Para investigar melhor o grau de diferenciação da espécie hipotética Itaparica B foi feita uma análise *multilocus* incluindo as amostras da Bahia (A e B), Florianópolis e *Anopheles bellator*. A Tabela 1 mostra os polimorfismos observados nas amostras de Itaparica B e de *Anopheles bellator*, sendo que os

valores para as amostras de Florianópolis e Itaparica A podem ser encontrados nos Capítulos 1 e 2. A Tabela 1 mostra também os valores do teste D de Tajima e dos testes D e F de Fu & Li de neutralidade, baseado no número total de mutações. Em nenhum caso os testes foram significativos, não indicando desvios ao modelo neutro de evolução ($P > 0.10$ em todos os casos).

A Tabela 2 mostra os valores de F_{ST} , as medidas de divergência bruta (D_{XY}) e líquida (D_A), o número de polimorfismos compartilhados (S_s) e de diferenças fixas (S_f) entre as populações analisadas e o número de sítios polimórficos exclusivos para cada população (S_1 e S_2).

A análise inicial que realizamos utilizando o gene *timeless* indicou altos valores de F_{ST} entre as três comparações feitas com Itaparica B. Além disso, a árvore NJ feita com as seqüências deste gene (Figura 2), mostrou que as quatro amostras analisadas formam grupos bem isolados entre si. No entanto, Itaparica B parece ser mais próxima de *Anopheles bellator* do que de Florianópolis e de Itaparica A, o que é consistente com os valores mais baixos de F_{ST} entre estes dois grupos. Esta maior similaridade entre Itaparica B e *An. bellator* foi confirmada com o seqüenciamento do mesmo fragmento do gene *cpr* estudado no Capítulo 3. Enquanto que a seqüência de Itaparica A é tão diferente que um alinhamento com as outras espécies do complexo não é possível (dados não mostrados), Itaparica B mostrou seqüências idênticas à algumas de *An. bellator* (Figura 3). Além disso, o padrão de repetições de dinucleotídeos na região do intron deste gene observado nas populações de *An. cruzii* do sul/sudeste do Brasil (Capítulo 3), não foi encontrado em Itaparica B e *An. bellator*.

Assim como em *timeless*, os genes *Clock*, *cycle*, *Rp49*, *RpS2* e *RpS29* mostraram altos valores de F_{ST} na maioria das comparações feitas com Itaparica B (Tabela 2). Nas comparações com *An. bellator*, os valores de diferenciação foram sempre os mais baixos, mas com exceção do gene *cycle*, todos foram significativos e variaram de ~0.2 a ~0.6 (Tabela 2).

As árvores NJ feitas com as seqüências de Florianópolis e das duas espécies de Itaparica, (Figura 2 e Figuras 4 a 8) mostram que estas três amostras formam grupos bem isolados. No entanto, a análise feita com os genes *cycle* e *RpS29* (Figuras 5 e 8) revelam que apesar de bem separadas, as espécies A e B de Itaparica apresentam evidência de introgressão (indivíduo 12). A hipótese de uma introgressão recente parece mais provável visto que os alelos introgredidos do indivíduo acba12, tanto no gene *cycle* quanto no *RpS29* (alelo acba12a em ambos os casos), é idêntico a alguns alelos de Itaparica A, não tendo tido tempo ainda de adquirir novas mutações.

Tabela 1. Polimorfismos observados nas populações de *An. bellator* e de Itaparica B.

População	<i>n</i>	<i>S</i>	<i>Eta</i>	<i>H</i>	θ	π	D_T	D_{FL}	F_{FL}
<i>timeless</i>									
<i>An. bellator</i>	24	20	21	15	0.01586	0.01837	0.65589	-0.03629	0.20508
Itaparica B	06	14	15	06	0.01918	0.01886	0.02876	0.18740	0.16677
<i>Clock</i>									
<i>An. bellator</i>	08	12	12	06	0.02951	0.03461	1.06437	0.95107	1.08440
Itaparica B	06	06	06	04	0.01642	0.01800	0.66564	0.56715	0.62900
<i>cycle</i>									
<i>An. bellator</i>	08	04	04	06	0.00713	0.00885	1.09121	1.31251	1.38654
Itaparica B	06	17	17	05	0.03552	0.02905	-0.92637	-0.95451	-1.03173
<i>Rp49</i>									
<i>An. bellator</i>	08	01	01	02	0.00144	0.00199	1.16650	0.88779	1.03160
Itaparica B	06	02	02	03	0.00329	0.00350	0.31063	0.06221	0.11951
<i>RpS2</i>									
<i>An. bellator</i>	08	08	08	04	0.01175	0.01463	1.26555	1.04971	1.21673
Itaparica B	06	10	10	06	0.01691	0.01338	-1.16050	-1.16055	-1.25330
<i>RpS29</i>									
<i>An. bellator</i>	08	10	10	07	0.01462	0.01293	-0.50375	-0.54286	-0.59142
Itaparica B	06	22	22	05	0.03747	0.02886	-1.23772	-1.24241	-1.35382

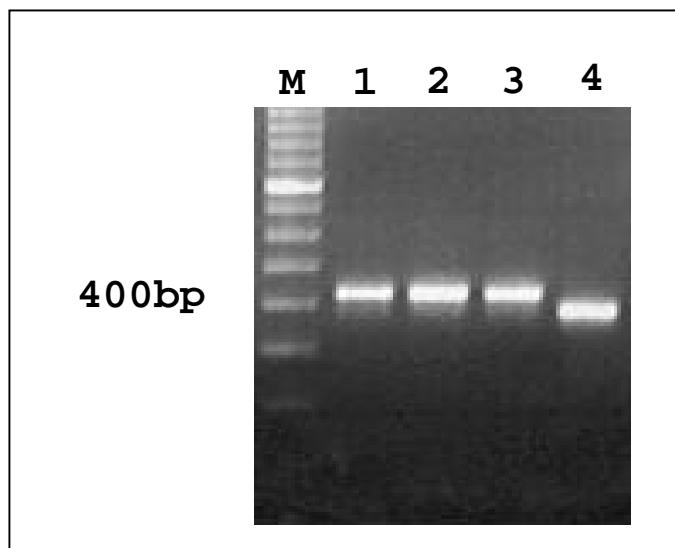
n, número de seqüências de DNA de cada amostra; *S*, número de sítios polimórficos em cada população; *h*, número de haplótipos encontrados em cada população; *Eta*, número total de mutações; θ , parâmetro neutro baseado no número de sítios polimórficos encontrados nas seqüências; π , diversidade nucleotílica, ou seja, número médio de diferenças par-a-par entre as seqüências; D_T , teste *D* de Tajima (1989); D_{FL} , teste *D* de Fu & Li (1993) e F_{FL} , teste *F* de Fu & Li (1993). Em nenhum caso os valores do teste *D* de Tajima e dos testes *D* e *F* de Fu & Li foram significativos ($P > 0.10$ em todos os casos). Estes testes foram calculados usando o número total de mutações.

Tabela 2. Medida da diferenciação genética (F_{ST}) entre as populações de *Anopheles bellator* e de *An. cruzii* provenientes de Florianópolis e Itaparica utilizando a análise *multilocus*. D_{XY} é a divergência bruta e D_A é a divergência líquida entre populações. S_s e S_f são os números de polimorfismos compartilhados e de diferenças fixas entre as populações. S_1 e S_2 são os números de sítios polimórficos exclusivos para cada população. S_1 são os sítios polimórficos exclusivos da primeira população da primeira coluna e S_2 são os sítios polimórficos da segunda população da primeira coluna.

Populações	F_{ST}	D_{XY}	D_A	S_s	S_f	S_1	S_2
<i>timeless</i>							
Florianópolis x Itaparica B	0.8154	0.1287	0.1050	01	30	47	14
Itaparica A x Itaparica B	0.8797	0.1155	0.1016	01	33	18	14
<i>An. bellator</i> x Itaparica B	0.6246	0.0490	0.0306	02	05	17	13
<i>Clock</i>							
Florianópolis x Itaparica B	0.8226	0.1006	0.0423	01	12	09	05
Itaparica A x Itaparica B	0.7898	0.0899	0.0710	00	09	08	04
<i>An. bellator</i> x Itaparica B	0.3634	0.0414	0.0150	01	00	11	05
<i>cycle</i>							
Florianópolis x Itaparica B	0.4350	0.0560	0.0243	03	00	19	14
Itaparica A x Itaparica B	0.5584	0.0434	0.0242	01	00	02	16
<i>An. bellator</i> x Itaparica B	0.0467 ^{ns}	0.0199	0.0009	03	00	01	14
<i>Rp49</i>							
Florianópolis x Itaparica B	0.9162	0.0808	0.0740	00	17	11	02
Itaparica A x Itaparica B	0.9016	0.0589	0.0531	00	13	09	02
<i>An. bellator</i> x Itaparica B	0.5700	0.0064	0.0037	00	00	01	02
<i>RpS2</i>							
Florianópolis x Itaparica B	0.8171	0.0767	0.0627	01	14	15	09
Itaparica A x Itaparica B	0.8458	0.0693	0.0586	00	12	07	10
<i>An. bellator</i> x Itaparica B	0.5383	0.0305	0.0164	00	00	10	22
<i>RpS29</i>							
Florianópolis x Itaparica B	0.6614	0.0723	0.0478	02	05	11	23
Itaparica A x Itaparica B	0.6495	0.0729	0.0474	05	00	16	21
<i>An. bellator</i> x Itaparica B	0.2233	0.8696	0.0269	00	03	08	10

A significância nos valores de F_{ST} foi avaliada com 1000 permutações. Todos os valores de $P < 0.001$, com exceção do valor seguido por ^{ns}, onde $P > 0.05$.

Figura 1: Produtos de PCR dos fragmentos gerados pelo gene *timeless* nos indivíduos da Bahia: Electroforese (agarose 2%) dos produtos de PCR do gene *timeless* do DNA genômico de *Anopheles cruzii* da Bahia utilizando os oligos 5'acbatim2a x 3'cruziitimid03. M – Marcador de peso molecular (100 pares de base). 1 – 3: indivíduos de Itaparica A. 4: indivíduo de Itaparica B.



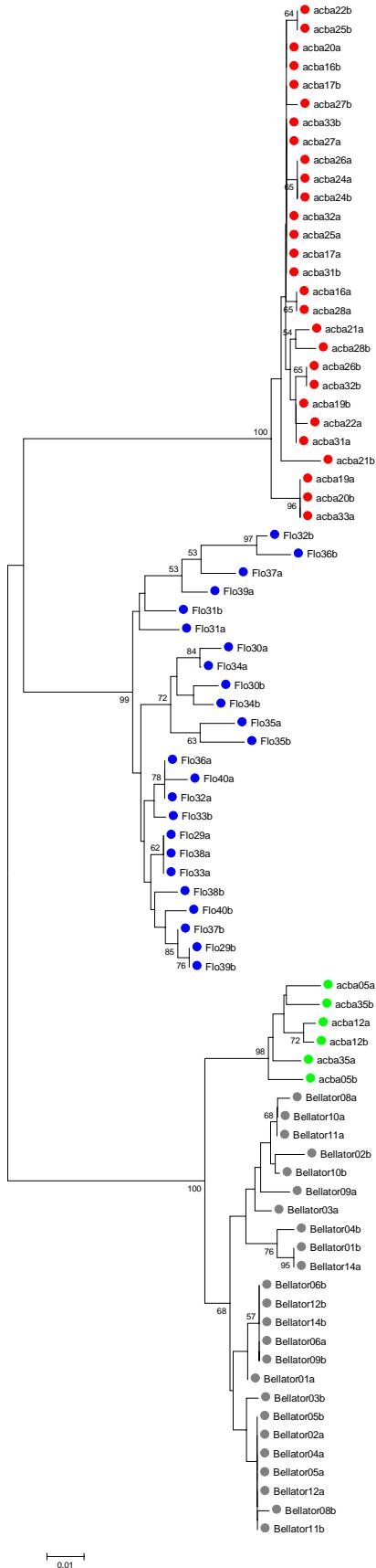


Figura 2. Árvore filogenética das seqüências de *An. cruzii* e *An. bellator* utilizando o fragmento do gene *timeless*, obtida com o método de Neighbor-Joining e distância Kimura 2-parâmetros. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações. Apenas os valores acima de 50% são mostrados. **Azul:** indivíduos de Florianópolis; **Cinza:** *Anopheles bellator*; **Vermelho:** Itaparica A; **Verde:** Itaparica B.

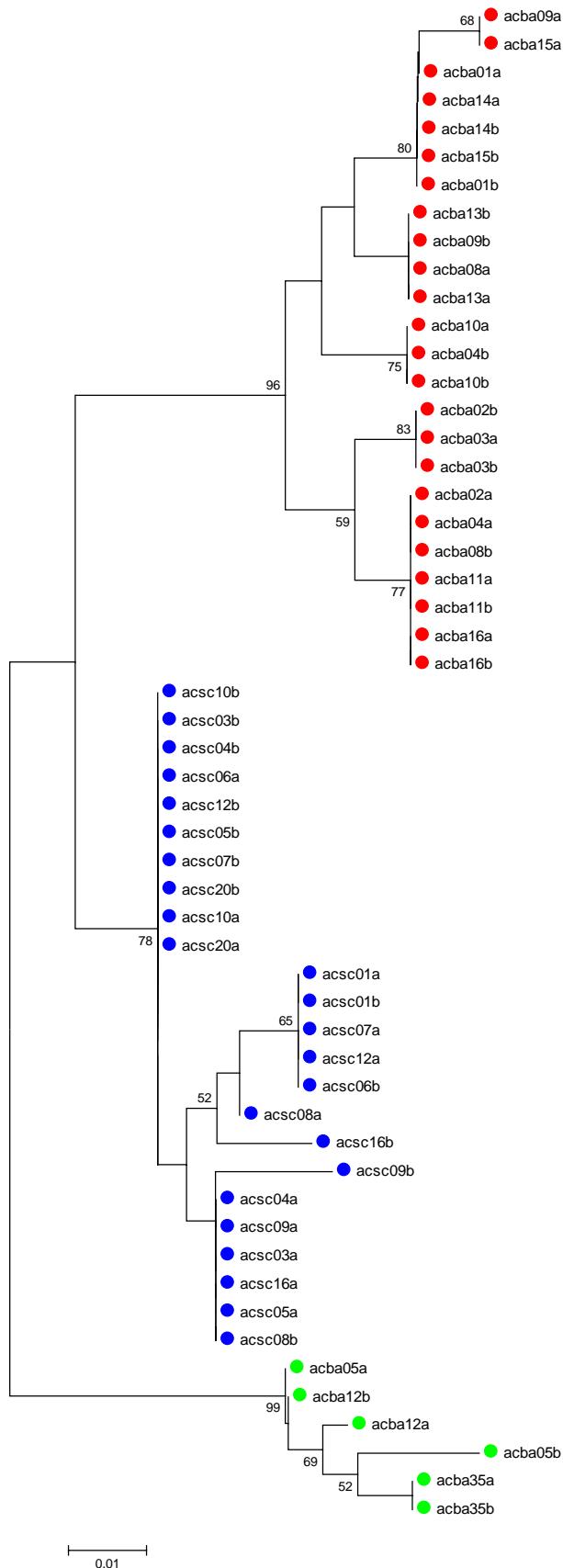


Figura 4. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene **Clock**, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações. **Azul:** indivíduos de Florianópolis (SC); **Vermelho:** Itaparica A; **Verde:** Itaparica B.

Figura 5. Árvore Neighbour-joining

das seqüências de *An. cruzii*

utilizando o fragmento do gene

cycle, obtida com a distância

Kimura 2-parâmetros, estimadas

par-a-par. Os números acima dos

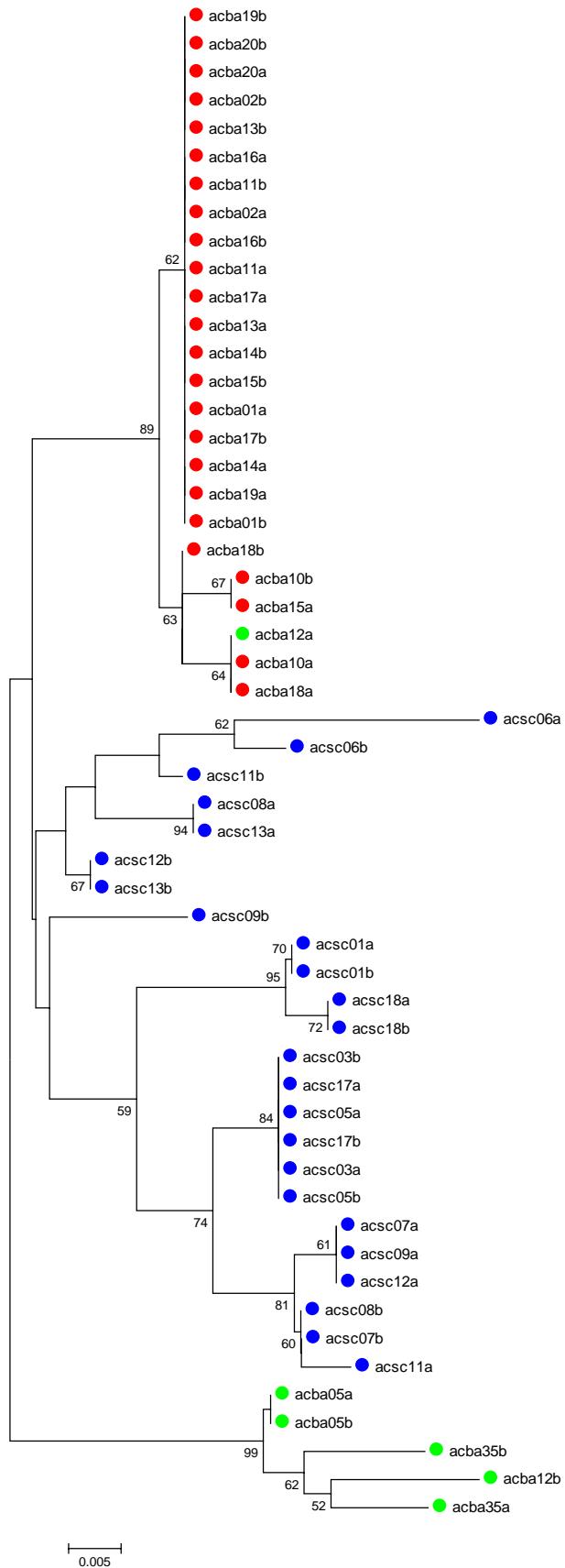
ramos são os valores de

“bootstrap”, baseados em 1000

replicações. **Azul**: indivíduos de

Florianópolis (SC); **Vermelho**:

Itaparica A; **Verde**: Itaparica B.



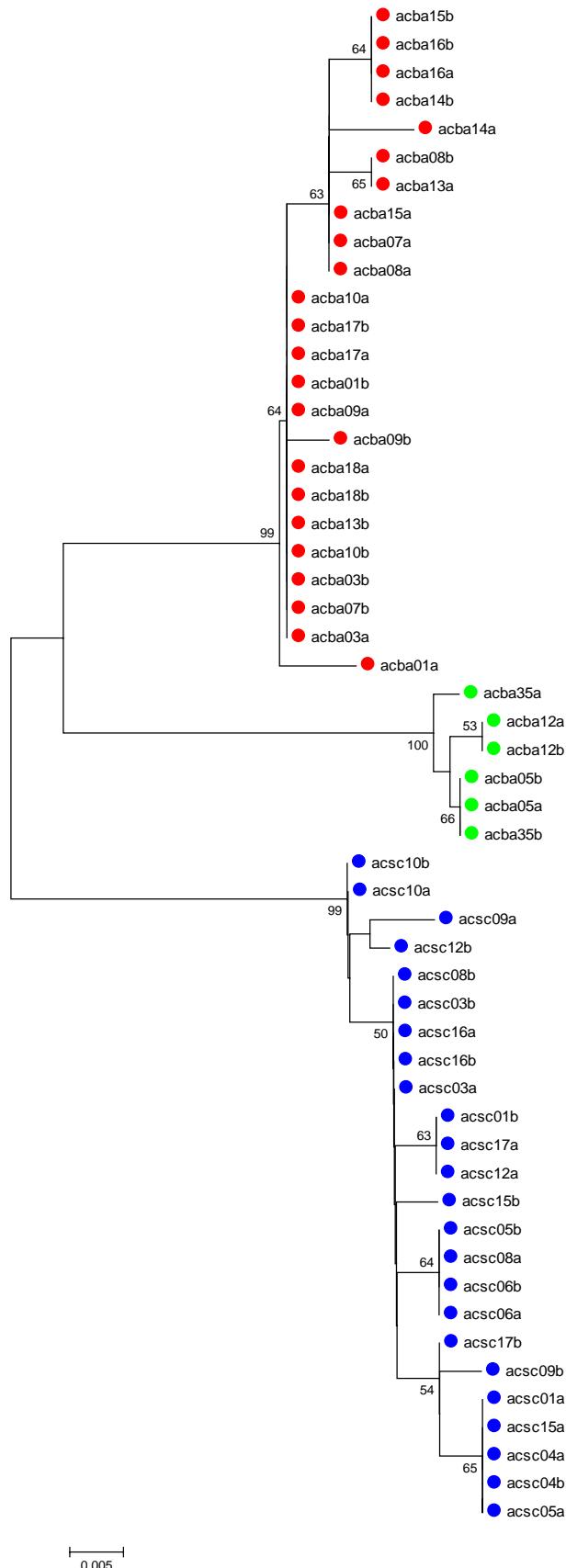


Figura 6. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene ***Rp49***, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações. **Azul:** indivíduos de Florianópolis (SC); **Vermelho:** Itaparica A; **Verde:** Itaparica B.

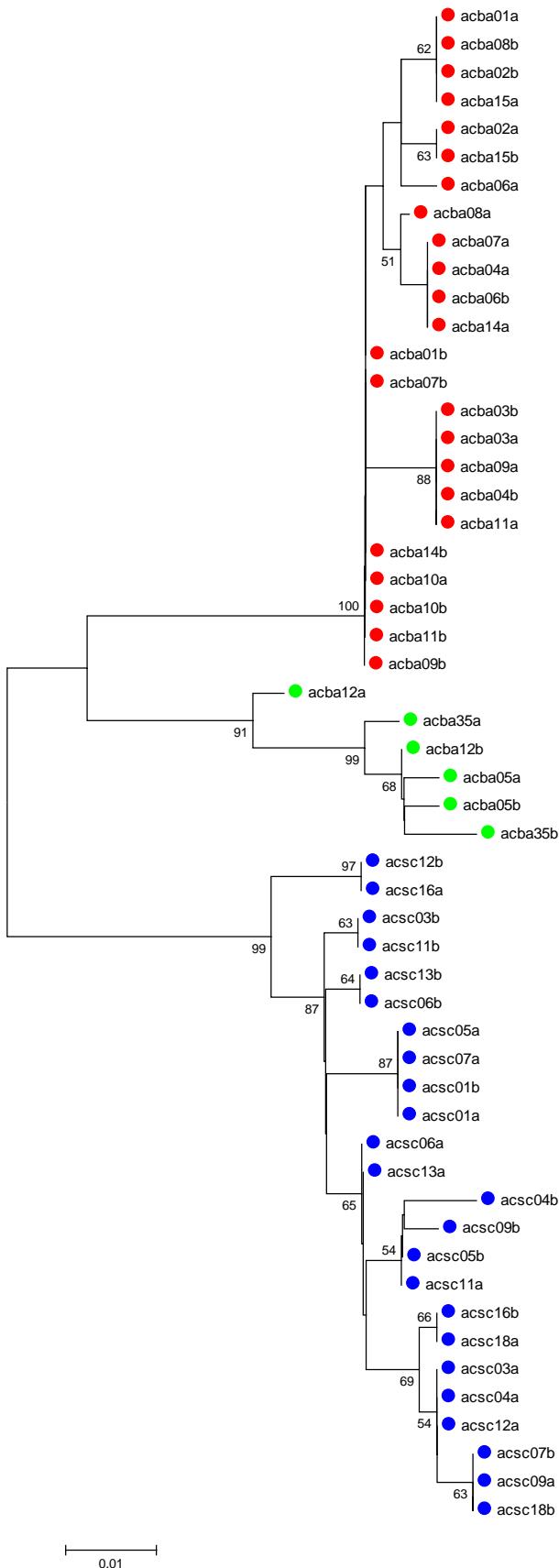


Figura 7. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene **RpS2**, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações. **Azul:** indivíduos de Florianópolis (SC); **Vermelho:** Itaparica A; **Verde:** Itaparica B.

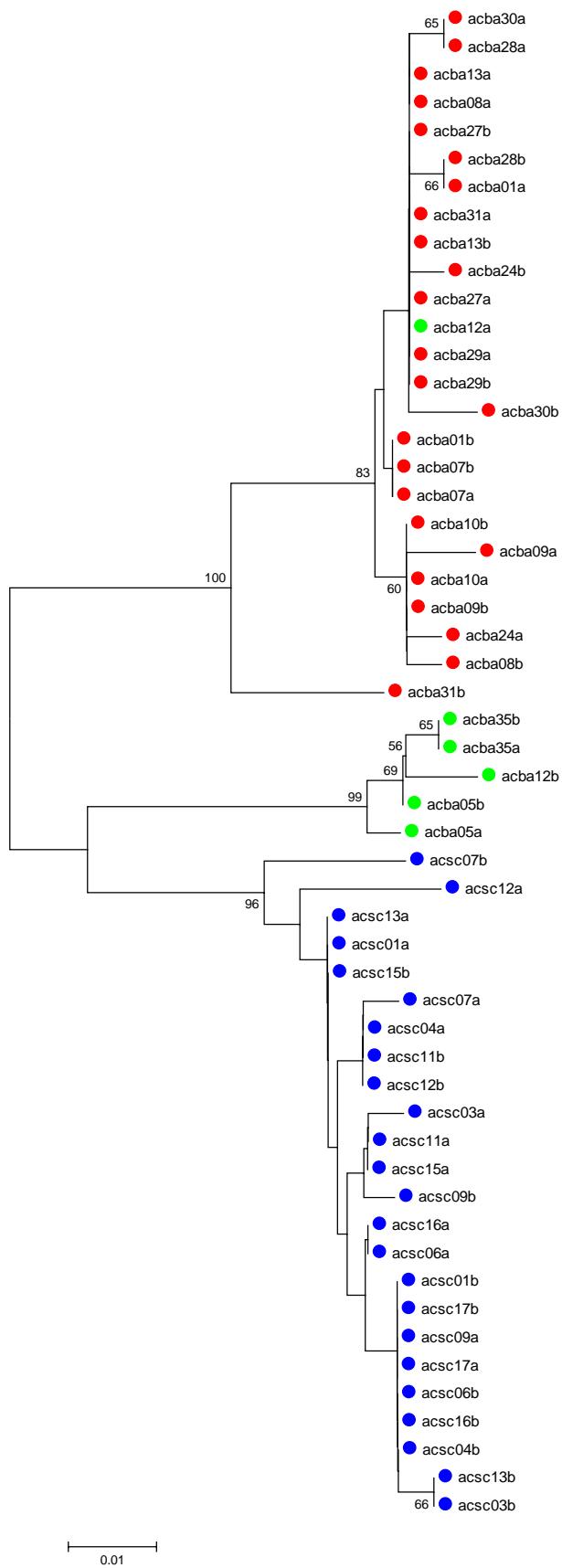


Figura 8. Árvore Neighbour-joining das seqüências de *An. cruzii* utilizando o fragmento do gene **RpS29**, obtida com a distância Kimura 2-parâmetros, estimadas par-a-par. Os números acima dos ramos são os valores de “bootstrap”, baseados em 1000 replicações. **Azul:** indivíduos de Florianópolis (SC); **Vermelho:** Itaparica A; **Verde:** Itaparica B.

RESUMO APÊNDICE 2

- Os resultados desta análise *multilocus* sugerem fortemente que Itaparica (BA) é constituída por duas espécies crípticas simpátricas, sendo que uma delas é mais próxima de *An. bellator*.

DISCUSSÃO

Desde que Zavortink (1973) observou diferenças morfológicas entre as larvas de *Anopheles cruzii* das populações provenientes dos Estados do Rio de Janeiro e de Santa Catarina, diversos trabalhos têm sido feitos tentando elucidar se *Anopheles cruzii* é um complexo de espécies (Ramirez & Dessen, 2000a, 2000b; Carvalho-Pinto & Lourenço-de-Oliveira, 2004; Calado *et al.*, 2006; Malafronte *et al.*, 2007; Rona *et al.*, 2009).

Os resultados com o gene *timeless* apresentados no Capítulo 1 confirmaram que Itaparica (BA) representa uma espécie críptica dentro do táxon *An. cruzii* e sugeriram uma estruturação incipiente da população de Itatiaia (RJ). Os resultados referentes a Itaparica (BA) serão discutidos com detalhes mais adiante. Ainda, utilizando o gene *cpr* (Capítulo 3), os resultados mostram que Itatiaia (RJ) pertence a um grupo geneticamente isolado das demais populações do sul/sudeste do Brasil.

Besansky, *et al.* (2003), utilizando uma abordagem *multilocus* para explorar os padrões de polimorfismos no complexo *Anopheles gambiae*, apresentaram um forte argumento para introgressão entre *An. gambiae* e *An. arabiensis*, que juntos revelaram genomas que são mosaicos com respeito ao fluxo gênico. Em contraste as seqüências ligadas ao cromossomo X mostraram diferenças fixas e divergências profundas quando houve comparação entre

estas espécies. Estes dados são consistentes com um modelo no qual existem barreiras semipermeáveis ao fluxo gênico entre *An. gambiae* e *An. arabiensis*, com interrupção na troca de seqüências ligadas ao cromossomo X. O gene *cpr* em *Anopheles gambiae*, está localizado no cromossomo X e o gene *timeless* é autossômico. Se estes genes tiverem a mesma localização em *An. cruzii*, podemos esperar que a divergência em *timeless* seja menor que em *cpr*, pois se o isolamento reprodutivo entre as espécies do complexo ainda não é completo e ocorre alguma troca de genes entre elas, este fluxo gênico seria maior no gene *timeless* do que na região do cromossomo X, já que a seleção contrária a eventos de introgressão seria maior neste último marcador, assim como acontece entre *An. gambiae* e *An. arabiensis*.

A análise com o gene *cpr* mostrou que em Itatiaia existem provavelmente dois grupos de indivíduos geneticamente separados, identificados pelo número de repetições ininterruptas AG (para mais detalhes, ver Capítulo 3), e classificados como Itatiaia A e Itatiaia B. Estes dois grupos mostraram uma alta diferenciação utilizando o gene *cpr*, e os resultados obtidos com o gene *timeless* confirmaram a mesma separação entre Itatiaia A e Itatiaia B. Os resultados obtidos utilizando os outros genes isolados em *An. cruzii* (Apêndice 1), também suportam a hipótese da existência de duas espécies simpátricas em Itatiaia. No entanto, as árvores NJ mostraram que alguns genes separam claramente estes dois grupos, e outros não mostram uma separação tão evidente, sugerindo que o processo de diferenciação entre Itatiaia A e Itatiaia B é muito recente e ainda existe a retenção de polimorfismos ancestrais. Apesar de em alguns genes

Itatiaia A e Itatiaia B não apresentarem uma separação evidente, não foi verificado nenhum haplótipo idêntico entre estes dois grupos, sugerindo que se houve introgressão, esta é antiga e alelos introgredidos já tiveram tempo de acumular diferenças. Os resultados obtidos com a análise da amostra de Itatiaia, portanto, mostraram que, apesar do pequeno tamanho amostral, existem duas populações sofrendo um processo de especiação incipiente. No futuro, seria interessante realizar novas coletas em Itatiaia (RJ) para aumentar o tamanho amostral destas populações.

Podemos encontrar um paralelo ao que está acontecendo entre Itatiaia A e Itatiaia B, em outro complexo de espécies já bem estudado, o complexo *Anopheles gambiae*, onde a diferenciação entre os membros desse complexo apresenta um modelo com genomas em forma de mosaico, onde o nível de diferenciação entre as espécies varia entre diferentes regiões cromossômicas (della Torre *et al.* 1997; Besansky *et al.* 2003; Slotman *et al.* 2005). Estes complexos de espécies parecem originar-se de um processo de especiação muito recente, com a persistência de polimorfismos compartilhados entre as unidades do complexo, devido a um ancestral comum recente, e devido também a baixos níveis de fluxo gênico (della Torre *et al.*, 2002). Talvez isto explique as diferenças nos resultados entre Itatiaia A e Itatiaia B dependendo do marcador utilizado, onde alguns marcadores detectam altos níveis de diferenciação e outros revelam níveis menores.

Um estudo utilizando análise isoenzimática em algumas populações de *An. cruzii* sugeriu que as populações do sul/sudeste do Brasil (SC, SP e RJ) são

geneticamente distintas da população do Estado da Bahia (Carvalho-Pinto & Lourenço-de-Oliveira, 2004). Com a análise do gene *timeless*, nossos resultados mostram que Itaparica (BA) é realmente diferente das populações do sul/sudeste do Brasil (Capítulo 1). Além disso, comparando Florianópolis e Itaparica utilizando os outros cinco marcadores moleculares, nossos resultados mostraram altos valores de F_{ST} , diferenças fixas e nenhum indício de fluxo gênico entre elas (Capítulo 2). Ainda, encontramos fortes evidências para confirmar a existência de mais de uma espécie no Estado da Bahia. Na verdade, encontramos na Bahia dois grupos geneticamente isolados das populações do sul e sudeste do Brasil, nomeados provisoriamente de Itaparica A e Itaparica B. Comparando Itaparica B, com Itaparica A, Florianópolis (SC) e *Anopheles bellator*, encontramos valores de F_{ST} altos, assim como um grande número de diferenças fixas, mostrando que no Estado da Bahia encontramos duas espécies diferentes do táxon *An. cruzii*, sendo Itaparica B mais próxima de *An. bellator* que das demais populações de *An. cruzii*, indicando que este complexo é provavelmente polifilético.

As seqüências das duas espécies da Bahia mostram separações claras nas árvores Neighbour-joining, formando grupos isolados. No entanto, os genes *RpS29* e *cycle* mostram que apesar de bem separadas, Itaparica A e B apresentam evidência de introgressão. Estes dois genes que geraram este resultado se localizam no cromossomo 2 em *An. gambiae*. Se esta mesma distribuição for verdadeira para *An. cruzii*, talvez os dois alelos do indivíduo 12 que se agrupam com Itaparica A possam estar localizados dentro de uma única

inversão cromossômica que tenha sofrido introgessão, que provavelmente foi recente pois o alelo introgredido de Itaparica B, tanto no gene *cycle* quanto no *RpS29*, é idêntico a alguns alelos de Itaparica A, não tendo tido tempo de adquirir novas mutações.

As inversões cromossômicas são muito importantes na adaptação dos mosquitos a diferentes habitats ecológicos (della Torre *et al.*, 2002). Sendo assim, a introgessão de inversões responsáveis pelo controle da adaptação a determinados ambientes pode ter um forte impacto no espalhamento de doenças transmitidas por vetores. De fato, evidências suportam o papel das inversões *2Rb* e *2La* em conferir tolerância a condições climáticas áridas e sugerem que elas tenham sido “capturadas” por *An. gambiae*, através de fluxo gênico, de *An. arabiensis* (Besansky *et al.*, 2003). Evidências de introgessão também foram encontradas entre duas espécies vetoras de leishmaniose, *Lutzomyia intermedia* e *L. whitmani*, utilizando uma análise *multilocus*, onde foi visto que aproximadamente um terço do genoma destes dois vetores sofrem algum fluxo gênico (Mazzoni *et al.*, 2008). Como já mencionado, esta introgessão em insetos vetores pode ter consequências epidemiológicas. Enquanto os genes envolvidos no isolamento reprodutivo podem estar protegidos contra o fluxo gênico, isto não é necessariamente verdade para genes que podem ter um importante papel na capacidade vetorial ou na adaptação a diferentes ambientes.

Concluindo, nossos resultados mostram que os mosquitos do Estado da Bahia constituem dois grupos geneticamente diferentes entre si e das

populações do sul/sudeste do Brasil. Além disso, os resultados também sugerem que Itatiaia é constituída por duas espécies incipientes que ocorrem em simpatria nomeadas de Itatiaia A e Itatiaia B.

Seria muito interessante coletar outras populações de *Anopheles cruzii* nas proximidades da Ilha de Itaparica (BA) e investigar até onde as duas espécies deste Estado se distribuem e também obter material suficiente para análise no limite norte da distribuição geográfica de *Anopheles cruzii*, no Estado do Sergipe, para verificar com qual das espécies do complexo *An. cruzii* esta amostra se assemelha. Seria importante também realizar novas coletas em Itatiaia para caracterizar melhor estas duas populações. Finalmente, seria importante explorar a localização no genoma de *An. cruzii* dos sete genes que analisamos, utilizando técnicas como hibridização *in situ*, e verificar se algum deles se encontra realmente dentro de alguma inversão cromossômica. Neste sentido, novos estudos utilizando a técnica de análise dos cromossomos politénicos, como os realizados no passado em *An. cruzii* (Ramirez & Dessen, 2000a, 2000b), associados ao tipo de análise que realizamos poderá contribuir muito para o melhor entendimento do complexo.

CONCLUSÕES

- Nossos resultados mostram que os mosquitos do Estado da Bahia constituem dois grupos geneticamente diferentes entre si e das populações do sul/sudeste do Brasil.
- Além disso, os resultados também sugerem que Itatiaia é constituída por duas espécies incipientes que ocorrem em simpatria nomeadas de Itatiaia A e Itatiaia B.

PERSPECTIVAS

- Coletar outras populações de *Anopheles cruzii* nas proximidades da Ilha de Itaparica (BA) e investigar até onde as duas espécies deste Estado se distribuem;
- Obter material suficiente para análise no limite norte da distribuição geográfica de *Anopheles cruzii*, no Estado do Sergipe, para verificar com qual das espécies do complexo *An. cruzii* esta amostra se assemelha;
- Realizar novas coletas em Itatiaia para caracterizar melhor estas duas populações;
- Explorar a localização no genoma de *An. cruzii* dos sete genes que analisamos, utilizando técnicas como hibridização *in situ*, e verificar se algum deles se encontra realmente dentro de alguma inversão cromossômica.

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