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ORIGINAL PAPER

Detecting number of clones, and their relative abundance, of a malaria parasite (*Plasmodium mexicanum*) infecting its vertebrate host

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Abstract Microsatellites, short tandem repeats of nucleotides in the genome, are useful markers to detect clonal diversity within *Plasmodium* infections. However, accuracy in determining number of clones and their relative proportions based on standard genetic analyzer instruments is poorly known. DNA extracted from lizards infected with a malaria parasite, *Plasmodium mexicanum*, provided template to genotype the parasite based on three microsatellite markers. Replicate genotyping of the same natural infections demonstrated strong repeatability of data from the instrument. Mixing DNA extracted from several infected lizards simulated mixed-clone infections with known clonal diversity and relative proportions of clones (N=56 simulations). The instrument readily detected at least four alleles (clones), even when DNA concentrations among clones differed up to tenfold, but alleles of similar size can be missed because they fall within the "stutter" artifact, and rarely does an allele fail to be detected. For simulations of infections that changed their relative proportions over time, changes in relative peak heights on the instrument output closely followed the known changes in relative proportions. Such data are useful for a broad range of studies on the ecology of malaria parasites.

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Introduction

The genetic diversity of malaria parasites (*Plasmodium*) comprises the number of asexually replicating clones within individual vertebrate and insect hosts and the overall diversity among hosts in a local region (Vardo and Schall 2007). Numerous studies reveal substantial clonal diversity within and among both vertebrate and insect hosts, although most studies center on clonal diversity within the vertebrate (e.g., Anderson et al. 2000; Ferreira et al. 2007; Imwong et al. 2006). Two kinds of information would be valuable when scoring the clonal diversity within an infection: the number of genetically distinct clones and their relative abundance. The first class of data is useful for studies on transmission intensity, genetic bottlenecks during drops in transmission, geographic differentiation, gene flow, and the force of selection (Paul et al. 1995, 1998; Anderson et al. 2000; Nair et al. 2003; Vardo and Schall 2007). Relative abundance of clones is relevant for studies on the dynamics of infection, how clones may interact, virulence, and gametocyte sex ratios (Read and Taylor 2001; Reece et al. 2008; Vardo-Zalik and Schall 2008, 2009).

Studies on *Plasmodium* clonal diversity have used surface protein loci (both repeat length and sequence variation) and microsatellites (review in Vardo and Schall 2007). Microsatellites (regions of the genome with tandem short repeats of nucleotides) are particularly helpful because they are common in the *Plasmodium* genome, assumed selectively neutral, and alleles differing in number of repeats are readily scored using genetic analyzer instruments (Bruce et al. 2007). The microsatellite region is amplified by polymerase chain reaction (PCR) in which a florescent dye is annealed to one primer, the product is run through a microcapillary gel system, and the instrument matches the size of the fragment(s) to a concurrently

running set of size standards (Selkoe and Tooner 2006). Because the parasite is haploid, each peak (allele) on the resulting pherogram represents one clone, and multiple peaks represent different genetic clones of the asexually replicating parasite.

Although these methods have become common in *Plasmodium* research, scoring microsatellite alleles has several technical difficulties that can hinder both ecological and genetic studies. The competitive nature of PCR for different lengths of DNA and the relative abundance of DNA fragments (clones for each marker), and vagaries in both the PCR and function of the instrument call into question the ability to determine accurately the number of clones present. For example, capillary-based instruments, such as the ABI Prism, tend to detect the shortest fragments more readily (highest peak will appear for the microsatellite allele with fewest repeats; Selkoe and Toonen 2006). When multiple clones are present, not all may be obvious on the pherogram, and the relative heights of these peaks may not indicate the true relative density of the parasite clones in the infection. A taller peak may simply represent a fragment that was amplified more efficiently because of its size or other more obscure qualities. Thus, it is unclear if the number of peaks and their relative heights provide clear information on clonal diversity and relative abundance of the clones within *Plasmodium* infections. Quantitative PCR detects the relative proportion of *Plasmodium* clones (Reece et al. 2008), but the technique is not feasible for microsatellite markers because each length allele does not have a unique flanking DNA sequence.

We have examined these issues for a malaria parasite of lizards, *Plasmodium mexicanum*. *P. mexicanum* falls within the clade of parasites infecting birds and squamate reptiles (Martinsen et al. 2008). *P. mexicanum* is useful for the study because host DNA is present in any blood sample in hundreds of times the density of parasite DNA (host erythrocytes are nucleated and the vertebrate genome is much larger than that of *Plasmodium*). Therefore, the problems noted above should be even more extreme for this parasite than for those infecting mammals, including humans. Our strategy follows the protocol of Contamin et al. (1995) of mixing extracted DNA from known single-clone infections to produce simulated infections with a manipulated clonal diversity and set relative concentrations.

Materials and methods

Infections

Western fence lizards (*Sceloporus occidentalis*) were collected at the Hopland Research and Extension Center in northern California USA, where lizards are commonly infected with *Plasmodium mexicanum*. This parasite–host system has been studied at the site for many years (reviews in Schall 1996, 2002). Drops of blood were taken (toe clip) to produce thin blood smears to be processed with Giemsa's stain, and several drops were dried on filter paper and stored frozen. Upon inspection of stained blood smears at 1,000×, infected samples were identified. Parasitemia of infections was determined by counting 1,000 erythrocytes and scoring the number of *P. mexicanum* meronts and gametocytes.

Genotyping infections

DNA from the dried dots taken from infected lizards was extracted with the DNeasy kit (Qiagen) using the protocol provided. Microsatellite markers were scored using PCR primers and conditions reported by Schall and Vardo (2007). All markers contained an ATT repeat region. One primer was labeled with a fluorescent marker, and the product run on the ABI Prism DNA analyzer instrument, and the results examined using ABI GeneMapper 3.5 software. The GeneMapper software processes data produced by the DNA analyzer and presents a graph, or pherogram, that shows the length of each fragment (microsatellite size allele). A size allele is represented by a peak with one or more smaller "stutter" peaks one repeat (three bases in this case) smaller and one larger (Fig. 1; Selkoe and Toonen 2006). The software allows those peaks to be enlarged to detect a second allele that may fall within the stutter peaks by visualizing even smaller stutter peaks. The software also shows the heights and areas of peaks on the pherograms. All analyses showed identical results using peak height or peak area; therefore, only results for peak heights are reported here.

Simulated mixed-clone infections

To simulate mixed infections with known number of clones, DNA from several infections was mixed, PCR amplified, and the product run on the Prism instrument. For some simulations that sought to score only number of alleles in mixed-clone infections, extracted DNA from infections with similar parasitemia was mixed without knowledge of the concentration of DNA in the extraction product. For other simulations that manipulated the relative proportions of each clone, the parasitemia (counts under the microscope) and concentration of total extracted DNA was determined (Nanodrop spectrophotometer) and the DNA product diluted to produce approximately equal concentrations of parasite DNA. This could only be an approximate measure because parasite cells could contain 1–12 copies of the genome (male gametocytes may contain several copies, and meronts up to 12), and the relative proportions of cell classes would vary among infections.

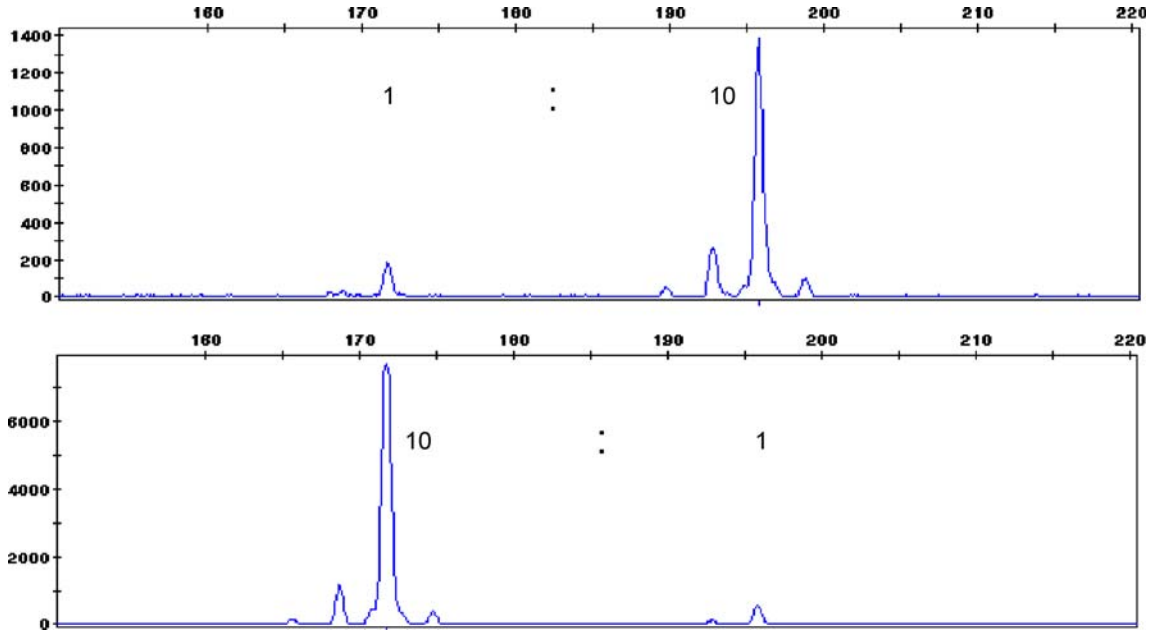

Fig. 1 Pherograms produced by GeneMapper 3.5 software and the ABI Prism genetic analyzer instrument for two simulated infections of *Plasmodium mexicanum* containing two clones of parasite. DNA from known single-clone infections was mixed to contain a ratio of 1:10 for two alleles. Peak heights show that the more common clone produces the highest peak even when that allele includes fewer repeats (total length differs by 24 bp). Note the extra "stutter" peaks adjacent to the taller peak on each pherogram which are taller than the peak for the less common allele. These stutter peaks are normal for the instrument and permits differentiation of true allele peaks from artifacts. The GeneMapper software allows expansion of the smaller true peaks to reveal the stutter there as well

Results

Reproducibility of results

Samples from five naturally infected lizards carrying one, two, two, four, and five alleles of microsatellite marker Pmx 306 were chosen, and DNA extracted each day for 4 days for these samples. Over the subsequent four days, each sample was amplified four times, and then over the last 4 days, each was run through the prism instrument. Thus, each sample was processed from extraction to genotyping four times over a 12-day period. For the 20 runs, all alleles were recorded for each sample; thus, of 60 alleles in the simulated infections, all were seen on the instrument's output. This indicates that minor differences in the laboratory procedures or functioning of the instrument did not produce variable results.

Scoring number of alleles for mixed-clone infections in equal proportions

Four trials mixed DNA from infections with known number of clones and with similar parasitemia. DNA was mixed in equal proportions for each donor infection. In Trial 1, DNA was mixed from six lizards harbouring a total of seven alleles of marker Pmx306. Sizes of these alleles were 170, 173, 182, 191, 194, 197, 206 bp. All seven alleles were seen on the pherograms. Allele length 170 fell within the stutter range of allele 173 but was clearly present when the peaks were expanded in size by the software. Allele 191, however, was small, fell within the stutter of allele 194, and could easily have been missed.

In Trial 2, DNA from five infections was mixed to produce four simulated infections that contained two, three, four, or six alleles for marker Pmx306, with allele sizes of 170, 173, 185, 194, 197, and 209 bp. All alleles were detected when 2–4 alleles were present. For the six allele infection, alleles within 3 bp in size could fall within the stutter of other alleles, but alleles were clearly visible when the peaks were expanded.

In Trials 3 and 4, five other infected lizards were used. DNA from infections carrying six alleles of marker Pmx306 and six alleles of marker Pmx747 (different groups of infected lizards) was combined. In Trial 3, in which Pmx306 allele sizes 170, 173, 185, 194, 200, and 206 bp were combined, five alleles were clearly visible (including allele 170 when the peaks were expanded), but allele 200 failed to amplify and was not visible on the pherogram. In Trial 4, Pmx747 allele sizes 160, 175, 187, 193, 199, and 208 bp were mixed; none of these would fall into the stutter range of other alleles. Four alleles were clearly visible, but the two largest alleles did not produce peaks on the pherogram.

In summary, seven simulated infections were initiated, carrying 2–7 alleles for two microsatellite markers. Of the total of 34 alleles in these infections, 31 were clearly visible on the pherograms. All of the three alleles that were missed were the larger sizes, suggesting that these amplify less efficiently when mixed with smaller length alleles.

Scoring number of alleles for mixed-clone infections in unequal proportions

The competitive nature of PCR may favor the more common DNA fragment and, thus, the more common clone in a mixed infection. Also, shorter fragments may both amplify and be taken into the genetic analyzer more readily as was suggested by the previous trials. Both biases may result in some alleles not appearing on the pherogram. To test for this possible effect, DNA from single-clone infections was combined in various unequal proportions to produce a total of 38 simulated infections. In the first series, DNA from five single-clone infections with Pmx306 alleles differing in length of 3–12 bp was combined to produce eight simulated infections each containing two alleles in 1:10 proportions. The mixed DNA was then used as template for PCR and examination of number of peaks and their heights on the resulting pherogram. Both alleles were seen in 7/8 simulated infections, with the allele in higher concentration producing the tallest peak (Fig. 1). Only one allele was seen in the last simulated infection; this was the allele with lower concentration and longer length (length 185 vs. 182).

In the second series, simulated infections were created using three markers (Pmx306, 747, 732). Three natural infections for each marker were chosen that contained a single allele for that marker. DNA was mixed to produce simulated infections with two alleles at concentrations of 1:1, 1:5, or 1:10 (total of ten simulated infections for each marker). Alleles were chosen with a broad size range for Pmx306 (24 bp), Pmx747 (24 bp), and Pmx732 (74 bp). Thus, alleles did not overlap in the stutter range, but alleles were chosen to accentuate any effect of amplifying two alleles of substantially different lengths. For marker Pmx306, all alleles were seen on the pherograms. Of the ten simulated infections for marker Pmx747, all but one allele was seen. Contrary to expectations, this was the smaller allele and in a higher concentration (169 vs. 193 bp; 5:1). One of the ten simulated infections for marker Pmx732 failed to amplify any alleles, and one showed one allele. Again, contrary to expectations, this was the shorter allele at higher concentration (270 vs. 279; 10:1).

In summary, of 76 alleles in 38 simulated infections, only five failed to appear on the pherograms, despite many simulations carrying alleles in substantially different proportions (10:1).

Detecting relative proportion of clones

Three single-clone infections (based on three microsatellite markers with one allele for each of Pmx306, 732, and 747) with similar parasitemias were chosen, and the DNA concentration was diluted to approximately equal proportions for each clone using overall DNA concentration and parasitemia to estimate relative amounts of parasite DNA present ("Materials and methods"). Relative peaks heights were examined for Pmx306, with the three alleles for that marker labeled as A, B, and C (these differed by 12, 12 and 24 bp in length, so the alleles did not fall within the stutter regions). In Trial 1, samples were mixed in proportions 1A:1B:1C; 1A:1B:2C; 1A:1B:5C; 2A:1B:1C; and 5A:1B:1C. In Trial 2, extracted DNA was mixed in ratios of 1A:1B:1C (twice); 1A:1B:2C (twice); 1A:1B:5C; 2A:1B:1C. The mixed DNA was used as template for PCR and examination for peak heights.

In both trials, all three alleles were seen for each simulated infection (Fig. 2). The ratio of actual peak heights for each trial was compared with the ratio expected based on the mixture of DNA. For example, for a mixture of 1A:1B:5C, the actual peak ratios for the three alleles were compared with the peaks expected based on those proportions of the mixed DNA. The results showed a significant relationship, with slope close to unity as expected, between actual peak height and known mixture of the DNA (r^2=0.442, slope=0.937, P<0.05). However, a substantial residual variation resulted from differing peak heights for the 1A:1B:1C proportions in an infection (Fig. 2).

Another goal of field studies would be to follow relative changes in clonal density within infections over time. In that case, the first sample would be used to calibrate subsequent results to detect changes in density among clones. This was simulated by assuming the sample mixtures were from the same infection but taken over time. The order of the samples can be arbitrarily chosen, but the qualitative result would be the same for any order. The ratio of actual peak heights was then used as the "observed" relative change in the clones from the first sample. For both simulation trials, the change in observed peak height closely followed the actual changes in relative density of alleles present (r^2=0.937, slope=1.096, P<0.01). Figure 3 shows the result for one of the two trials, following the sample order of simulated infections shown in Fig. 2.

Discussion

Information on both the number and relative proportion of *Plasmodium* clones coexisting in the vertebrate host gives insight into a diverse array of questions in ecological parasitology. One class of markers for measuring *Plasmodium*

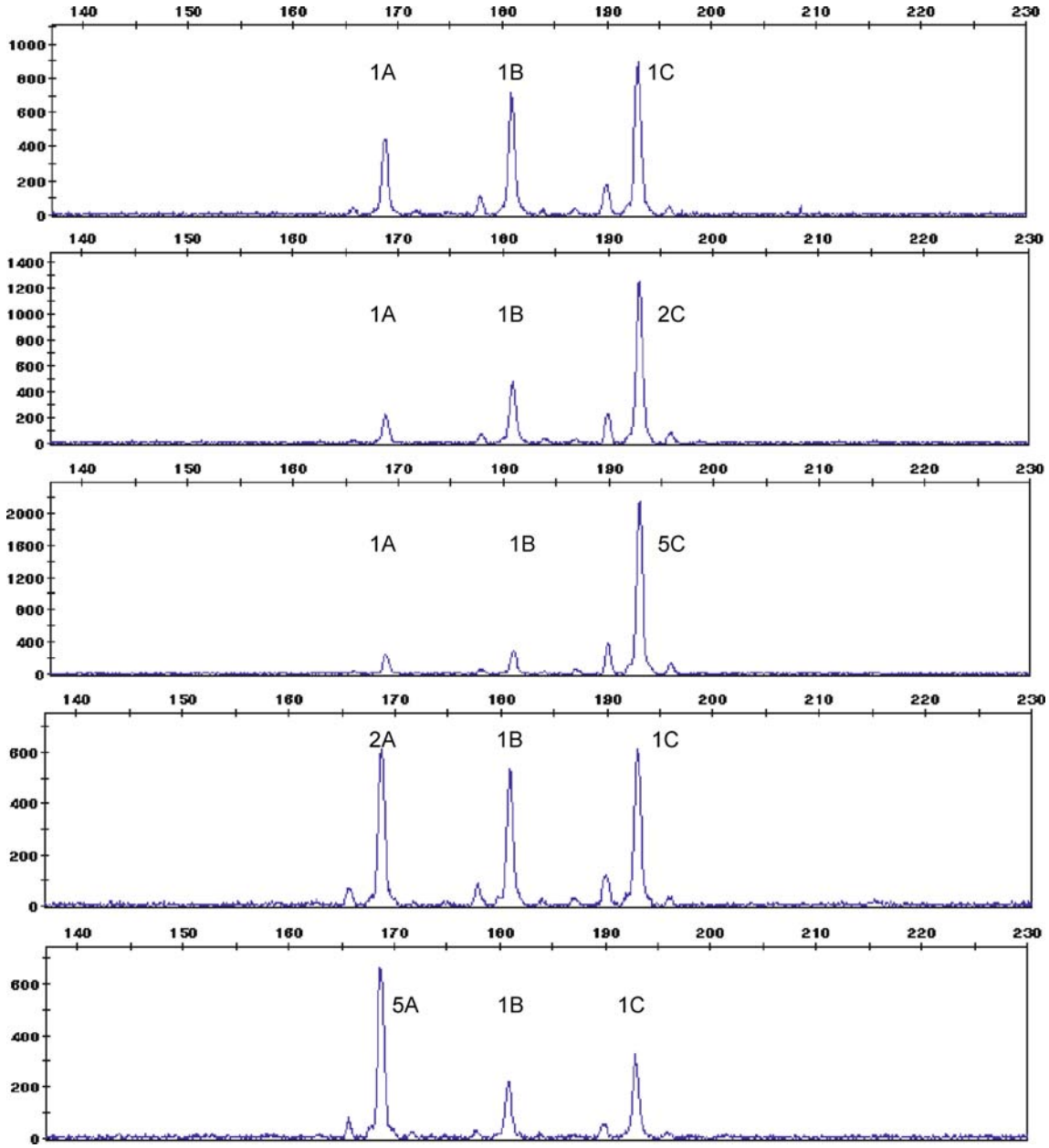

Fig. 2 Pherograms produced by GeneMapper 3.5 software and the ABI Prism genetic analyzer instrument for five simulated infections created by mixing DNA from known single-clone infections of *Plasmodium mexicanum* in various proportions. Extra small peaks adjacent to the main peaks are "stutter" expected with the instrument

clonal diversity are microsatellites, the sort tandem nucleotide repeats that are common in the parasite's genome. These markers have proven useful for studies in population genetics, virulence, infection dynamics, and the force of selection (Anderson et al. 2000; Nair et al. 2003; Bruce et al. 2007; Vardo and Schall 2007; Vardo-Zalik and Schall 2008, 2009). However, two major technical challenges may obstruct interpretation of data on microsatellite markers for *Plasmodium*. First, the PCR and instrument artifacts may underestimate the number of clones in genetically complex infections. Some alleles can fall within the stutter range of other alleles, and PCR can favor the amplification of alleles with fewer repeats. Second, although it is tempting to use peak heights in mixed-clone infections to score relative proportions of the clones, unknown biases in both PCR and the instrument function could yield spurious conclusions. These potential problems result in most studies using only some of the potential information presented by instrument data. In most studies, only the tallest peak is scored for any marker for each sample (e.g., Anderson et al. 2000; Machado et al. 2004). Our goal here was to determine if complex infections are revealed by the pherograms and if peak heights contain useful information.

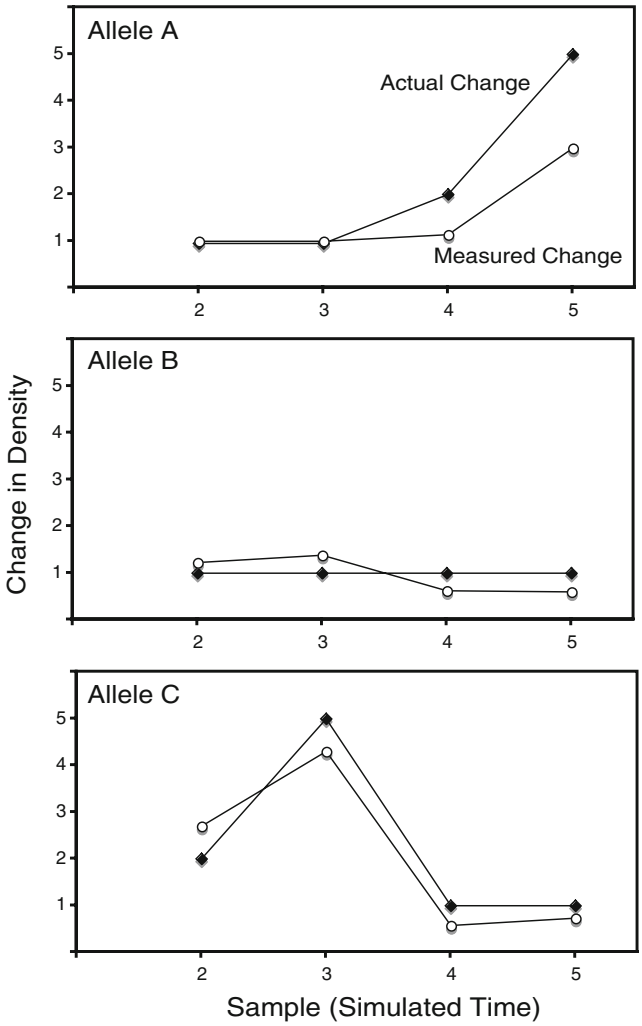

Fig. 3 Data from Fig. 2 are used to compare known changes in relative proportions of three clones of *Plasmodium mexicanum* (based on differences in relative proportions of DNA mixed to create simulated infections) with observed changes in relative peak heights resulting from analysis with GeneMapper 3.5 software and data from the ABI Prism genetic analyzer instrument. Sequence of samples shown in Fig. 2 is used to simulate multiple samples from the same infection over time. For example, the second sample simulates no change in clones A and B, but a doubling of Clone C, with closed points showing the actual change in relative proportion of DNA from each clone, and open points the relative proportion determined by measuring peak heights on the pherogram. Throughout the series, the measured peaks heights closely matches the known change in relative proportion of DNA from the three clones

The trials reported here follow a protocol devised by Contamin et al. (1995) who scored number of alleles of *P. falciparum* surface proteins in mixtures of DNA from natural infections. We simulated infections of the lizard malaria parasite, *P. mexicanum*, by mixing DNA from known genotype infections based on three microsatellite markers. Both number of peaks and their heights were determined by examination of pherograms produced by an ABI Prism genetic analyzer instrument. We first demonstrated that the results were highly reproducible. Repeated processing of the same samples from DNA extraction to final genotyping revealed the same number of peaks on each pherogram. Thus, variation in preparing materials for PCR and in the function of the instrument over several days did not alter the final outcome. Although this reliability is always assumed in population genetics studies using microsatellite data, we suggest that a verification run be conducted for each study to assure the continuity of results.

We next asked if all clones within genetically complex infections could be detected, either when the clones were in approximately equal proportions or if some were five or ten times more abundant than others. Contamin et al. (1995) found a limit to the number of clones detected for *P. falciparum*, using the surface protein loci. We examined simulated infections with two to six clones in proportions from 1:1 to 10:1 for different alleles. Microsatellite alleles falling within one repeat unit of another allele are notoriously problematic in scoring (Selkoe and Toonen 2006). However, in most cases, we were able to distinguish alleles falling within one repeat unit in size by expanding the pherogram peaks using the GeneMapper software. Nonetheless, of a total of 110 alleles in the simulated infections, eight failed to amplify or were confused as stutter. Therefore, in our ecological studies, we are cautious in examining infections, preferring to cast infections within bins as one, two, three, or >3 alleles (Vardo et al. 2007; Vardo and Schall 2007; Vardo-Zalik and Schall 2008, 2009). Also, scoring only the tallest peak on pherograms versus scoring all clear peaks gives similar qualitative results in population genetics studies, but scoring all peaks is vital for determining the proportion of mixed-clone infections (Vardo and Schall 2007).

The final set of simulated infections sought to determine if peak heights provide valid data on relative proportions of parasite clones within infections. In following both natural and experimental infections of *P. mexicanum* over time, we have noted major shifts in the peak heights for coexisting clones (data not shown), reminiscent of such changes for mixed-species *Plasmodium* infections in humans (Bruce et al. 2000). If such changes in peak heights represent actual shifts in relative proportion of the parasites, this would open a novel window into studies on the dynamics of infection. But, using peak heights as an indication of the relative abundance of *Plasmodium* clones within an infection can be questioned. Studies on heterozygotes for diploid organisms reveal that the shorter DNA fragment (fewer repeats) will amplify preferentially (Selkoe and Toonen 2006). Also, unknown mechanisms may favor particular fragments in the PCR and in their being recorded by the instrument. Such factors may overshadow the target effect for studies on relative proportions of each parasite clone. In our simulated infections, if one allele was in an

estimated ten times concentration over another, that allele peak was substantially taller on the pherogram regardless of its repeat length (Fig. 1). This result suggested that peak height may well contain information on the relative density of each genotype of parasite in an infection.

In the final trials, we attempted to generate simulated infections with equal proportions of DNA from each parasite clone. Peak heights for such simulations varied, perhaps either because we were not able to precisely match the DNA density of each clone (almost certainly true) and/or the size alleles were amplified and read unequally. When combining DNA in different concentrations, observed peak heights were correlated with predicted heights (the regression had a slope near one), but a substantial residual variation remained. Thus, peak heights may give only a weak indication of actual relative proportions in an infection. However, when we simulated a single infection changing over time in relative proportions of clones, the first sample could be used as a baseline for the three coexisting allele, and an excellent match was found between observed and predicted peaks for the simulated infections, with slope of the regression again nearly one and with a high proportion of variation explained (r^2=0.937). Thus, changes over time within an infection in relative abundance of the three coexisting alleles are revealed by changes in the relative peak heights.

These results demonstrate that pherograms for single samples of an infected host will give some idea of the relative proportions of clones, but only if the differences are substantial. Any quantitative estimates are highly questionable for single samples. In contrast, the simulated infections show that genotyping an infected host over time does supply valuable information on changes in the proportion of clones. The first sample is used to calibrate results for subsequent samples, and changes in relative peak heights reflect actual changes in the proportion of each allele and, thus, clone. Such results would be very valuable in detecting changes in clonal density over time as the infection increases, as well as possible competitive interactions among clones or response to attack of the immune system.

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