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Abstract

Genome sizes and the organization of repetitive DNA were determined in the hard ticks *Ixodes scapularis* and *Boophilus microplus* using reassociation kinetics. The *I. scapularis* genome contains ~2.15 pg (2.1×10^3 Mbp) of DNA and consists of no foldback (FB), 27% highly repetitive (HR), 39% moderately repetitive (MR), and 34% unique DNA. The *B. microplus* genome contains 7.5 pg (7.1×10^3 Mbp) DNA, and consists of 0.82% FB, 31% HR, 38% MR, and 30% unique DNA. In both species, repetitive sequences occur in a mixture of long and short period interspersion but most (65–80%) of the DNA follows a pattern of short period interspersion. Genome size and organization in the three tick species so far examined are distinct from other arthropods in having a greater proportion of MR, a lower proportion of unique and HR DNA of very low sequence complexity.

Keywords: genome size, genome organization, hard ticks, reassociation kinetics.

Introduction

Ticks are second only to mosquitoes in the ability to transmit pathogens to vertebrates (Sonenshine, 1991). *Ixodes scapularis*, the blacklegged tick, is an important vector of the aetiologic agents causing several human diseases including Lyme disease (*Borrelia burgdorferi*), human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), and human babesiosis (*Babesia microti*) (Belongia, 2002; Krause, 2002). In addition, potential pathogens such as a variant of Powassan virus have been detected in *I. scapularis* (Ebel *et al*. 2001). *Boophilus microplus*, the Southern cattle tick, has long been recognized as one of the most important vectors of the veterinary disease agents that cause bovine babesiosis in tropical and subtropical regions of the world (Bram *et al*., 2002). Despite their tremendous impact on human and animal health, little is understood about the genetics underlying phenotypic characteristics such as host seeking, host selection, attachment and feeding, salivation, oral and transovarial transmission of pathogens, development, physiology and reproduction. A number of insect genomes have been or are in the process of being sequenced. Furthermore a wide range of tools have been developed for assessing gene function in insects. However, it has been *c.* 500 million years (MY) since arthropods in the subphylum Mandibulata [containing the order Hexapoda (insects)] shared a common ancestor with species in the subphylum Chelicerata [containing the order Acari, suborder Ixodida (ticks)]. It is likely that significant divergence has occurred between ticks and insects in aspects of genome structure, promoters, transcription and translation and gene function.

There are *c.* 830 species of ticks (Sonenshine, 1991). These are distributed in two families: Argasidae (soft ticks) with *c.* 170 spp. and Ixodidae (hard ticks) with *c.* 660 spp. The Ixodidae consists of two major subdivisions: the Prostriata (*c.* 240 spp. in the subfamily Ixodinae) and the Metastriata; *c.* 420 spp. in four subfamilies: Bothriocrotinae (Klompen *et al*., 2002), Amblyomminae, Haemaphysalinae, and Rhipicephalinae containing *Hyalomma* spp. (Klompen *et al*., 2000). Most soft and hard tick species are ectoparasites on wildlife but *c.* fifty species are important pests acting either as vectors of pathogens in epizootic or enzootic cycles or as causes of exsanguination (Sonenshine, 1991). Because of their tremendous impact on human and animal health, respectively, *I. scapularis* and *B. microplus* are two hard ticks for which white papers for whole genome sequencing have or are in the process of being approved. The *I. scapularis*

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genome has recently been selected for sequencing (Catherine Hill, International *Ixodes scapularis* Sequencing Committee, pers. comm.).

Determination of genome size and the organization of repetitive elements in a given genome is an important precursor to whole genome sequencing. Knowledge of genome size indicates the numbers of libraries and the amount of shotgun sequencing that will be needed to obtain complete genome coverage. Eukaryotic genomes consist of unique (single copy) sequences interspersed within a variety of repetitive sequences (Davidson *et al.*, 1975). Repetitive sequences are composed of foldback (FB), highly repetitive (HR) and moderately repetitive (MR) elements. FB DNA is composed of tandem repeated sequences with inverted repeats that fold back on themselves more rapidly than predicted by two-strand reassociation kinetics. HR DNA is composed of sequence families that exist as tandem or dispersed repeats in a wide range of abundances from 1000 to > 100 000 copies. MR sequences have a lower abundance (from 10 to 1000 copies) and include transposable elements and members of multigene families (e.g. ribosomal RNA cistron).

Two basic patterns of HR and MR organization are found in eukaryotes (Davidson *et al.*, 1975). Short period interspersion describes a pattern of single-copy sequences 1000–2000 bp in length that alternate with short (200–600 bp) and moderately long (400–1000 bp) repetitive sequences. This pattern of organization is characteristic of the majority of animal species. Long period interspersion describes a pattern of long (5600 bp) repeats alternating with very long (13 kbp) uninterrupted stretches of unique sequences. The long repeats generally consist of a mixture of long and short HR and MR elements (Crain *et al.*, 1976). Long period interspersion is characteristic of most species with small genomes (0.10–0.50 pg) (Samols & Swift, 1979), however, avian genomes are relatively large (1–2 pg) and exhibit long period interspersion (Epplen *et al.*, 1978, 1979). As genome size increases repetitive DNA generally occurs in a continuum between long and short period interspersion (Black & Rai, 1988). Knowledge of HR and MR amount and organization predicts the frequency with which unique, presumably coding, sequences will be found in genomes.

Genome size has already been reported in the metastriate tick *Amblyomma americanum* to be *c.* 1.08 pg (1.04×10^3 Mbp) (Palmer *et al.*, 1994). This is a relatively large genome for an arthropod (Palmer & Black, 1997), furthermore repetitive elements were distributed in a pattern of long period interspersion which is unusual for such a large genome. Herein we describe genome size and the organization of repetitive elements in *I. scapularis* and *B. microplus.*

Results and discussion

The reassociation rate of *Escherichia coli* DNA was measured to calibrate our methods and equipment and to act as a genome size standard. Reassociation rate is typically expressed in terms of molarity of single-stranded DNA multiplied by the time of reassociation in seconds. For comparison purposes, the $C_ot_{1/2}$ is usually reported. This is the number of mole-seconds at which 50% of the single stranded DNA is reassociated for a particular species or in a particular fraction of the genome in a species. The $C_ot_{1/2}$ of 2.67 mol s estimated in the present study (Table 1) falls well within the 2.2–2.8 mol s range of estimates from previous studies (Lewin, 1980).

Ixodes scapularis and *B. microplus* fragments sonicated to 300 or 1800 bp were allowed to reassociate, and the proportions of single and double stranded DNA at increasing C_ot values were assayed by hydroxyapatite chromatography (Britten *et al.*, 1974). Nonlinear least squares regression was used to estimate reassociation rates and proportions of FB, HR, MR and unique sequences. The best fit for short fragments in both species was obtained with a model consisting of four components. The Y-intercept below a $\log_{10}$ (EC_ot) of –2 estimates the amount of FB sequences. The fastest reassociating fraction between –2 and 0 $\log_{10}(EC_ot)$ consists primarily of HR DNA. The fraction between 0 and 2 $\log_{10}(EC_ot)$ consists primarily of MR DNA. The slowest reassociation between 2 and 4 $\log_{10}(EC_ot)$ occurs primarily among unique sequences (Palmer & Black, 1997). For both species, the best fit with long fragments was obtained with a three component model consisting of FB, HR and unique DNA. Reassociation data and the least squares fit for short and long DNA fragments of *I. scapularis* and *B. microplus* are plotted in Figs 1 and 2. Amounts and reassociation rates of FB, HR, MR and unique sequences appear in Table 1.

The short fragment curve indicates that the *I. scapularis* genome contains 66% repetitive DNA (27% HR and 39%

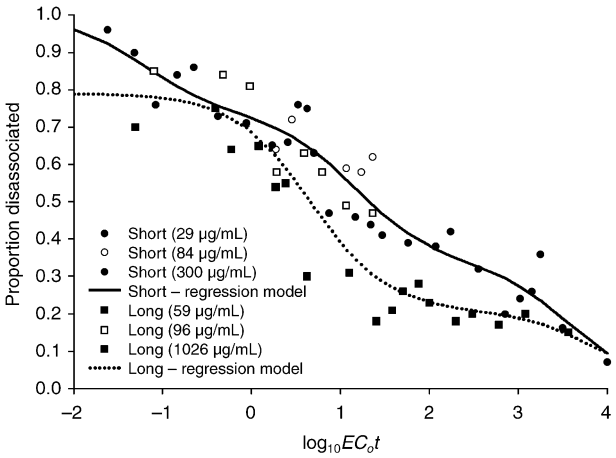

Figure 1. Reassociation of *Ixodes scapularis* DNA sheared to short and long fragment sizes. The short curve represents the least squares solution with four components [foldback (FB), highly repetitive (HR), moderately repetitive (MR), and unique sequences]. The long curve represents the least squares solution with three components (FB, HR, and unique sequences).

Table 1. DNA reassociation kinetic analysis of *E. coli*, *I. scapularis* and *B. microplus*

Species	Proportions of fragments (%)	Reassociation rate(1/mol s)	$C_ot_{1/2}$ mix (mol s)	$C_ot_{1/2}$ pure (mol s)
Escherichia coli				
Fragments unreassociated = 1.05%				
Fragment size = 400				
[Range = 190–820 bp]				
Unique Asymptotic 95% CI	100.00	0.375 (0.23–0.52)	2.67	2.67
Ixodes scapularis				
Short fragments				
Fragments unreassociated = 7%				
Fragment size = 300 bp				
[Range = 150–920 bp]				
Highly repetitive Asymptotic 95% CI	26.7 (7.22–46.13)	60.75 (0–209.4)	0.02	0.004
Middle repetitive Asymptotic 95% CI	39.5 (26.61–52.29)	0.1734 (0–0.4029)	5.77	2.28
Unique Asymptotic 95% CI	33.9 (21.96–45.89)	0.000765 (0–0.00167)	1307.19	443.40
Long fragments				
Fragments unreassociated = 15%				
Fragment size = 1800 bp				
[Range = 900–7200 bp]				
Highly repetitive Asymptotic 95% CI	58.3 (46.42–70.15)	0.3755 (0.0465–0.7045)	0.94	0.38
Unique Asymptotic 95% CI	20.8 (11.49–30.22)	0.000584 (0–0.00309)	4854.37	800.97
Boophilus microplus				
Short fragments				
Fragments unreassociated = 7.0%				
Fragment size = 300 bp				
[Range = 80–950]				
Foldback	0.82			
Highly repetitive Asymptotic 95% CI	30.92 (3.11–58.72)	33.66 (0–84.1720)	0.03	0.01
Middle repetitive Asymptotic 95% CI	38.14 (31.51–44.78)	0.0488 (0.0134–0.0842)	20.49	7.82
Unique Asymptotic 95% CI	30.12 (23.70–36.55)	0.000220 (0.000029–0.000410)	4545.46	1369.09
Long fragments				
Fragments unreassociated = 8.0%				
Fragment size = 1800				
[Range = 800–8200]				
Highly repetitive Asymptotic 95% CI	46.06 (29.83–62.29)	5.3374 (0–11.3720)	0.19	0.09
Unique Asymptotic 95% CI	51.93 (44.31–59.55)	0.0160 (0.0064–0.0255)	62.50	32.46

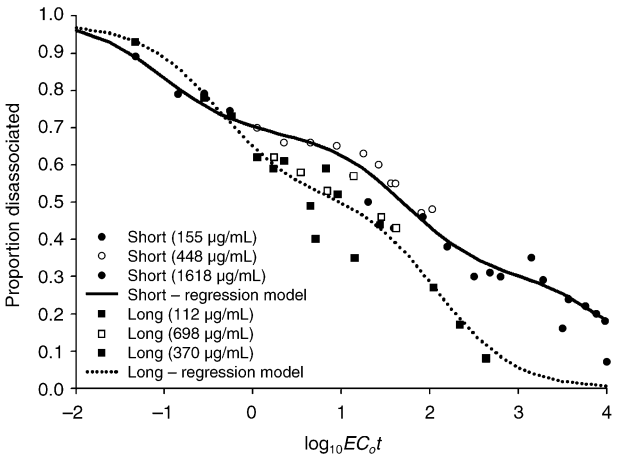

Figure 2. Reassociation of *Boophilus microplus* DNA sheared to short and long fragment sizes. The short curve represents the least squares solution with four components [foldback (FB), highly repetitive (HR), moderately repetitive (MR), and unique sequences]. The long curve represents the least squares solution with three components (FB, HR, and unique sequences).

MR) (Fig. 1). Estimates of genome size were obtained with the equation:

$$\text{genome size} = C_ot_{1/2}\,(\text{unique}) \times (0.0044\ \text{pg or}\ 4.2 \times 10^6\ \text{bp DNA})/C_ot_{1/2}\,(\textit{E. coli}) \quad (1)$$

The *I. scapularis* $C_ot_{1/2}$ (unique) = 1307 mol s and thus the haploid genome contains *c.* 2.15 pg (2.1×10^3 Mbp) of DNA with (95% CI: 0.978 pg – ∞; 9.42×10^2 Mbp – ∞). A haploid genome size of 2.37 pg (2.3×10^3 Mbp) was estimated for *I. scapularis* using cell flow cytometry (C. Hill, S. Wikel & S. Johnston, pers. comm.).

If a genome is of the long period interspersion type, the reassociation rate of long unique fragments can be predicted using the estimated reassociation rate of short fragments in the equation:

$$\text{Predicted rate (long)} = \text{Estimated rate (short)} \times \sqrt{L2/L1} \quad (2)$$

where *L*1 and *L*2 are, respectively, the average lengths of the short and long fragments. If, however, the observed reassociation rate of long unique fragments is substantially

different than predicted this suggests a pattern of short period interspersion. The observed rate can be faster than predicted if *L2* lengths occur in a narrow size range. A homogeneous distribution of long fragments each containing both unique and repetitive DNA will reassociate at the same rate as repetitive sequences. However, obtaining long fragments of uniform size requires gel purification or collection of fractions on a CsCl gradient and is seldom achieved. For these reasons, the rate with which long fragments reassociate is usually slower than predicted. Substantial hybridization seen late in the long fragment curve either represents reassociation of unique fragments that are shorter than the mean size of the long fragments or is indicative of a mixture of both long and short interspersed elements.

The observed reassociation rate of long fragments in *I. scapularis* (0.0006/mol s; $\log_{10}(EC_ot_{1/2})$ = 3.23 mol s) is substantially different than predicted (0.0019/mol s; $\log_{10}(EC_ot_{1/2})$ = 2.73 mol s), suggesting primarily a short period interspersion pattern. At 3.23 mol s, *c.* 80% of the long DNA had reassociated. Note that the reassociation of the long fragments parallels the reassociation seen with short fragments (compare short and long regression models between 3 and 4 $\log_{10}(EC_ot_{1/2})$ in Fig. 1. The remaining *c.* 20% of fragments probably therefore consisted of unique DNA. This observation suggests that the *I. scapularis* genome contains a mixture of both long and short interspersed elements.

The *B. microplus* genome also contains a high percentage (69%) of repetitive DNA (31% HR and 38% MR) (Fig. 2). The *B. microplus* $C_ot_{1/2}$ (unique) = 4545 mol s and the haploid genome thus contains *c.* 7.5 pg or 7.1×10^9 bp (95% CI 4.02–56.8 pg; 3.84×10^3–54.2×10^3 Mbp). In contrast with *I. scapularis,* the observed reassociation rate of long fragments (0.0160/mol s; $\log_{10}(EC_ot_{1/2})$ = 1.80 mol s) is much faster than predicted (0.0005/mol s; $\log_{10}(EC_ot_{1/2})$ = 3.27 mol s), characteristic of a short period interspersion pattern. By 1.80 mol s, *c.* 65% of the long DNA had reassociated suggesting that *c.* 35% of remaining fragments consisted of short and long unique DNA indicative again of a mixture of long and short period interspersion.

Repeat frequency and complexity estimates for HR, MR, and unique fractions of both *I. scapularis* and *B. microplus* are presented in Table 2. The *I. scapularis* genome contains HR DNA with low complexity; HR elements occur with an average frequency of *c.* 80 000 copies/haploid genome. MR elements have greater complexity with an average frequency of *c.* 230 copies/haploid genome. The *B. microplus* genome contains HR DNA with very low complexity; with an average frequency of *c.* 153 000 copies/haploid genome. MR elements occur with an average frequency of *c.* 220 copies/haploid genome.

Amblyomma americanum is the other metastriate tick in which genome size and organization have been determined. The size of that genome is 1.04×10^3 Mbp composed of four kinetic components (4% FB, 18% HR, 42% MR and 36% unique DNA) (Palmer *et al.*, 1994). In contrast with the two ticks examined in the present study, HR and MR elements are distributed in a long period interspersion pattern. The HR DNA of *A. americanum* is of extremely low complexity with *c.* 238 000 copies/haploid genome.

The genome size and organization data we generated for *I. scapularis* and *B. microplus* were examined with respect to the same data collected in *A. americanum* as well as in eighteen insects (Palmer & Black, 1997). The percentages of each genome consisting of FB + HR, MR, and unique DNA are plotted in three dimensions for each of the twenty-one arthropod species (Fig. 3). Tick genomes fall into a cluster separate from insects. Tick genomes have a higher percentage of MR DNA and a lower percentage of unique DNA. Canonical discriminant analysis (PROC CANDISC SAS 9.1; SAS, 2002) showed that canonical discriminants corresponding to %MR, and %unique DNA accounted for 90.6% of the total variance and that the three clusters depicted in Fig. 3 are significantly differentiated (Wilks' $\lambda = 0.148$, $F = 13.57$, $P_{(4,34\ df)} < 0.0001$). MR elements in both tick and insect genomes have similar complexities of *c.* 100–500 copies/haploid genome. Only genome sequencing will reveal what proportion of this large amount of MR DNA in ticks consists of multigene families and transposable elements.

Table 2. Amount and repeat number for repetitive and unique sequences

Species	Amount (pg)	Complexity (bp)*	Complexity (pg)	Repetitive frequency
Ixodes scapularis				
Foldback	–	–		–
Highly repetitive	0.573	6.91×10^3 ($3.47 \times 10^3 - \infty$)	7.23×10^{-6} ($3.63 \times 10^{-6} - \infty$)	80 000
Middle repetitive	0.848	3.58×10^6 ($2.04 \times 10^6 - \infty$)	3.75×10^{-3} ($2.14 \times 10^{-3} - \infty$)	227
Unique	0.729	6.97×10^8 ($4.32 \times 10^8 - \infty$)	7.31×10^{-1} ($4.53 \times 10^{-1} - \infty$)	1.0
Boophilus microplus				
Foldback	0.061			
Highly repetitive	2.316	1.45×10^4 ($1.1 \times 10^4 - \infty$)	1.51×10^{-5} ($1.11 \times 10^{-5} - \infty$)	153 000
Middle repetitive	2.857	1.23×10^7 (8.37×10^6–3.7×10^7)	1.29×10^{-2} (8.76×10^{-3}–3.88×10^{-2})	222
Unique	2.256	2.15×10^9 (1.40×10^9–1.29×10^{10})	2.26 (1.47–1.35×10^1)	1

*The complexity in base pairs of each sequence class is calculated based on a genome size in *E. coli* of 4.2×10^6 bp which reassociates with a $C_ot_{1/2}$ of 2.67 mol s.

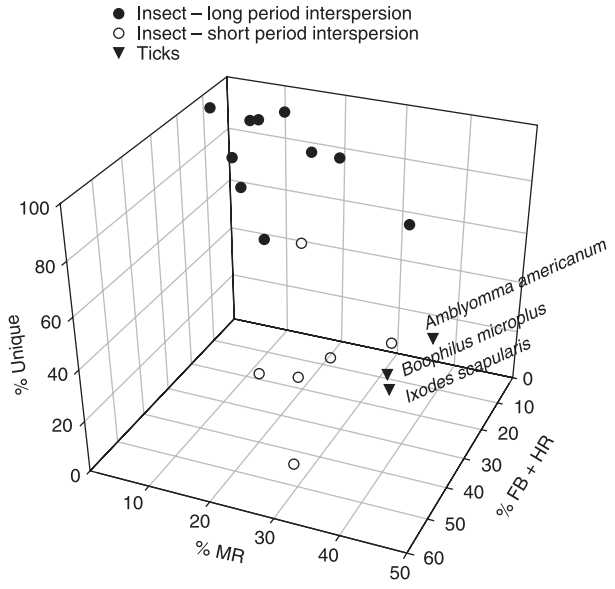

Figure 3. Comparison of the genome composition in *Ixodes scapularis*, *Boophilus microplus*, and *Amblyomma americanum* with eighteen other insect species published to date.

The HR component of tick genomes has a much lower complexity (80 000–238 000 copies/haploid genome) than the HR of insects. The lowest complexity in insects was detected in a strain of *Aedes albopictus* from Calcutta, India with 49 900 copies/haploid genome (Black & Rai, 1988). The low complexity of HR DNA in ticks is likely to be manifested in the upcoming genome projects as an abundance of HR elements of very similar sequence. This will be of benefit in terms of being able to easily identify repetitive elements in genomic sequences but may be a drawback in the identification of overlapping sequences or overlapping BAC ends. The overall length of the *I. scapularis* linkage map (Ullmann *et al.*, 2003) was estimated to be 3166 cM, providing a resolution of 663 kb/cM (2.1×10^3 Mbp/3166 cM). A resolution of *c.* 700 kb/cM compares favourably to the average 1100 kb/cM resolution in *Anopheles gambiae* (Dimopoulos *et al.*, 1996) or the 1000–3400 kb resolution for *Aedes aegypti* (Brown *et al.*, 2001). A map of this resolution has a higher probability of being successfully used in mapped-based positional cloning of candidate genes in *I. scapularis.*

Enormous variation exists in the amounts and organization of repetitive DNA at the family level in hard ticks. Ten-fold variation in genome size was also seen in mosquitoes (Black & Rai, 1988). However, a major difference is that the smallest tick genome is equal in size to the largest mosquito genome. Cavalier-Smith (1978) found a positive correlation between genome size, cell cycle length, and overall development time. Most ticks develop slowly from eggs to adults over a period of months and years while the average mosquito life cycle is generally completed in weeks. However, the *B. microplus* genome is three times larger than the *I. scapularis* genome and yet its life cycle can be completed in 3–4 weeks while the *I. scapularis* life cycle requires at least 18 months. The most that can be predicted from the three tick genome measurements made to date is that tick genomes will be large, highly variable in size and consist largely of MR DNA.

Experimental procedures

Preparation of DNA

Field-collected *Ixodes scapularis* females (NWS Earle, NJ) were allowed to oviposit and the resulting egg batches were collected. Frozen eggs from the Deutsch strain and the Munoz strain were utilized for DNA isolation in *Boophilus microplus*. Egg batches were divided into 50 mg samples that were subsequently frozen and ground in liquid nitrogen. Genomic DNA was isolated utilizing the CTAB (hexadecyltrimethylammonium bromide) method (Black & DuTeau, 1997). DNA was then purified on caesium chloride gradients (Palmer & Black, 1997). These procedures yielded *c.* 4.5 mg *I. scapularis* DNA and *c.* 2.9 mg *B. microplus* DNA. *Escherichia coli* DNA (strain B) was purchased from Sigma (D-2001; St Louis, MO).

Preparation and sizing of DNA fragments

Tick and *E. coli* DNA (1–2 mg) were dissolved in 5 ml 1 × sonication buffer (Sambrook *et al.*, 1989) in a 15 ml conical polypropylene tube. A mean fragment length of 1800 bp was obtained by cooling the sonication mixture on a dry ice/ethanol bath and sonicating (Branson sonifier, Model S110; Danbury, CT) for 30 s at the lowest setting. A mean fragment length of 300 bp was obtained by sonicating for a total of 45 cycles of 30 s bursts, with cooling of the DNA between cycles in a dry ice/ethanol bath. Average fragment length was determined on 0.75% agarose gels.

DNA reassociation

Following sonication, DNA fragments were ethanol precipitated and resuspended in 0.12 M phosphate buffer of pH 7.0 (PB) at approximate concentrations of 100 μg DNA/ml for low $C_o t$ values, and 500 μg/ml for mid $C_o t$ values. DNA was dissolved in 0.4 M PB at a concentration of *c.* 1000 μg/ml for high $C_o t$ values. When reassociating in 0.4 M PB, equivalent $C_o t$ values were calculated by multiplying the $C_o t$ value by 4.9 (Britten *et al.*, 1974). Approximately 40 μg DNA was used for each point on the $C_o t$ curve and 7–13 points were collected for each of the three reassociation conditions.

Optimal conditions for hydroxyapatite chromatography were determined using *E. coli* DNA (Palmer & Black, 1997). Conditions optimized were: (1) rate of air flow to push elutant through a column; (2) 0.3 M PB wash volume (6–12 ml in increments of 2 ml); (3) elution volume of 0.12 M PB and 0.4 M PB (4–10 ml in increments of 2 ml); (4) hydroxyapatite packed bed volume (1–3 ml in increments of 0.5 ml). Final conditions used a low air flow rate, 12 ml wash volume (2 × 6 ml washes), 8 ml elution volume (2 × 4 ml washes), and 2 ml packed hydroxyapatite bed volume. Kinetic parameters were estimated with non-linear regression (PROC NLIN SAS 9.1) using the source code described by (Palmer & Black, 1997).

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