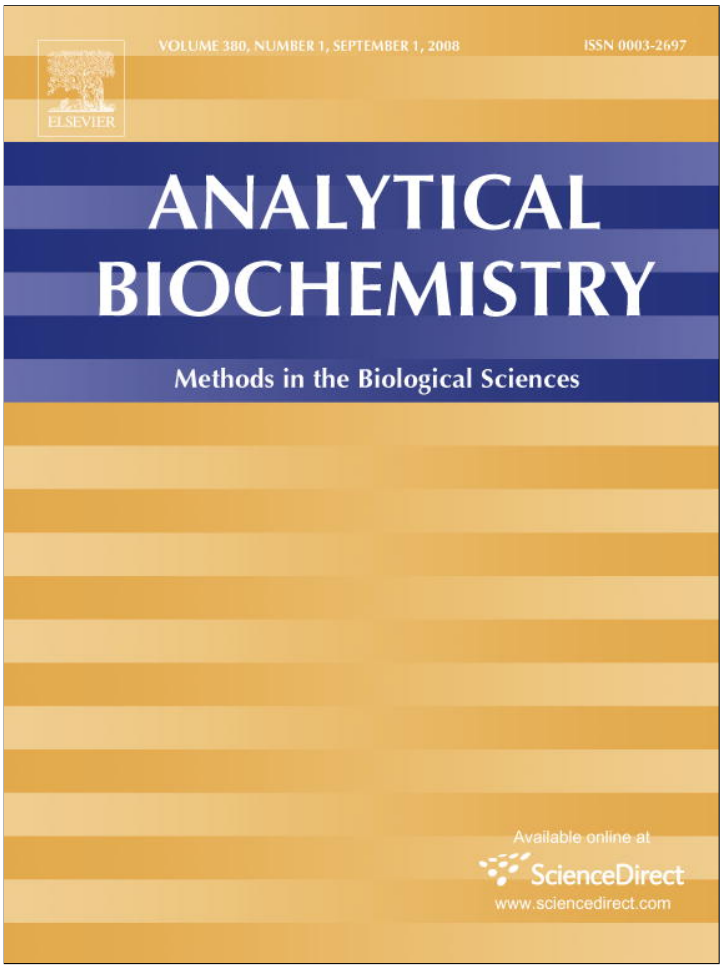

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Identification of repeat structure in large genomes using repeat probability clouds

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ABSTRACT

The identification of repeat structure in eukaryotic genomes can be time-consuming and difficult because of the large amount of information ($\sim 3 \times 10^9$ bp) that needs to be processed and compared. We introduce a new approach based on exact word counts to evaluate, *de novo*, the repeat structure present within large eukaryotic genomes. This approach avoids sequence alignment and similarity search, two of the most time-consuming components of traditional methods for repeat identification. Algorithms were implemented to efficiently calculate exact counts for any length oligonucleotide in large genomes. Based on these oligonucleotide counts, oligonucleotide excess probability clouds, or "*P-clouds*," were constructed. *P-clouds* are composed of clusters of related oligonucleotides that occur, as a group, more often than expected by chance. After construction, *P-clouds* were mapped back onto the genome, and regions of high *P-cloud* density were identified as repetitive regions based on a sliding window approach. This efficient method is capable of analyzing the repeat content of the entire human genome on a single desktop computer in less than half a day, at least 10-fold faster than current approaches. The predicted repetitive regions strongly overlap with known repeat elements as well as other repetitive regions such as gene families, pseudogenes, and segmental duplicons. This method should be extremely useful as a tool for use in *de novo* identification of repeat structure in large newly sequenced genomes.

Eukaryotic genomes contain many repetitive sequences, and understanding genome structure depends crucially on their identification [1–3]. The predominant repeat annotation approach, implemented in *RepeatMasker* [4], focuses on the identification of repeat element sequences based on their alignment with consensus sequences and relies on a curated library of known repeat families provided by Repbase [5]. This approach is presumably most effective for the human genome, which has attracted the greatest interest and the longest curation history, whereas the necessary libraries for more recently sequenced genomes may be substantially less complete or nonexistent. It is unknown how effective this common approach is overall, however, because there is no "gold standard" to determine the proportion of true repeats that have been identified, and this approach has simply been implemented on an *ad hoc* basis.

Methods for the *de novo* analysis of repeat structure have also been developed to annotate repeat elements in newly sequenced genomes independent of an a priori established repeat library. Such approaches have been implemented in RepeatFinder [6], RECON [7], *RepeatScout* [8], and PILER [9]. These methods essentially construct a repeat library by assembling genome alignments and use sequence similarity searches to annotate repeat elements in the genome (analogous to *RepeatMasker*). All require extensive computational effort and/or capability that limit the ability of individual genomic researchers to extensively investigate repeat structure, particularly for mammalian and other large genomes [10].

Repeat structure in large genomes has been analyzed without first constructing consensus repeat family sequences [11,12], including the use of oligonucleotide (hereafter "oligo") or lmer similarity, rather than sequence similarity [13,14], and analytical counting methods such as RAP [15] and the method of Healy and coworkers [16]. There has been some statistical evaluation of oligo-based repeat region identification using these methods [15,16], but no comprehensive genomic annotation approaches have been developed for oligo-based repeat analysis.

Here we describe the implementation of a new approach for the identification of repetitive regions of large genomes using oligo frequencies. Our goal was to develop a fast algorithm for *de novo* identification of repeated structures applicable to entire eukaryotic genomes that could be reasonably implemented using existing desktop computers. The resulting approach is computationally efficient for analyzing large genomes and is effective at identifying repeat elements. The principal novelty behind our approach arises from the realization that repetitive elements are likely to have given rise to clusters of similar oligos and that it may be statistically

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easier to detect clusters of related oligos than to determine whether each oligo is individually repeated more often than expected by chance.

To elaborate, duplicated sequences are identical at first but will tend to diverge over time. Given this simple fact, it is clear that many duplicated sequences will be more closely related to each other than are random sequences but perhaps will not be identical. Thus, it occurred to us that clusters of related sequences may be observed more often than expected by chance and might be more easily detected than searching only for overabundant identical sequences. Furthermore, it is not necessary to identify repetitive elements prior to assessing these clusters, or "clouds," of related sequences observed at higher than expected frequencies. By tuning parameters of the process for assembling these clouds of related sequences, the stringency of the identification process can be controlled. If the oligos are long enough, individual oligo sequences belonging to overrepresented clusters should have a high probability of originating from a duplication event.

There are three main steps to our approach: counting oligo occurrences in a genome, creating clusters of similar repeated oligos, and demarcating boundaries of predicted repeat structure in the genome based on the relative density of occurrence of repeated oligos. The algorithm that classifies repetitive oligos into clusters of related similar sequences that are observed more often than expected by chance is heuristic (*ad hoc*) and requires approximately as much computational time as does the oligo counting method. We refer to these clusters as probability clouds, or "*P-clouds*," because we view them as loose clouds of potentially related sequences that probably would not have formed by chance. Once constructed, the *P-clouds* are mapped back onto the genome and regions of high *P-cloud* density are demarcated (mapped) as repeat regions. The method is adjustable, with a controllable number of expected false positives, and annotations overlap with a diversity of repeated genomic regions based on empirical observations. The speed, accuracy, and sensitivity of this new method were also evaluated.

Materials and methods

Counting the observance of oligo words

The first step in our method entails calculation of the number of occurrences of each specific oligo in a large genome, which is a moderate computational challenge. To determine a reasonable oligo length (W) for analyses of different length genomes (n), we used $W = \log_4(n) + 1$, which has reasonable sensitivity and specificity, assuming that oligo sequences are sampled approximately randomly [8,15]. This rough approximation predicts that individual oligo words of length W are expected to occur less than one time in the genome by random chance. For example, mammalian genomes are typically around 3 billion base pairs (Gbp),[1] and the expected oligo count for an oligo of length 16 is 0.7, assuming equal base frequencies. Note that this approximation is used only to choose an oligo length and that later statistical assessments are based on observed dinucleotide frequencies.

A rapid approach for counting oligos in genomes is to use an integer counting array for every possible oligo word and then increment the appropriate site in the array by one each time a particular word is encountered; we refer to this as the "direct count" method. This requires prohibitively large amounts of physical memory for long words. Modern computers commonly have 1 gigabyte (Gb) of physical memory (random access memory [RAM]), which limits oligo lengths to 13 (13mers) with this direct count method regardless of the genome size. Analyzing 16mers with this method would require more than 16 Gb of RAM, well beyond the capacity of most current desktop computers.

We reduced RAM requirements for oligo counting in two ways, both of which capitalize on the fact that we were not interested in oligos that were observed less than twice. In the first method, the "mixed" approach, an array of bits corresponded to each oligo word. Because a single bit array can count only up to 1, a hash index was also included to count words that occur more than once. Under the assumption of equal nucleotide frequencies, fewer than 16% of oligos (0.155) are expected to be observed at least twice, and for unequal frequencies the number is even smaller. Nevertheless, for large genomes the memory size required for the hash plus the bit array exceeded 1 Gb in practice. Thus, when physical memory was full, the hash was copied to the hard disk and emptied. For analyses of human chromosomes 1 and X, no memory dumps were required and this "mixed method" was only slightly slower than the direct count method (see Results).

The size of the bit array limits the mixed method to 16mers or less if a RAM memory limit of 1 Gb is imposed, so we also tested what we call the "overlap" method for longer oligos. This method relies on the fact that for a particular 17mer to have more than one copy in the genome, the 16mer corresponding to the first 16 nucleotides (nt) of the 17mer must also have more than one copy. If a hash of all 16mers with more than one copy is created, then it is necessary to create hash entries only for those 17mers that have a multicopy 16mer beginning. The overlap method, therefore, requires successive passes through the genome but can be extended to any length oligo. It requires many hash comparisons, however, and the second pass is much slower than the direct count method. Although we did not require the overlap method in the analyses presented here, it might be useful for some implementations (e.g., unassembled whole eukaryotic genomes). More extensive rationale and details of implementation for the counting methods (direct count, mixed method, and overlap method) are described in the supplementary material.

The mixed method was used for all analyses other than the initial evaluation and comparison of the three methods. All speed calculations were assessed on human chromosome 1 using an affordable modern desktop computer (a single 3.0-gigahertz [GHz] Pentium processor, 1 Gb RAM, running RedHat Enterprise Linux 3.0 with kernel 2.4.21-20.ELsmp) unless otherwise noted.

P-cloud construction

Prior to genome annotation, groups of similar oligos that occurred more often than expected by simple chance were clustered. For example, based on the assumptions of equal base frequencies and the Poisson distribution, the probability that any 16mer would occur by chance 10 times or more in a 3 Gbp genome is only 4×10^{-9}, and the probability that any oligo will occur more than 10 times is only approximately 50%. The oligos that are high frequency by chance are unlikely to cluster, whereas oligos arising from the biological processes of duplication and divergence are likely to cluster. Thus, our basic presumption is that we might be able to use the tendency of biologically related sequences to cluster as a means of predicting whether medium-frequency oligos arose from a duplication process. We refer to these oligo clusters as *P-clouds* because they involve cloud-like clusters of oligos that are not expected from simple probability calculations and also because an approximation to this concept was suggested by Price and coworkers [8]. This step requires only the oligo counts, not the original genome sequence.

[1] *Abbreviations used:* Gbp, billion base pairs; Gb, gigabyte; RAM, random access memory; nt, nucleotides; GHz, gigahertz; SSR, simple sequence repeat; Mbp, million base pairs; BAC, bacterial artificial chromosome.

P-clouds were constructed using the highest frequency oligo to initiate a cloud and then expanding the cloud by adding similar high-frequency oligos to form a *P-cloud* "core." Here "similar" was defined to mean differences of up to 3 nt from a previously identified core oligo (depending on the magnitude of the highest frequency oligo), but the definitions of "similar" and "high frequency" were free parameters or adjustable "cutoffs" (see below). It is worth pointing out that our choices of parameters are *ad hoc* in that there is no theory to establish the "optimal" parameter settings, and a good theory might not even be possible given that the best parameter choices will probably depend primarily on the unknown phylogenetic relationships of all the (mostly unknown) repetitive elements that make up a newly sequenced genome. Preferred parameter settings for *P-cloud* construction were determined based on analyses of the sensitivity and accuracy estimated for each set of parameters (see below).

Multiple *P-clouds* were created by removing the oligos that belong to an identified *P-cloud* and repeating the core identification and core expansion process with the remainder until no oligos remained with counts greater than the "core cutoff." The core cutoff (which was set at between 5 and 200 observations in different runs) and the numbers of repeats required for inclusion of oligos in a *P-cloud* were also separately adjustable parameters.

Following expansion of the *P-cloud* cores, the "outer" layer of each *P-cloud* was created by attaching any medium-copy oligos that were similar to an oligo in the core set. The "lower cutoff," which ranged from 20 down to 2 copies, determined the definition of "medium copy" and, thereby, which oligos were potentially included in the outer layer. Furthermore, the definition of "similar" varied among *P-clouds*, depending on the highest copy oligo in the core. For most *P-clouds*, a candidate oligo for the outer layer needed to have only a single difference from a core oligo, but if a core oligo in the *P-cloud* had more than 200 copies (the secondary cutoff), for example, a difference of 2 nt was sufficient for inclusion. We also sometimes included an even higher tertiary cutoff (e.g., there must be an oligo with 2000 copies in the core layer) that would allow oligos with up to 3 nt difference to be included. In the process of *P-cloud* construction, when a given oligo might have belonged to two or more different *P-clouds*, it was assigned to the *P-cloud* with the highest frequency oligo in its core.

The appropriate setting for the cutoffs depends predominantly on oligo length and the length of the genome segment under consideration, but it also depends on how much divergence has occurred between the core oligos and related elements (i.e., how old the duplication events responsible for the cloud were). Most clusters of related oligos presumably arose from the duplication of repetitive elements and, thus, will reflect the evolutionary history of those elements, but even within the same repetitive element family different regions of the repetitive element may have evolved differently. The core cutoff was chosen to conservatively identify repetitive clusters, whereas outer layer extension cutoffs were chosen to limit the size of the outer cloud, extending it broadly only in cases where the core sequences were particularly frequent and, thus, likely to have spawned more copies of divergent nucleotides. Note, however, that because the method is designed to detect repetitive sequences in the absence of knowledge about repetitive element structure, and in the face of an unknown mixture of repetitive element phylogenetic histories, the choice of parameter settings currently is a purely empirical decision.

The parameter settings defining various cutoff values used in *P-cloud* construction are the lower and core cutoffs and the three core sizes (primary, secondary, and tertiary cutoffs) used to define outer layer extension distances. Suites of parameter settings are abbreviated by their core cutoff values: C^5 (2, 5, 10, 100, and 1000), C^8 (2, 8, 16, 160, and 1600), C^{10} (2, 10, 20, 200, and 2000), C^{20} (2, 20, 40, 400, and 4000), C^{40} (4, 40, 80, 800, and 8000), C^{100} (10, 100, 200, 2000, and 20,000), and C^{200} (20, 200, 400, 4000, and 40,000), with the numbers in parentheses referring to lower, core, primary, secondary, and tertiary cutoffs, respectively.

Because we were not certain whether simple sequence repeats (SSRs) would confound the construction of the *P-clouds*, low-complexity oligos, such as 1-, 2-, 3-, and 4-nt tandem repeats, were excluded prior to *P-cloud* construction.

Repeat region annotation

Given an alignment of repeat elements in a repeat family, each consecutive oligo in the alignment would be ideally included in one *P-cloud* and each repetitive element would be covered by consecutive *P-clouds*. In practice, we have found that related oligos from different repetitive elements often overlap each other and that *P-clouds* contain oligos arising from multiple repetitive elements. Nevertheless, contiguous stretches of the genome containing many oligos that belong to *P-clouds* are more likely to have arisen from repetitive elements (or other repeated regions). Hence, high-density *P-cloud* regions are obvious targets for stronger prediction of repetitive element membership.

To identify high-density *P-cloud* regions, oligos that were members of *P-clouds* were mapped back to the original genome sequence and segments of the genome with high *P-cloud* oligo density were demarcated as "repeated regions." A smoothing algorithm was used to eliminate very short *P-cloud* stretches and merge short *P-cloud* gaps into otherwise dense *P-cloud* regions. Our criterion was that 80% of every 10 consecutive oligos (using a sliding window) must be composed of *P-cloud* oligos, yielding a minimum demarcated region length of 25 bp if 16mer oligos are used. We chose the 80% *P-cloud* annotation criterion to prevent excessive false positives, but this criterion is fully adjustable in the program.

Comparison of annotation speed across programs

To compare the speed of repeat structure identification of the *P-cloud* method with other (non-word-counting) *de novo* repeat identification tools [7,8], we analyzed human chromosome X (123.8 million base pairs [Mbp]). *P-clouds* were built from 14mer counts and then mapped to the chromosome according to the *P-cloud* assignment of each oligo to identify genomic repeat structure. Based on preliminary experimentation, parameter set C^{10} was used.

Sensitivity and accuracy estimation

For a range of *P-cloud* parameters, the relationship between sensitivity (the fraction of known repetitive elements detected) and accuracy (the presumed true positive rate) was evaluated. The purpose was to identify the parameter settings that optimally balanced these two measures of performance because preferred parameter settings are otherwise uncertain. Here "detection" of a repetitive element was defined as overlap of an demarcated *P-cloud* region with a *RepeatMasker*-annotated region. Although we note that there is no known exhaustive or comprehensive standard set of all segments of a genome that are derived from repetitive elements, *RepeatMasker*-annotated regions at least represent a minimal (i.e., conservative) set of likely repeat elements. To estimate the sensitivity of the *P-cloud* method, we calculated the proportion of known *RepeatMasker*-annotated repeat elements that were identified by the *P-cloud* method. To estimate the probability of false positive identification of repeat regions in human chromosomes 1 and X, we simulated a random genome sequence that was the same size as these two human chromosomes. This simulated

dataset was constrained to have the same dinucleotide frequencies within 1-Mbp windows as the original chromosomes. Repeat regions demarcated in these simulated data provide an estimate of the false positive rate of the *P-cloud* method. The "accuracy" of the *P-cloud* method is the proportion of estimated true repeat regions in the repeat demarcated regions: 1 – false positive rate = accuracy.

P-cloud performance on known repeat sequences

To evaluate the fine scale repeat mapping performance of the *P-cloud* method on real genomic data, we tested the identification success of the method on *Alu* elements. A total of 100 known *Alu* elements were randomly chosen from human chromosomes 1 and X and then analyzed with the *P-cloud* method under various settings. The genomic location and classification of each *Alu* are listed in Supplementary Table S1. To visualize the results, a multiple sequence alignment of these *Alu* elements (and the flanking 15-bp segments) was assembled using ClustalX [17], and *P-cloud* density and demarcation were mapped along this alignment.

Results

Computational efficiency of the P-cloud method

Because the *P-cloud* method begins with oligo counting, it appeared worthwhile to consider different possibilities for this simple initial task. A direct count method of memorizing counts for all possible 16-mers (the preferred size for mammalian genomes [see Materials and Methods]) requires 4 Gb of RAM. Methods for counting and storing counts of oligos, therefore, were developed to facilitate analyses on standard desktop computers with only 1 Gb of RAM; these are the mixed method and overlap method (see Materials and Methods and the supplementary material).

The counting methods were applied to human chromosome 1 (245.5 Mbp), and the speed and memory requirements were compared with published results for other counting methods [8,15]. The direct count method was the fastest (as expected) but was limited to words of length 13 bp or less under the 1-Gb memory constraint (Table 1). The RAP method uses an algorithm similar to our direct count method and, as expected, achieves similar speeds (after compensating for different computer speeds and their use of dual processors) for words up to length 16 bp, but it requires 8 Gb of memory [15].

The mixed method (combining bit array and hash storage) worked well for oligos of length 16 bp or less but was approximately four times slower than the direct count method (Table 1). Compared with the mixed method for oligos of length 16 bp, the overlap method was half as fast for oligos of length 17 bp, with linearly increasing computational time as oligo word length increases (Table 1). In comparison, the suffix tree compression method of Healy and coworkers [16] should theoretically maintain similar speeds for oligos of any size, but our overlap method (compensating for differences in processor speeds) would be more than 10-fold faster for oligos of length 20 bp, more than 2-fold faster for oligos of length 30 bp, and of comparable speed for oligos of length 45 bp. The memory required for the suffix tree method is also much larger than our 1-Gb target memory size.

Based on these computation speed results, we subsequently used the mixed method for all analyses (oligos were length 15 or 16 bp). *P-cloud* construction and repeat annotation were performed on human chromosome X (123.8 Mbp) using 15-bp oligos to allow comparison with published results from the fastest current method, namely, *RepeatScout* [8]. The time required to complete *P-cloud* analysis was 46 min and includes the entire *P-cloud* process: constructing *P-clouds*, mapping *P-clouds* to the chromosome, and annotating repeated regions based on *P-cloud* density. This is relatively rapid compared with other methods, especially standard methods that do not employ word counting. There is no comparable report on the time required for *RECON* implementation on human chromosome X, but *RECON* required 39 h to analyze a 9-Mbp segment of the human genome (< 7.3% the size of the X chromosome [7]). *RepeatScout* [8] is orders of magnitude faster than *RECON*, but it required 8 h to analyze human chromosome X (*P-clouds* can be constructed for the entire human genome during that time). Thus, even including the 10 min required to obtain repeat counts, the *P-cloud* method is approximately 10 times faster than the fastest existing approach.

Sensitivity and accuracy under varying P-cloud parameter settings

Human chromosomes 1 and X were analyzed based on 15mer *P-clouds*. Parameter settings were varied to identify the best set of parameters for further analyses. A total of 66,449,854 15mers were observed two or more times, and *P-clouds* were constructed after the exclusion of 154 oligos containing tandem repeated nucleotide patterns. The higher values assessed for the core and lower cutoffs appear to be overly strict, with relatively few oligos included in the *P-clouds* (Fig. 1). For lower cutoffs, the percentage of oligos included in *P-clouds* was far greater, up to nearly 60% of all observed 15mers (Fig. 1).

Based on detection rates in simulated sequences with the same dinucleotide structure as the human 1 and X chromosomes, the false positive rate for the *P-cloud* method is low (and, thus, the accuracy is high) under a broad range of parameter settings (Fig. 2). Even when the core cutoff was set as low as 8 (C^8), *P-clouds* maintained a false positive rate of less than 4%. The sensitivity of the method for detecting *RepeatMasker*-annotated regions decreases substantially, however, when the core cutoff is set to larger values (Fig. 2). The relationship between accuracy and sensitivity suggests that different parameter settings may ideally suit different applications of the *P-cloud* method, depending on the relative importance of exhaustive re-

Table 1
Comparison of computations required to count oligos in human chromosome 1

Program	Algorithm	Oligo length	Speed (min/100 Mbp)	Hardware configuration
P-clouds[a]	Direct count method	⩽13	1.4	3-GHz processor, 1 Gb RAM
	Mixed method	14–16	6.0	
	Overlap method	⩾17	+ 7.0 per additional nucleotide	
RAP[b]	Direct pattern index array	⩽16	0.7	1 U dual Opteron 146 workstation, 8 Gb RAM
Healy[c]	Suffix tree and Burrows–Wheeler transform compression	Any size	100	1-GHz dual processor, 4 Gb RAM

[a] Algorithm descriptions for *P-clouds* methods are provided in the supplementary material.
[b] RAP method [15] (applied to the whole *Caenorhabditis elegans* genome and to mammalian genomes).
[c] Method of Healy and coworkers [16].

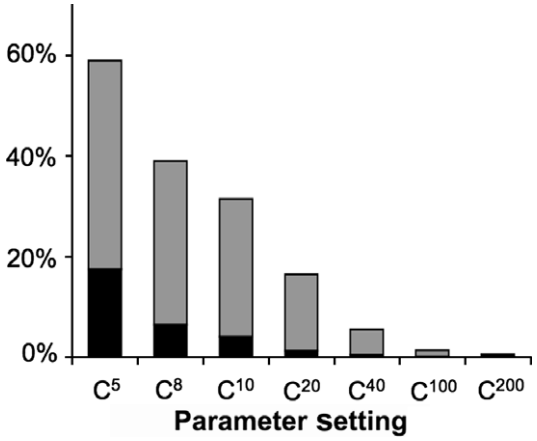

Fig. 1. Percentage of multiple copy 15mers included in *P-clouds* under different parameter settings. The percentage in the core layer is in black, and the outer layer is in gray. Suites of parameter settings are abbreviated by their core cutoff values as C^5 (2, 5, 10, 100, and 1000), C^8 (2, 8, 16, 160, and 1600), C^{10} (2, 10, 20, 200, and 2000), C^{20} (2, 20, 40, 400, and 4000), C^{40} (4, 40, 80, 800, and 8000), C^{100} (10, 100, 200, 2000, and 20,000), and C^{200} (20, 200, 400, 4000, and 40,000), with the numbers in parentheses referring to lower, core, primary, secondary, and tertiary cutoffs, respectively.

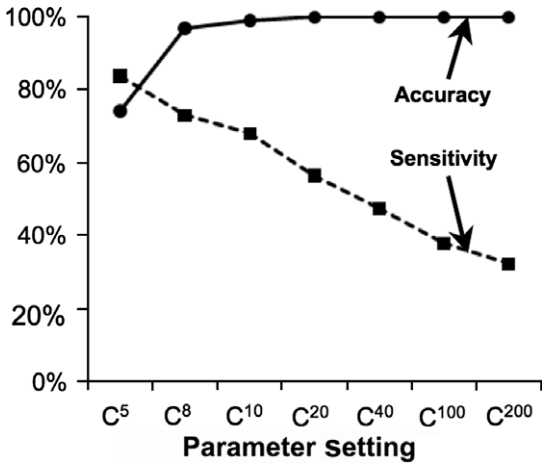

Fig. 2. Accuracy and sensitivity of the *P-cloud* annotation under different parameter settings. Accuracy is labeled with circles and a solid line, and sensitivity is labeled with squares and a dotted line. The parameter settings of each point are the same as in Fig. 1. Accuracy is defined as 1 minus the estimated false positive rate (based on whole genome simulation), and sensitivity is defined as the percentage of *RepeatMasker* repeat elements that were annotated by the *P-cloud* method.

peat annotation versus a minimal rate of false positive annotations. The C^8 parameter conditions appear to be reasonably conservative for basic analyses, and the simulated false positive rate can be used for corrections.

Evaluation of P-cloud annotations on Alu elements

To more thoroughly evaluate the ability of *P-cloud* annotations to identify known repetitive elements, 100 random *Alu* elements were aligned and their average *P-cloud* coverage was assessed. Despite discontinuous initial *P-cloud* coverage of some regions (Fig. 3A), the secondary sliding window identification step substantially increases the continuity and consistency of the *P-cloud* repeat element mapping (Fig. 3B). Nearly 100% of all *Alu* element regions were identified as repetitive regions, even under the most stringent parameter settings (Fig. 3B). Based on these results, the *P-cloud* mapping and demarcation process appears to be effective,

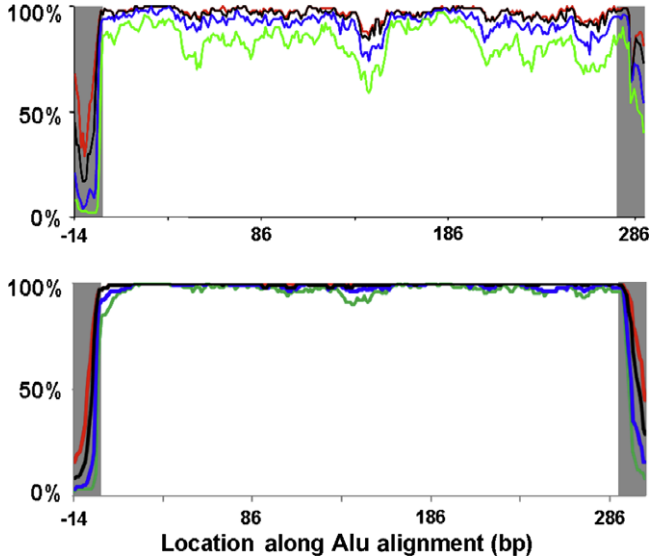

Fig. 3. *P-cloud* coverage of *Alu* elements. The percentages of 100 randomly chosen and aligned *Alu* elements that belonged to *P-clouds* (A) and that were annotated based on sliding window detection of contiguous *P-cloud* segments (B) are shown. These are shown for various *P-cloud* parameter settings: C^5 (red), C^{10} (black), C^{40} (blue), and C^{200} (green). The 15 bp flanking each *Alu* region was not realigned and is shown in gray. Gray shading indicates the boundaries of *Alu* elements. Note that although the *Alu* alignment on which these annotations were visualized was 292 bp, the end of the white unshaded region in panel A marks the last 15mer that is pure *Alu* at alignment site 292 bp alignment – 15 bp oligo length = 277 bp, and the alignment ends at 292 bp alignment + 15 bp flank – 15 bp oligo length = 292 bp. In panel B, each nucleotide is either annotated or not, based on whether it is located in a contiguous region of *P-clouds*, so the gray region begins after the end of the alignment at 293 bp. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and known boundaries of *Alu* repeat elements are well defined by the *P-cloud* predictions (Fig. 3B).

Overlap between P-cloud demarcations and RepeatMasker annotations on human chromosomes

P-cloud mapping of all repeat elements in human chromosomes 1 and X under C^8 parameter conditions was compared with *RepeatMasker* annotations. Examples of strong overlap between *P-cloud* demarcations and *RepeatMasker* annotation of repetitive elements were numerous and clearly observable (Fig. 4). 38% of the genome was identified by both the *P-cloud* method and *RepeatMasker* (Fig. 5), whereas 13.3% of the genome was identified by *RepeatMasker* but not *P-clouds* (Fig. 5), partly because the selected parameter settings may have been overly conservative. *P-clouds* usually identified at least part of each whole repeat element, but they occasionally missed parts of the more divergent regions (Fig. 4). Only 3.4% (22,547 of 663,879) of known repeat elements in human chromosomes 1 and X were completely missed by the *P-cloud* method.

Notably, 14.7% (58.74 Mbp) of human chromosomes 1 and X was mapped by the *P-cloud* method but not annotated by *RepeatMasker*. The *P-cloud* method was designed to identify repetitive regions that originate from any duplication events, not necessarily constrained to identifying only traditional repetitive or transposable elements (e.g., *Alu*), as is *RepeatMasker*. Thus, a portion of these regions identified by *P-clouds* but not by *RepeatMasker* may represent other duplicated sequences, such as tandem duplications, multigene families, and segmental duplications, and this is verified by empirical observations. There are many examples of strong overlap between *P-cloud* demarcated regions and multigene family members (Supplementary Fig. S1A), pseudogenes (Supplementary Fig. S1B), or segmental duplications (Supplementary Fig. S1C). However, such regions rep-

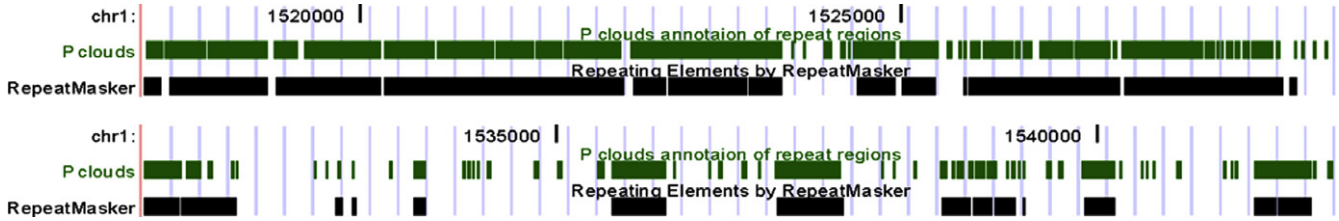

Fig. 4. *P-cloud* and *RepeatMasker* annotation. Two example regions are shown to compare *P-cloud* annotation of repeated regions (green) with *RepeatMasker* annotation of repetitive elements (black). The human genome browser views are based on the May 2004 version. Visualizations are from the UCSC genome browser (http://genome.ucsc.edu). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

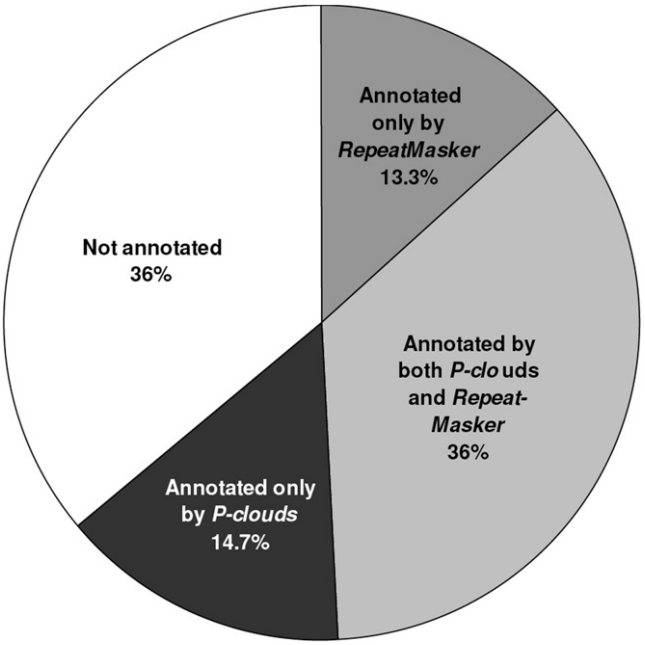

Fig. 5. Overlap of *P-cloud* and *RepeatMasker* annotation in human chromosomes 1 and X. The percentages of the nucleotides in the genome annotated by either method, both methods, or neither method are shown.

resent just a small fraction of the regions identified only by the *P-cloud* method.

It is an interesting question whether a substantial fraction of regions uniquely identified by *P-clouds* are repetitive elements that were unidentified previously. If so, it is possible that they may represent new repeat families not included in Repbase, but it is more likely that a notable fraction of them represent known repeat elements that the *RepeatMasker* procedure failed to annotate. Although this is primarily a methods article and this question will be addressed in detail later, it is worth noting that the *P-cloud* method identified repeat structure in regions that were not previously characterized as either known repeat families, gene families, pseudogenes, or segmental duplications (Supplementary Fig. S1D). The hypothesis that *P-clouds* can help to identify undiscovered repetitive elements is consistent with the observation that the region shown in Supplementary Fig. S1D was not annotated by *RepeatMasker* in the May 2004 human genome annotation that we originally used but was subsequently annotated as a LTR element in the current (March 2006) release of the *RepeatMasker* annotation. We note that we do not see any particular reason to believe that the *RepeatMasker* annotation of repetitive elements is itself completely exhaustive.

Discussion

The *P-cloud* approach represents an attractive alternative tool for mapping of genomic repeat structure. It is capable of jointly analyzing two human chromosomes (1 and X) on a standard desktop computer in approximately 2 h and of analyzing the entire human genome in less than half a day. It does not require prior assessment of repetitive element families and is not restricted to identifying transposable elements. The false positive rate and sensitivity can be controlled by adjusting algorithm parameters and, thus, may be set to best fit the goals of specific research applications. The *P-cloud* method is well suited for *de novo* analysis of newly sequenced large eukaryotic genomes and is likely to complement other methods in identification of new repeat families. It may also augment analyses of even well-characterized genomes such as the human genome because it is possible that repeat libraries in Repbase might not be complete even for the intensively studied human genome [8].

The ability of the *P-cloud* method to rapidly conduct *de novo* repeat structure analysis for large complete genomes on a standard desktop computer is unique, providing a significant step toward making computational genomic research more tractable for a broader set of researchers. The method does not require large-scale alignments or a priori knowledge of repeat families, further extending its versatility. Instead, it relies on the observation that many repetitive families are fairly large and that divergent evolution subsequent to duplication has created large clouds of related oligos. In contrast to consensus sequence matching algorithms used by existing annotation tools, the *P-cloud* method is effective even for relatively small repeated segments (as short as 25 bp based on adjustable annotation criteria). The *P-cloud* approach rapidly identifies a majority of the repeat regions annotated by *RepeatMasker*, the latter of which required substantially more computation and extensive manual curation of repeat databases. Clearly, further research and empirical study is required to fully optimize the tuning of parameters, understand the false negative and false positive rates of parameter settings, and more practically interpret the impacts of parameter settings within the *P-cloud* approach. Limited empirical analyses presented here (and more extensive unpublished empirical research) indicate that the method appears to work remarkably well in accurately predicting repeat structure, especially considering the tremendous (10- to 100-fold) increase in computational efficiency of the *P-cloud* method versus comparable repeat annotation methods.

The *P-cloud* method has clear potential for enabling more detailed dissection of repeat structure in eukaryotic genomes. Putative regions of repeat origin identified by *P-clouds* can be verified by alignment using standard methods, but the speed of *P-clouds* to work with newly sequenced genomes has the potential to dramatically accelerate the repeat discovery and demarcation process. *P-clouds* may also be applicable for comparative analysis of repeat structure among multiple vertebrate genomes. Furthermore, it could easily be used for analysis of local repetitive structures in more moderately sized genomic regions even prior to genome assembly, including regions cloned into bacterial artificial chromosomes (BACs), for which it can be important to have an immediate understanding of repeat structure prior to the development of genome-specific repeat libraries [18].

Although we have compared the *P-cloud* method primarily with other repeat identification tools, the basis of the method is designed to provide a broad perspective on how the process of duplication has shaped the content and structure of large genomes. Given its accuracy, efficiency, and flexibility, we expect that the availability of *P-cloud* maps will make complete comparative analysis of genomic repeat structure more accessible to a broader diversity of genomic researchers. Thus, this new computational feasibility should enable a new generation of in-depth genomic analyses contributing to our understanding of the function, diversity, and evolution of eukaryotic genomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2008.05.015.

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