Microarrays **2013**, *2*, 1-x manuscripts; doi:10.3390/microarrays20x000x

OPEN ACCESS

microarrays

ISSN 2076-3905

www.mdpi.com/journal/microarrays

Type of the Paper (Article, Review, Communication, etc.)

Pigeons: A Novel GUI Software for Analysing and Parsing High Density Heterologous Oligonucleotide Microarray Probe Level Data

Hung-Ming Lai [1,4,†], Sean T. May [1,2] and Sean Mayes [1,3,*]

[1] School of Biosciences, University of Nottingham, Sutton Bonington, Loughborough, LE12 5RD, United Kingdom

[2] Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, Sutton Bonington, Loughborough, LE12 5RD, United Kingdom; E-Mail: sean@arabidopsis.org.uk

[3] Crops for the Future Research Centre, University of Nottingham Malaysia Campus (UNMC), Jalan Broga, Semenyih, 43500, Malaysia

[4] Department of Informatics, King's College London, Strand, London, WC2R 2LS, United Kingdom; E-Mail: hung-ming.lai@kcl.ac.uk

† The first author worked at the University of Nottingham and now is with King's College London.

* Author to whom correspondence should be addressed; E-Mail: sean.mayes@nottingham.ac.uk; Tel.: +44-115-951-6234; Fax: +44-115-951-6060.

Received: / Accepted: / Published:

Abstract: Genomic DNA-based probe selection by using high density oligonucleotide arrays has recently been applied to heterologous species (Xspecies). With the advent of this new approach, researchers are able to study the genome and transcriptome of a non-model or an underutilised crop species through current state-of-the-art microarray platforms. However, a software package with graphical user interface (GUI) to analyse and parse the oligonucleotide probe pair level data is still lacking when an experiment is designed on the basis of this cross species approach. Most recently, a novel computer program called Pigeons has been developed for customised array data analysis to allow the user to import and analyse Affymetrix GeneChip® probe level data. One can determine empirical boundaries for removing poor probes based on genomic hybridisation of the test species to the Xspecies array, followed by making a species-specific Cell Description File (CDF) file for transcriptomics in the heterologous species, or pigeons can be used to examine an experimental design to identify potential Single-Feature Polymorphisms (SFPs) at the DNA

or RNA level. Pigeons is not only a GUI program but also focused around visualization and interactive studies of the datasets. The software with its manual (the current release number 1.2.1) is freely available at http://affymetrix.arabidopsis.info/xspecies/pigeons

Keywords: Affymetrix; heterologous microarray; oligonucleotide probe selection; Pigeons; probe pair data analysis; SFPs; Xspecies

1. Introduction

Microarrays have become a powerful and widely exploited tool when studying complete gene expression profiles of a multitude of cells and complex tissues in many different organisms. The major technical advance was the hybridization of RNA from tissues or cells to either cDNA or oligonucleotides fixed on glass slides or on a nylon membrane [1]. High-density oligonucleotide gene expression arrays have recently been applied to many areas of biomedical research to assess the abundance of mRNA transcripts for many genes at the same time [2]. Affymetrix (Santa Clara, CA, USA) generated GeneChip® arrays and dominated the market of high-density microarray for many years. Although there is a great deal of informative, reproducible, uniform and precise data generated by the use of a GeneChip® for large scale expression profiling, the Affymetrix chips are only available for a limited number of species of eukaryotes and a small number of model/commercial plant species including *Arabidopsis thaliana*, barley, rice, maize, tomato, soybean, sugar cane, grape and wheat [3,4]. A genomic DNA-based probe selection technology, known as the Xspecies approach, has been developed to research the transcriptomes of heterologous plants and to allow the sensitivity of high-density oligonucleotide microarrays to be applied to species where chips have not yet been designed [3,4]. The approach begins with a genomic DNA/DNA hybridization, hybridising DNA from species X onto an appropriate Affymetrix GeneChip® of a heterologous species. The next step uses a Script to parse an Affymetrix CDF file of the selected chip. The parser takes the CDF file of the chip and the CEL file of the hybridization to identify and remove 'bad' oligoprobes whose perfect match probe intensities are below a cut-off value defined by the user, eventually making a 'new' CDF file for Species X [5]. The new probe–masking file, namely the species X.CDF, can be used for Xspecies transcriptomic analysis of RNA hybridizations. Hammond *et al*. [3] showed that the Xspecies approach had been successfully applied to analyzing the transcriptome of *Brassica oleracea* L. by labeling gDNA from *B. oleracea* and hybridizing it to the ATH1-121501 (ATH1) GeneChip® array. The approach with heterologous oligonucleotide microarray was also utilised to profile and to compare the transcriptional level of the *Thlaspi caerulescens* and *Thlaspi arvense*, both being species where no GeneChip® is available [4]. A further application of this novel approach was to examine the evidence for neutral transcriptome evolution in plants by quantifying more than 18,000 genes transcripts at the level of 14 taxa from the cabbage family [6]. However, the original script parser has a specific limitation in choosing the cut-off – the selection of the value is essentially arbitrary, although a more recent iteration does allow a degree of sub-sampling to suggest thresholds. One method to improve on this approach is to generate many custom CDF files according to different cut-offs, from low to high. Then, a range of good probes pairs and probe-sets with respect to the chosen specific cut-offs are obtained. The researcher, using a

spreadsheet, plots these data as background information and uses them to finally decide the optimal value of the cut-off and the corresponding CDF file [7]. The approach is valid but is still human-dependant since people choose the threshold based on their observations and experience when looking at the plot. Recently, oligonucleotide arrays have been used to recognise allelic variation, the variants being termed single-feature polymorphisms (SFPs), in model species. The polymorphism is often detected by a single probe in an oligonucleotide array - the so-called "features". There is no *a priori* understanding of the DNA nature of the polymorphism, simply that it is a reproducible polymorphism. With this cross-species approach using Affymetrix GeneChip®s, people have the opportunity to interrogate signals for potential SFP markers that exist in minor species. Thus, it is essential to design biological and algorithmic approaches for heterologous oligonucleotide microarray analysis, to help facilitate the genomic investigation of minor plants and animals. Here, we have developed an innovative software package 'Pigeons', abbreviated from "Photographically InteGrated En-suite for the OligoNucleotide Screening", to work towards a solution to the issues mentioned above. Pigeons allows the user to input and analyse microarray data from the Xspecies microarray approach. This can be DNA hybridisations across species, to determine the empirical boundaries for custom CDF files for Xspecies transcriptomics or to examine an experimental design to identify SFPs at single oligonucleotides within the probe sets, either at the DNA or RNA level. To allow intuitive interaction and a final selection, we have also developed a specific visualization interface to facilitate navigation through the hundreds of thousands of Affymetrix oligonucleotides.

2. Methods and Algorithms

In this paper, there are three algorithms (ATM, DFC, POST) presented to fulfil the needs of analysing and parsing the Xspecies microarray data at the probe level. We aim to improve on current Xspecies parser scripts by using several traditional and modern computing techniques including interpolation, projection and clustering [8,9]. Meanwhile, recent microarray gene selection approaches, such as a fold-change (FC) analysis and a variety of statistical tests [10–12], have also been extended and modified to address the issue of searching for the single oligonucleotides containing the feature of interest.

2.1. ATM

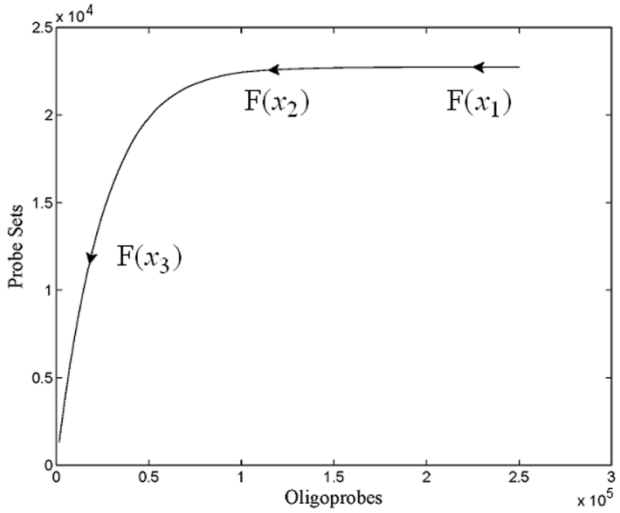
As a mixed model of numerical analysis and a soft computing technique, a heuristic method called Automated Threshold Mapping (ATM) was proposed to improve on the human-dependant cut-off selection of poorly hybridising oligonucleotide probes. First of all, a vector-valued function is introduced to perform an in-depth analysis of the problem. Let X be a scalar variable and Y be a vector variable with two dimensions. A vector function $\mathrm{F}: \mathbb{R} \to \mathbb{R}^2$ is defined as follows:

$$\mathrm{Y} = \mathrm{F}(\mathrm{X}) = \langle f_1(x), f_2(x) \rangle = \begin{pmatrix} \mathrm{Y}_1 \\ \mathrm{Y}_2 \end{pmatrix}, \quad (1)$$

where X is a set of cut-offs and the component functions f_1 and f_2 are real-valued functions of the parameter *x*. The two components of Y, Y_1 and Y_2, are therefore viewed as sets of retained oligoprobes and probe-sets, respectively, when a cut-off is given. Using the vector-valued retention function F, we can easily trace the graph of a curve to know the relationship between cut-off and the retention units.

The point of the position vector F(x) coincides with the point (y_1, y_2) on the plane curve given by the component equations, as shown in Figure 1. The arrowhead on the curve represents the curve's orientation by pointing in the direction of increasing values of x, namely $x_3 > x_2 > x_1$. Due to the nature of the problem, the retention function F monotonically decreases in the direction of the point (0, 0). The characteristic reveals that the mapping from y_1 to y_2 is also a monotone function (increasing), and moreover, it is actually like a learning curve with a stagnant occurrence.

Figure 1. Plane Curve. A vector valued function traced out by retention unit with respect to the cut-off of poorly hybridising oligonucleotides using the heterologous GeneChip® platform.



Tangent vector based numerical analysis could be applied to the evaluation and the differentiation of the function at a given point. For example, a turning point $\mathrm{F}(x_{tp})$ can be defined as the intersection between a tangent to the stagnant phase of the curve and the tangent to the linear-like decrease portion of the curve, and the inverse of this point $\mathrm{F}^{-1}(\mathrm{F}(x_{tp}))$ will be selected as a threshold value. However, the cut-off decision problem is not deterministic, and it usually needs to take biological sense into account and needs more tolerance for selection. The ATM has offered a turning area (TA) covering the turning point and derived from a closed interval I from which the feasible thresholds can be retrieved. Let I be the surrounding area of $x_{tp} \in \mathrm{X}$ such that $\mathrm{F}(x_{tp})$ is the turning point, and then we construct the turning area by $\mathrm{TA} = \{\mathrm{F}(x): x \in \mathrm{I}\}$. The construction leads to the fact that the careful definitions of a lower bound (x_{lb}) and an upper bound (x_{ub}) of I are required, with a view to fulfilling the idea of the flexible region rather than the turning point. Since the well-defined function F is a one to one and onto function on the interval I and is a monotonically decreasing function; in theory, we can define x_{lb} and x_{ub} to satisfy that $\mathrm{F}(x_{lb})$ would be in the terminating phase of plateau and $\mathrm{F}(x_{ub})$ would be in the earliest phase of sharp decline, respectively.

The ATM is a data-driven mapping method using a two-stage unsupervised learning process for deconstruction of the cut-off plane curve. Orthogonal projection is carried out at the first stage in order to spotlight the turning area. To achieve this, we consider an inner-product vector space $\mathcal{V} = \mathbb{R}^3$, let $\mathcal{U}$ be an r-dimensional subspace of $\mathcal{V}$ and $\mathcal{U}^{\perp}$ be the orthogonal complement of $\mathcal{U}$. Given a matrix $\mathbf{B}_{3\times r}$ such that the column space of $\mathbf{B}$ is $\mathcal{U}$, and then for $\forall v = (v_1, v_2, v_3) \in \mathcal{V}$, $v_1 \in \mathrm{X}$, $v_2 \in \mathrm{Y}_1$ and $v_3 \in \mathrm{Y}_2$ there exists a projector $\mathbf{P}$ to project v onto $\mathcal{U}$ along $\mathcal{U}^{\perp}$, i.e. $\mathbf{P}v = u$, $u \in \mathcal{U}$. The

unique linear operator $\mathbf{P}$ can be acquired by $\mathbf{P} = \mathbf{B}(\mathbf{B}^{\mathrm{T}}\mathbf{B})^{-1}\mathbf{B}^{\mathrm{T}}$, in particular, if $\mathbf{B}$ constitutes orthonormal bases, then $\mathbf{P} = \mathbf{B}\mathbf{B}^{\mathrm{T}}$. Through simplification of the system, the goal at this stage is to minimize the loss of information relevant to the problem of concern. As a consequence, given n numbers of data points of thresholds and after linear transformation of each vector $v_i \in \mathcal{V}$, $i = 1, \cdots, n$, we will then gain a learning data set $\mathrm{D} = \{u_i : \mathbf{P}v_i = u_i \in \mathcal{U}\}$ that ideally has the most informative feature for the turning area discovery. Suppose that all the data vectors in TA have been projected onto a particular area, we define the area as a hotspot D' such that

$$\mathrm{D} \supset \mathrm{D}' = \{u_i\}_{i \in \mathrm{J}} \tag{2}$$

$$\mathrm{I} = \left[x_{\inf(\mathrm{J})}, x_{\sup(\mathrm{J})}\right] \tag{3}$$

where J is an index set to collect and distinguish the elements of the hotspot, and $\inf(\mathrm{J})$ & $\sup(\mathrm{J})$ denote infimum and supremum of J respectively. Obviously, J is a subset of $\{1, \cdots, n\}$, and both D' and J are well-ordered closed sets.

In other words, the task at the second stage is towards hotspot identification to discover the feasible selection of cut-offs. To do this, grouping methods would be rather appropriate as the object of clustering is to group a set of data vectors that leads each cluster to include only those vectors which are similar to each other. Although there are similarities between the data points of the target group to a certain extent, it is also believed that the similarities might be more or less across groups. This is due to the design of a flexible choice of thresholds. Some elements of the turning area are closer to the end of plateau, others are the neighbours of the beginning of linear-like decline, and still others are nearby the turning point. Depicting the hotspot is to capture the "grayness" of the cross-cluster similarities so it is essential to allow some degree of uncertainty in its description. The ATM applies Fuzzy c-Means (FCM) clustering to this issue since the FCM allows us to build clusters with vague boundaries that some overlapping clusters are in possession of the same object to a certain degree [13]. Based on an objective function or performance index $\mathcal{J}_{fcm}$, the weighted within-class sum of squares, to quantify how good the quality of clustering models is, the FCM attempts to find the best allocation of data to clusters with a gradual membership matrix $\mathbf{M}$. Given a number of clusters c ($1 < c < n$), then the learning data set $\mathrm{D} \subseteq \mathbb{R}^r$ is dominated by fuzzy sets $\tilde{C} = \{\tilde{C}_i : i = 1, \cdots, c\}$ and the fuzzy partition matrix $\mathbf{M} = \left[m_{ij}\right]_{c \times n}$, where $\bigcup_{i=1}^{c} \tilde{C}_i = \mathrm{D}$ and $\forall i \; \emptyset \subset \tilde{C}_i \subset \mathrm{D}$. For the individual entries in $\mathbf{M}$, m_{ij} are the membership degree of element $u_j \in \mathrm{D}$ to cluster i, i.e. $m_{ij} = \tilde{C}_i(u_j) \in [0, 1]$. Let $\Omega = \{\omega_i : i = 1, \cdots, c \text{ and } \omega_i \in \mathbb{R}^r\}$ be a set of cluster prototypes so that each cluster $\tilde{C}_i$ is represented with a cluster centre vector ω_i, and the objective function with two constraints can then be defined as below:

$$\mathcal{J}_{fcm}(\mathrm{D}, \mathbf{M}, \Omega) = \sum_{i=1}^{c} \sum_{j=1}^{n} m_{ij}^{q} d_{ij}^{2} \tag{4}$$

$$\sum_{i=1}^{c} m_{ij} = 1, \forall j \tag{5}$$

$$0 < \sum_{j=1}^{n} m_{ij} < n, \forall i. \tag{6}$$

Here, $q \in \mathbb{R}_{>1}$ is termed the 'fuzzifier' or weighting exponent, and d_{ij} is the distance between object u_j and cluster centre ω_i, within ATM, the Euclidean inner product norm denoted by $\|\cdot\|$ is taken, i.e. $d_{ij} = \|u_j - \omega_i\|$. The purpose of the clustering algorithm is to obtain the solution $\mathbf{M}$ and Ω

minimizing the cost function $\mathcal{J}_{fcm}$, and this can be carried out by

$$m_{ij} = \left[\sum_{k=1}^{c}\left(\frac{d_{ij}}{d_{kj}}\right)^{\frac{2}{q-1}}\right]^{-1} \tag{7}$$

$$\omega_i = \left[\sum_{j=1}^{n} m_{ij}^{q} u_j\right] / \sum_{j=1}^{n} m_{ij}^{q}; \tag{8}$$

namely the FCM proceeds with two events: the computation of cluster centroids and the allocation of data elements to these centroids. In practice, the cost function $\mathcal{J}_{fcm}$ is minimized by an alternating optimization (AO) scheme, i.e., the membership degrees are first optimized given recently fixed cluster parameters, followed by optimizing the cluster prototypes given currently fixed membership degrees. This procedure will be repeated until the cluster centres have reached equilibrium which is equivalent in mathematics to the optimal objective function $\mathcal{J}_{fcm}$.

After the grouping scheme is accomplished, the hotspot D' can be deciphered by defining the greatest lower bound and the least upper bound of its index set. The first two clusters ($\tilde{C}_1$, $\tilde{C}_2$) are concentrated for the purpose of deciphering since most of elements of $\tilde{C}_1$ are very likely to be projected from vectors in stagnant phase while data points near the beginning of the sharp drop have mostly fallen into $\tilde{C}_2$. Thus, we let D' be the subset of the union of the two clusters and set the infimum and the supremum of J according to the objects whose membership values are the maximum of $\tilde{C}_1$ and $\tilde{C}_2$, i.e.

$$\inf(\mathrm{J}) = \arg\max_{\forall j} \tilde{C}_1(u_j), \sup(\mathrm{J}) = \arg\max_{\forall j} \tilde{C}_2(u_j). \tag{9}$$

Not only do the above equations define the index set J, but also they reveal that the tolerance interval I has been established. Besides the selection of feasible cut-offs, the ATM also provides an automated threshold value x_{ATM} and a target interval I' for the selection of candidate cut-offs. Both x_{ATM} and I' are evaluated through the fuzzy boundary between the first two fuzzy sets. The elements in the boundary imply that $\tilde{C}_1$ and $\tilde{C}_2$ have them in common to some extent. Owing to the grayness characteristic and the continuity of the learning-like curve, we believe that a good threshold value for parsing the Affymetrix cell description files would come from a projected object that simultaneously belongs to the two clusters with remarkable membership degrees. As a result, the fuzzy boundary can enable us to offer a more reasonable selection of cut-offs. Two indices, *l* and *k*, are utilised to lead to the evaluation of the highly likely cut-offs and the automated threshold value, determined by

$$l = \arg\min_{\mathrm{j}\in\mathrm{J}} \tilde{C}_2(u_j)_{>\epsilon}, k = \arg\min_{\mathrm{j}\in\mathrm{J}} \tilde{C}_1(u_j)_{>\epsilon}. \tag{10}$$

Here ϵ is a small number to assess the possibility of the overlap between the two clusters. By this definition, the fuzzy boundary is then portrayed as the set of $\tilde{C}_1 \cap \tilde{C}_2 = \{u_j \in \mathrm{D}: l \le j \le k\}$ and another closed interval $[x_l, x_k]$ is constructed as the target interval I'. Let $\bar{\mathrm{u}}$ be the arithmetic mean of the elements of $\tilde{C}_1 \cap \tilde{C}_2$, and what is more, x_{ATM} can also be calculated by linear interpolation or by the Lagrange polynomial, as shown in the following formulae:

$$x_{ATM} = \mathrm{p}(\bar{\mathrm{u}}) = \sum_{j=l}^{k} x_j \mathrm{L}_j^{l,k}, \ \mathrm{L}_j^{l,k} = \prod_{\substack{i=l \\ i\neq j}}^{k} \frac{\bar{\mathrm{u}}-u_i}{u_j-u_i}. \tag{11}$$

In summary, the ATM returns a 3-tuple $(x_{ATM}, \mathrm{I}', \mathrm{I})$ for the issue of the cut-off choices. The suggested cut-off given by the ATM, x_{ATM}, can directly be exploited to remove the weak intensity signals while

any values within a target interval, $\mathrm{I}' = [x_l, x_k]$, can be taken as the potential cut-offs. The design of the target interval gives users a chance of picking a scientifically reasonable value on their own. Those values in a tolerance interval, i.e. $x \in \mathrm{I}=\left[x_{\inf\,(\mathrm{J})}, x_{\sup\,(\mathrm{J})}\right]$, can be used as feasible thresholds and values outside the interval are viewed as infeasible choices.

2.2. DFC

Dual fold-change analysis (DFC) is an approach to seek potential single-feature polymorphism markers through screening all of the 25-mer oligonucleotide probes of the heterologous microarray. Initially, there are two groups (G_1 and G_2) under the design of the single trait experiment. While two distinct parental genotype gDNAs are involved in G_1, G_2 is composed of two different phenotypically based F_2 bulk segregant pools, derived from a hybrid between the two parental genotypes We then label the four Xspecies chips with $\mathfrak{B}_1$ & $\mathfrak{B}_2$ for two parent samples and with $\mathfrak{B}_3$ & $\mathfrak{B}_4$ for two F_2 bulks. In practice these F_2 genotypes will be bulks from the defined parental cross and self-pollination of F_1 offspring collected according to the trait of interest. The phenotype classification is a necessary prerequisite for the numerical analysis of potential SFP markers using the high throughput technology. Generally speaking, $\mathfrak{B}_1$ and $\mathfrak{B}_3$ are classified into one type under a single trait experiment whereas $\mathfrak{B}_2$ and $\mathfrak{B}_4$ belong to the other - the prerequisite can be denoted as $\mathfrak{B}_1 \mathcal{R} \mathfrak{B}_3 \sim \mathfrak{B}_2 \mathcal{R} \mathfrak{B}_4$. Let N be the number of genes and #($\mathcal{B}$) be the cardinal number of a probe-set $\mathcal{B}$ then each chip can be represented as follows:

$$\mathfrak{B}_m = \bigcup_{i=1}^{N} \mathcal{B}_i \quad m = 1, \cdots, 4 \tag{12}$$

$$\mathcal{B}_i = \left\{ b_{ij}^m \in \mathbb{R} : j = 1, \cdots, \#(\mathcal{B}_i) \right\} \tag{13}$$

where b_{ij}^m denotes the *j*-th signal intensity of the *i*-th probe-set in the *m*-th chip. Let $\mathrm{Q}_{ij}^1 = b_{ij}^1 / b_{ij}^2$ and $\mathrm{Q}_{ij}^2 = b_{ij}^3 / b_{ij}^4$ be the intensity ratio of G_1 and G_2, respectively, thus the value of one is for unchanged hybridisation and less than or greater than one is for differentially hybridised oligonucleotides. To generate a symmetric distribution of intensity ratios, the fold-change ratio is defined by

$$\mathrm{FC}_{ij}^1 = \begin{cases} \mathrm{Q}_{ij}^1, & \mathrm{Q}_{ij}^1 \geq 1 \\ \frac{1}{\mathrm{Q}_{ij}^1}, & \mathrm{Q}_{ij}^1 < 1. \end{cases} \tag{14}$$

Where FC_{ij}^1 is to assess the differential probe hybridisation of the parental group. For the evaluation of the offspring group, FC_{ij}^2 is denoted and is calculated in the same way as FC_{ij}^1 which simply replaces Q_{ij}^1 with Q_{ij}^2. Given the threshold of weak signals x_{ATM}, the cut-off of a fold-change between the parents $\epsilon_1 \in \mathbb{R}_{>1}$ and that between the offspring $\epsilon_2 \in \mathbb{R}_{>1}$, a number of logical criteria are performed to globally screen and search Affymetrix's single oligoprobes for SFP markers. For $\forall m, i, j$, let the first condition be $b_{ij}^m > x_{ATM}$ since any signals whose intensities are below the threshold should not be used for good probes in the analysis of heterologous data - this satisfies the demand of the across species technology. When the first criterion holds, the DFC enables the procedure to run the second condition with the two fold-changes FC_{ij}^1 and FC_{ij}^2, $\mathrm{FC}_{ij}^1 \geq \epsilon_1$ and $\mathrm{FC}_{ij}^2 \geq \epsilon_2$, to measure whether $\mathfrak{B}_1 \mathcal{R} \mathfrak{B}_3 \sim \mathfrak{B}_2 \mathcal{R} \mathfrak{B}_4$ still holds at the genomic level. The FC approach is commonly used in microarray data analysis to identify differentially expressed genes (DEGs) between

a treatment and a control. Calculated as the ratio of two conditions/samples, the FC gives the absolute ratio of normalized intensities without log scale. We extend the same concept in our approach by introducing an additional FC - one ratio is as an assessment for the differential hybridisation within G_1 and the other is for that within G_2. The design of the extra FC is due to the idea that the difference in phenotype could result from a difference in genotype at a single locus. Therefore, when there are any differentially hybridised oligonucleotides for the feature of interest between the two parental types, the inherited attribute of $\mathfrak{B}_1\mathcal{R}\mathfrak{B}_3 \sim \mathfrak{B}_2\mathcal{R}\mathfrak{B}_4$ would imply that we could expect those differential oligonucleotides hybridisations to have also been transmitted into the F_2 hybrids. In a word, the fold-change of F_2 is introduced herein as a cross-check mechanism for identifying probable SFPs highly related to the trait in question. The mixture of F_2 genotypes (which are bulked according to the trait difference which segregates within the cross) should mean that the attribute difference is only detected when the location of the parental SFP is close to the gene controlling the trait difference. If any oligoprobes have satisfied the second criterion, they are likely to be used as potential SFP markers between the two distinct phenotypes and could be used for genetic mapping of the gene controlling the phenotypic difference.

2.3. POST

The FC is typically viewed to be significant if there is at least a two-fold difference [10]. However, the strength of signal intensities of the F_2 hybrid bulks often tends to have weakened against that of the parents. In addition, the FC is selected arbitrarily and does not involve any assessment of statistical confidence so using the FC approach alone could not be optimal [11,14]. Although it is straightforward and intuitive way to detect oligonucleotides using a dual fold-change criterion, the approach does not engage any evaluation of the significance of differential hybridisation in the presence of biological and experimental variation, which might differ from probe to probe. We have therefore developed inferential statistics herein through a method called the probewise one-sample statistical test (POST) for the assessment of the differential oligoprobe variation in terms of statistical power and measures of confidence. We first define an MA-value ρ_{ij} for the examination of signal variation in the single trait experiment, for $\forall i, j$ the value is calculated by the following formula:

$$\rho_{ij} = \begin{cases} \log_2 \left(\frac{b_{ij}^1 b_{ij}^3}{b_{ij}^2 b_{ij}^4} \right)^{\frac{1}{2}}, & (\log_2 \mathrm{Q}_{ij}^1)\,(\log_2 \mathrm{Q}_{ij}^2) > 0 \\ 0, & (\log_2 \mathrm{Q}_{ij}^1)\,(\log_2 \mathrm{Q}_{ij}^2) \leq 0 \end{cases} \tag{15}$$

to exactly correspond with the experimental attribute of $\mathfrak{B}_1\mathcal{R}\mathfrak{B}_3 \sim \mathfrak{B}_2\mathcal{R}\mathfrak{B}_4$. The MA-value is named after the MA plot, a very useful tool in cDNA and GeneChip® microarray data analysis [15–17], and it is actually an average intensity ratio between parental samples and F_2 hybrid bulks in the base 2 logarithmic scale with a mnemonic for subtraction and a mnemonic for addition. The POST then uses the MA-value and the single sample *t*-test to assess differentially hybridised oligonucleotides between parent group and offspring group and to test in a probe-set *i* whether or not there is significant difference between an interrogated probe *k* and the other probes in terms of their log ratios. As a test statistic, the average of the MA-values of each of the oligoprobes except the probe *k* is denoted by $\bar{\rho}_{ik}$ and determined by:

$$\bar{\rho}_{ik} = \frac{1}{n_i} \sum_{\substack{j=1 \\ j \neq k}}^{\#(\mathcal{B}_i)} \rho_{ij} \tag{16}$$

where $n_i = \#(\mathcal{B}_i) - 1$ is the sample size in the examined probe-set *i*. Suppose that the sampling distribution of $\bar{\rho}_{ik}$ is normal so that the random variable

$$T_{ik} = \frac{\sqrt{n_i}(\bar{\rho}_{ik} - \rho_{ik})}{S_{ik}} \tag{17}$$

has a Student's *t*-distribution with $n_i - 1$ degrees of freedom. Where S_{ik} is the standard deviation of the sample of the log ratios in the *i*-th probe-set excluding the MA-value of the oligoprobe *k*. The last step performed by the POST is to asymptotically compute the p-value converting the value of T_{ik} into a probability that expresses how likely the oligonucleotides in question are to be differentially hybridised. To customise this probewise testing of single oligonucleotides, a visualization filter with a Volcano Plot output was also developed. The volcano plot is an effective and easy-to-interpret scatter plot in the selection of DEGs [11]. In the POST, the plot shows the negative common logarithm (base 10) of the p-value versus the average intensity ratio in the form of the binary logarithm (base 2), i.e. average fold-change ratio. Oligoprobes with large log ratios and low p-values are easily detectable in the view and a list of potential SFP markers can be acquired.

Another approach for statistical inference using a different measure, intensity difference, has also been involved in the POST to evaluate significantly variable oligonucleotides within an experimental group. Basically, the approach is a methodology analogous to that of testing between two groups, but it is more focused on variation within a single group. Since a potential SFP marker could be due to oligonucleotides with deletions or duplications or nucleotide differences, we propose using intensity difference rather than the traditional intensity ratio to measure the significant difference in intensity between the signal of an array and the same signal of the other within either the parent group G_1 or the offspring group G_2. We name the intensity difference the D-value, in contrast to the MA-value, and define it in compliance with the trait of interest as below:

$$\delta_{ij} = \begin{cases} b_{ij}^1 - b_{ij}^2, & \forall i,j \in G_1 \\ b_{ij}^3 - b_{ij}^4, & \forall i,j \in G_2. \end{cases} \tag{18}$$

Similar to statistical tests between groups, the sample mean of the D-value would be the statistic to test whether the intensity difference of the oligoprobe of interrogation is significantly different from that of the other signals in the same set of a cell line; meanwhile, an *ad hoc* test procedure within G1 or G2 also assumes that the population distribution is at least approximately normal and proceeds with the probewise strategy. However, there are practical issues that ought to be addressed. As an overwhelming majority of intensity signals are poorly hybridised in the heterologous oligonucleotide microarray, most interesting probe-sets could just have one potential SFP. Thus, the sample mean is in general a good estimator for the central value of the data distribution of δ_{ij} when the statistical testing is performed in the way of the probewise strategy. But for those probe-sets which have two or more possible markers, the mean is no longer an appropriate measure of location under the probewise procedure since it will be susceptible to an extreme value. Accordingly, the γ-trimmed mean $(0 < \gamma < 0.5)$ is employed instead of the mean as the statistic in this version of POST. More

mathematically, let $\Delta_i^k = \{\delta_{ij} \in \mathbb{R}: j = 1, \cdots, k-1, k+1, \cdots, \#(\mathcal{B}_i)\}$ and let $\delta_{i(1)} \leq \delta_{i(2)} \leq \cdots \leq \delta_{i(n_i)}$ be the observations of Δ_i^k written in ascending order. We define the sample γ-trimmed mean $\bar{\delta}_{ik}$ to account for probe-specific fluctuations in a probe-set *i* and its value is calculated by

$$\bar{\delta}_{ik} = \frac{1}{n_i(1-2\gamma)} \sum_{j=h+1}^{n_i-h} \delta_{i(j)} + (h - \gamma n_i)\left(\delta_{i(h)} + \delta_{i(n_i-h+1)}\right) \quad (19)$$

, where $h = [\gamma n_i]$ is the value of γn_i rounded down to the nearest integer. Then, let s_{ik}^2 be the sample γ-Winsorized variance in the data of Δ_i^k and consider the finite-sample Student-*t* statistic analogue, the γ-trimmed mean can be studentized by s_{ik} as the form of

$$t_{ik} = \frac{(1-2\gamma)\sqrt{n_i}(\bar{\delta}_{ik} - \delta_{ik})}{s_{ik}}. \quad (20)$$

Tukey and McLaughlin [18] suggested a reasonably accurate approximation of the distribution of t_{ik} using a Student's *t*-distribution with $n_i - 2h - 1$ degrees of freedom. Also, Patel *et al*. [19] further introduced a scaled Student-*t* variate $a(n_i, h)t_{ik}$ and proposed approximating the distribution of $a(n_i, h)t_{ik}$ with a Student's *t* distribution having $v(n_i, h)$ degrees of freedom, where $a(n_i, h) = 1 + 16h^{0.5}e^{2h-n_i}$ for small-sample ($n_i < 18$) *t*-type statistics and $v(n_i, h)$ has a slight variation depending on γ in their investigation. Given γ = 0.05, 0.10, 0.15, 0.20 or 0.25 we apply the Tukey-McLaughlin suggestion and Patel's refined approximation to each of t_{ik} for the calculation of the p-value, and the asymptotic p-value accompanied with the intensity difference can therefore be prepared for the volcano plot filter. To better reveal detection of large-magnitude changes in the view, the POST has been performed with the *square-root-transformation* of the D-value into the fold-change difference FCD_{ij} defined as follows:

$$\mathrm{FCD}_{ij} = \begin{cases} \sqrt{\delta_{ij}}, & \delta_{ij} \geq 0 \\ -\sqrt{-\delta_{ij}}, & \delta_{ij} < 0 \end{cases} \quad (21)$$

which produces a symmetric distribution of intensity differences with the assumption that most oligonucleotides are not differentially hybridised so that the modified volcano plot using fold-change differences is still able to plot changes in both directions showing equidistance from the centre. Due to the experiment of design, the POST tests the inferential statistics on individual oligonucleotides within the parent group and the offspring group respectively, colouring the plotted points in accordance with the group to which they belong. The colour scheme can be employed as the third dimensional information for ease of filtering and parameter setting. By constructing the coloured volcano plots of G_1 and G_2, one can quickly identify the most-meaningful changes of hybridisation relying on the feature of interest.

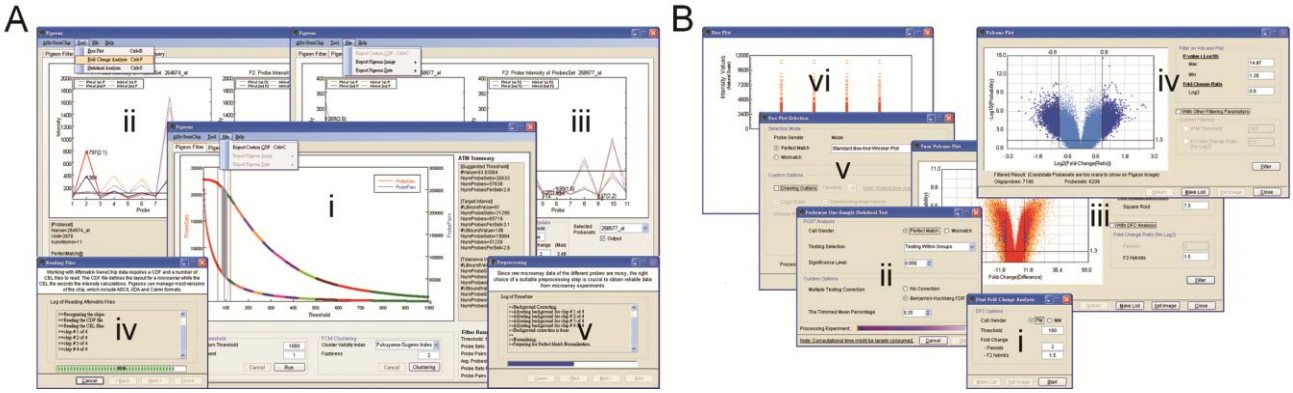
3. Results and Discussion

3.1. Software Implementations

Pigeons is a standalone GUI program for the Windows platform under the .NET framework to analyze Affymetrix GeneChip® data generated from cross-species experiments and the current version number is 1.2.1, released in late-June 2012. The software is able to read most recent or current Affymetrix .CEL file types, including version 3, version 4 and Command Console version 1 (the latest one at the time of the program development) and is focused around visualization and interaction

studies of data (Fig. 2). This computer program is a freeware license so it is free of charge to download and to fully execute for research uses. The .NET Framework version 3.5 or greater is required to install the program. 2MB of free hard disk space is the minimum to execute the program while 200MB would be better if data/image file export is required. The golden rule of thumb is that the more RAM the better the capacity, and the faster the microprocessor the quicker the response. At least 1GB RAM & Intel® Pentium® M-class processors or better are recommended, although slower CPU speeds with 512MB system memory will still work in most circumstances. This computer software has successfully been tested on Windows 2000, Windows XP, Windows Vista and Windows 7.

Figure 2. Software Snapshots. Pigeons is a tab-page based standalone GUI program. There are three tab-pages for the three main applications in the main form for different purposes. Each application can be used either separately or jointly. Other tools in a menu strip are also tab-page associated, that is, their availability depends on the main application currently being performed. (**A**) Central Applications. Three main applications are: i. Pigeon Filter, ii. Pigeon Mining/Image and iii. Pigeon Query; they are executed after the completion of two core components, iv. File Reading and v. Data Preprocessing. (**B**) Statistical Analyses. Several essential tools can also be called from the menu strip. They are: i. DFC, ii. POST, iii. Twin Volcano Plot, iv. Volcano Plot, v. Box Plot dialog-box, and vi. Box Plot output.



Pigeons is a tab-page-reliant program with the availability of the functions in the main form depending on the tab-page currently presented. There are three tab-pages inside the windows form. Pigeon Filter is an application to implement the ATM method for the removal of poorly hybridising oligoprobes and to make a probe–masking CDF file (Fig. 2A-i). Pigeon Mining & Image is developed to perform the DFC approach and the POST statistical filters for finding potential SFP markers (Fig. 2A-ii, 2B-i). There are two POST-based graphical summary tools within Pigeon Mining (Fig. 2B-ii). While the Volcano Plot (VP) is to test differential variation between groups of parents and F_2 hybrids using the binary average fold-change ratio (Fig. 2B-iv), the Twin Volcano Plot (TVP) has been designed based on statistical tests within the groups (Fig. 2B-iii). Results acquired by either the DFC or the POST can be exported as lists and as graphical representations for probe-sets to assist in the interpretation of oligo-level data at the DNA or RNA level. Pigeon Query is an interface for quick probe-set retrieval from datasets (Fig. 2A-iii). Besides the three main applications, a couple of essential upstream tools are also involved in this software package – they are data preprocessing (Fig.

2A-v) and a box-and-whisker plot (Fig. 2B-v, 2B-vi). The Exponential-Normal Convolution Model was utilised for background correction in this program to adjust for systematic effects that arise from variation in the Affymetrix platform [16]. Pigeons employs quantile normalization to address the comparability of intensity distributions between arrays [17]. Then, one can use the box-and-whisker plot, a significant quality control tool, to examine the data before and after data preprocessing. This exploratory data analysis conducts a check for evaluating any extraordinary chip distributions and to verify if a normalization procedure has been effective. A user manual has been provided and built within an installer program so that users can access it from the start menu of MS Windows after the Pigeons has successfully been installed on a local machine.

3.2. Case Studies of ATM

Table 1. Summaries of case studies.

	Species	Selected Cut-off	Automated Threshold Mapping (ATM)				Reference
			%	Suggested Cut-off	Target Interval	Tolerance Interval	
1.	*Brassica oleracea* L.	400	2.17	391.34[a]	[351,426][a]	[272,454][a]	Hammond *et al*. 2005
2.	*Thlaspi caerulescens*	300	10.54	331.63[a]	[297,363][a]	[234,387][a]	Hammond *et al*. 2006
3.	*Musa* (Banana)	550	10.47	492.40[b]	[399,586][b]	[305,698][b]	Davey *et al*. 2009
4.	Equine (Horse)	100	5.93	94.07[a]	[82,106][a]	[65,119][a]	Graham *et al*. 2010
5.	Ovine (Sheep)	450	6.93	481.20[b]	[381,582][b]	[284,694][b]	Graham *et al*. 2011

The cut-off values to mask the poor intensity signals were examined from 0 to 1000 with an increment of 1 in all cases. These data sets were then tested under the ATM framework with cluster validation methods to generate the ATM three-tuple result for comparison to the previous publications. % denotes relative difference in cut-off and was calculated by the absolute value of difference between the selected and the suggested cut-off divided by the selected cut-off value.

[a]ATM was accompanied by a cluster validation procedure using Fukuyama-Sugeno's index.

[b]The partition entropy was applied as a cluster validity index into the ATM algorithm.

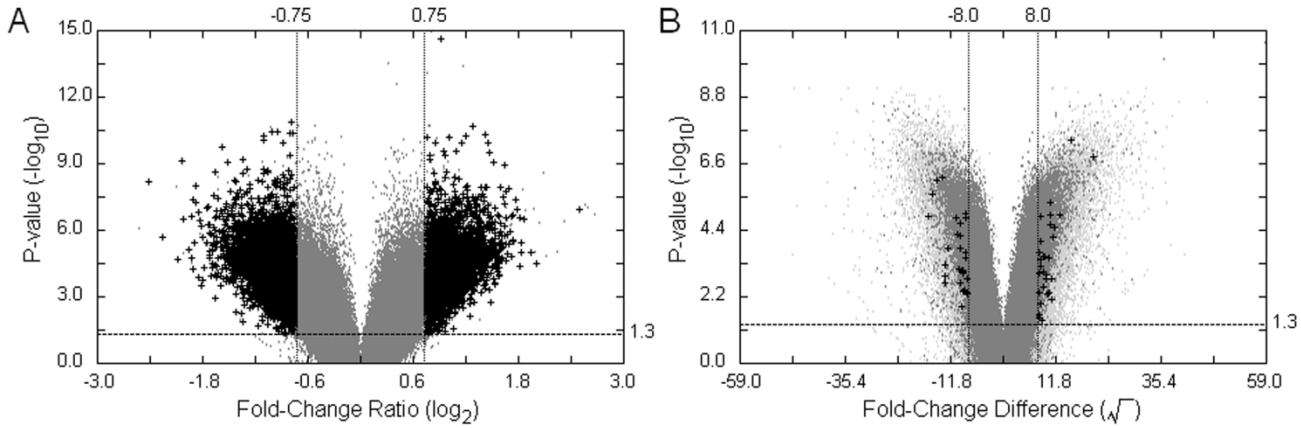
Here, we surveyed a number of previous studies which focused on the transcriptome analysis of heterologous species through the across species microarray approach, and compared the cut-off values chosen to make species-specific CDF files in those studies with the ATM's suggestion on gDNA hybridisation intensity thresholds (Table 1). The *Brassica oleracea* L. (case 1) and *Thlaspi caerulescens* (case 2) were hybridised on the *Arabidopsis thaliana* ATH1-121501 GeneChip® Arrays [3,4] whereas the two animals (case 4 and 5) were hybridised on Human U133 Plus 2.0 Genome Arrays [20,21]. Also, in the third case the Affymetrix Rice Genome Array was used to investigate transcriptomic profiling related to drought stress in *Musa* [7]. In the original research of the Xspecies approach, i.e. the first case, a probe mask created at a cut-off value of 400 was determined systemically and empirically by generating 13 custom CDF files with a series of gDNA hybridisation intensity thresholds and assessment of them in turn. The probe mask file excluded 68% of the probe-pairs but retained 96% of the available probe-sets, and was used to study transcriptional response under phosphorus stress. This empirical method of determining the cut-off value was also

applied to the second and the fourth cases, reporting the preferred hybridisation intensity threshold of 300 and of 100, respectively. The same probe selection strategy but subtly different considerations were taken in the third and fifth cases. The authors of these two studies determined the hybridisation intensity threshold used to create a probe mask file that was able to detect the maximum possible number of Differentially Expressed Genes (DEGs) even though Hammond *et al*. showed that there was a significant loss of available probe-sets for transcriptomics profiling at the higher end of the cut-off value [3]. As a result, the selected cut-offs used in Banana and Sheep were at the value of 550 and of 450 respectively; both were higher than those which were decided not just based on the number of DEGs but using the current considerations.

Since FCM is an unsupervised process, we introduce two cluster validity indices to accompany the ATM framework to indicate the reliability of clustering results and to cover two different aspects of choosing gDNA hybridisation intensity thresholds. The two cluster validation measures are Fukuyama-Sugeno's index [22] and partition entropy [13]. In our studies, the first index was exploited in case 1, 2 and 4 whilst the second one was utilised where there was a desire to gain a larger number of differentially regulated transcripts such as in case 3 and 5; the 3-tuple results of ATM were summarised in Table 1. We found that the hybridisation intensity thresholds selected to understand the transcriptome results in the five cases were all located in the target interval, and they were generally in the vicinity of cut-off values suggested by ATM. The relative difference in the hybridisation intensity cut-offs were from 2.2% up to 10.5%. Out of the five species, the numerical suggestion of 391.34 by ATM was very close to the biologist's choice of 400 in *Brassica oleracea* L. – the original research paper presenting the heterologous gDNA hybridisation probe selection approach. Determined by the restriction of less than 3% removed probe-sets, the optimal cut-off selected in the second case was 300. This imposed constraint led to the fact that the value of the researcher's selection was not very close to the suggested value given by ATM (331.63) but was very near the value of 297, the low end of the target area. ATM is initially developed to find the optimal cut-off of a vector valued retention function (Fig. 1), and in practice, the probe mask filter using this numerical optimum has a high sensitivity to discover changes of gene expression in heterologous species. The practical consequence can substantially be shown by means of the above studies, particularly the first, second and forth cases. The difference in thresholds between the experienced researcher selection and the ATM's suggestion in Banana/Sheep were by 6.93% and 10.47%. Not surprisingly, both were higher than those in the other three species, and this is due to the stringent criterion for detecting the number of differentially expressed transcripts. By having studied the five non-model plants/animals using model species oligonucleotide arrays, we believe that ATM is valid for the determination of gDNA hybridisation intensity thresholds. The proposed approach can provide fast, reasonable and rational number thresholds in comparison with the empirical method. When ATM is in operation, we strongly recommend making use of the Fukuyama-Sugeno's index for transcriptomics and genomics analysis. More specifically, the index is best for research activities where there is no direct interest in the expressed genes in a designed experiment, for example, as with finding SFP markers. If the number of DEGs is, however, the major consideration, the partition entropy will be a good cluster validity index for this biological purpose.

3.3. Examples of an SFP Screen

Figure 3. Filtering on Volcano Plots. The customised Volcano-plot tools depicting estimated fold-change (x-axis) and statistical significance (–log10P-value, y-axis) were created by means of the POST inferential statistics for filtering on the screen of the single oligonucleotides related to the trait of interest. Each point represents an oligonucleotide probe, and the black crosses corresponded to large fold-changes with a p-value less than the significance level or the user-defined value under a number of filtering criteria. (**A**) VP. This is an example of applying the POST approach to test between groups of parents and F_2 hybrid bulks using the binary average fold-change ratio, the MA-value. (**B**) TVP. This is an illustration of another version of POST - testing oligonucleotide probes within a parental group and within an offspring group, respectively, followed by plotting the two graphical summaries together in different colours. Light-gray spots were the output of the parental group and gray ones represented the group of F_2 hybrid bulks. The fold-change difference was defined by reasonably transforming the intensity difference D-value into its square root, and was used as a measure to assess the significant intensity difference on the plot.



Besides the provision of the optimal probe mask, a complete solution containing biological and algorithmic approaches to SFP interrogation has been proposed in this article. While DFC is a biology-oriented method and conventionally uses two fold-changes with a gDNA hybridisation intensity threshold, POST is a statistically-based and newly-developed procedure with graphical summarisation filters from two aspects of test approach. To show these approaches to be functional, we examined bambara groundnut genotypes with an F_2 offspring derived from a cross between two contrasting parents with offspring bulked according to the trait 'number of branches per plant'. Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is an underutilised indigenous African crop species and an important food legume grown widely in sub-Saharan Africa and is highly inbreeding. At present, very limited sequence resources exist, which means that the Xspecies is a valid approach. The gDNA-based probe-selection using heterologous oligonucleotide microarrays allows us to interrogate thousands of single-feature polymorphisms in parallel and, through the current design, should allow us to efficiently discover markers in a genomic region linked to a phenotype. As an illustration of this point, we selected the agronomic trait 'number of branches per plant' in a cross between a wild accession with a spreading habit and a cultivated accession with a bunched habit. Cross-hybridisation of bambara groundnut DNA from two parental landraces VSSP11 (few stem per plant) and DipC (many stem per plant) were conducted on the Affymetrix Arabidopsis ATH1

GeneChip®. Meanwhile, two bulks from F_2 individuals (10 individuals each, representing the extremes from 96 individual F_2 plants) were also constructed for high and low stem number and hybridised (separately) onto Arabidopsis ATH1 GeneChip® arrays. The experiment was therefore composed of four gDNA hybridisation chips and their relationship could be represented as $\mathfrak{B}_1\mathcal{R}\mathfrak{B}_3 \sim \mathfrak{B}_2\mathcal{R}\mathfrak{B}_4$, defined in the methodology section. The probe-level raw data were then background-adjusted and quantile-normalized using the RMA method [16,17] so that these preprocessed intensity signals could be carried over into high level analyses.

Figure 3 illustrates two graphical filters, VP and TVP, generated by the two POST's editions on the interrogation of statistically differential hybridisation between the two bulks of bambara groundnut in relation to stem number. To correct for multiple testing, we implemented an approach based on controlling false discovery rate, as proposed by Benjamini and Hochberg [23]. The BH adjusted P-values were transformed into inverse significances in both VP and TVP, and the suspected SFPs could then be filtered and highlighted in the graphical outputs under a number of conditions. Since the samples of F_2 offspring play a role as a cross-check mechanism in our experimental design, the fold-change of the offspring (FCF_2) is necessarily used as one of filtering parameters. Additionally, the optimal cut-off of gDNA hybridisation intensity produced by ATM and the cut-off of the parental fold-change used in DFC could be optionally selected to increase the sensitivity of the graphical filters. The 7903 differentially hybridised signals were summarised (BH adjusted $P < 0.05,\ -0.75 \leq MA \leq 0.75,\ FCF_2 \geq 1.5$) when the POST procedure was performed between the group of parents and of F_2 samples (Fig. 3A). The lower levels of hybridisation of features will be more likely to show a significant difference between parental genotypes by chance than high level hybridisation, although the latter could represent repetitive elements within the bambara groundnut genome. Due to the scale of binary fold-change ratio, this phenomenon is quite common in microarray data analysis. The same preprocessed data set was tested using the other version of POST to examine intensities within groups, followed by filtering potential SFPs on the coloured TVP (Fig. 3B). Interestingly, there were only 59 oligoprobes (BH adjusted $P < 0.05$, $-8 \leq FCD \leq 8$, $FCF_2 \geq 1.5$) detected as statistically differentially hybridised using the probewise strategy. The sharply reduced number from thousands to dozens shows that the D-value is highly resistant to low intensity signals and that the design of TVP, disjointed testing on two groups with a process of filtering in relation to each other, was much more sensitive than the approach of VP based on the average fold-change ratio.

To have a deeper understanding of the practical effects of using different approaches for SFP detection, various conditions of VP, TVP and DFC were systemically examined and are briefly described in Table 2. Two-fold change is normally the cut-off in microarray analysis. However, the value of 1.5 was adopted rather than 2 for the cut-off of F_2 in our illustration since the stringent conditions used led to nearly nothing in dual fold-change analysis and hybridisation in this case is genomic DNA, rather than expression values for RNA. There were four instances inspected using VP and TVP respectively whereas two cases were considered in DFC. Initial filtering parameters were fixed in the four instances of VP (BH adjusted $P < 0.05,\ -0.75 \leq MA \leq 0.75$) and TVP (10% trimmed mean, BH adjusted $P < 0.05,\ -8 \leq FCD \leq 8$) and in the two instances of DFC ($FCP \geq 2$, $FCF_2 \geq 1.5$). ATM with Fukuyama-Sugeno's index produced the three-tuple suggestion (93.04, [81,106], [63,120]) of gDNA hybridisation intensity cut-offs for the cases of VP3, 4 and DFC2. Only the perfect match features of the ATH1 GeneChip® was considered in these examinations. When

filtering on VP and TVP using initial conditions of x and y axis without extra parameters, we found that VP1 identified more than ten thousand potential SFPs. This was eight times the number using TVP1. This large difference was similar to our findings in Figure 3. We also noticed that the number of differentially hybridised features significantly declined from VP1 to VP2 and very dramatically dropped from VP1 to VP3. The results revealed that the gDNA hybridisation intensity threshold was an essential parameter in the VP filter and low signal hybridised oligoprobes were largely involved in the experiment. This is consistent with the phylogenetic distance between *Vigna subterranea* L and *Arabidopsis thaliana*. When all conditions were applied in VP4 and TVP4, there were approximately equivalent numbers of potential SFPs identified in the two cases, 10 and 8, respectively. An analogous consequence between VP1 and VP3 could be found in the investigation of DFC as well. While 3360 differentially hybridised features were detected in DFC1, very surprisingly, there were just 5 probable SFPs discovered in DFC2 - the lowest number out of ten examined conditions. It implies that dual fold-change analysis would be the most stringent approach among the three methods. From the outcomes of VP4, TVP4 and DFC2, few identified SFPs suggested that Affymetrix ATH1 might not be the best array for heterologous genomic DNA hybridisation with a view to the interrogation of bambara groundnut genomes, due to a more distant genetic relationship between *Arabidopsis thaliana* and bambara groundnut.

Table 2. Screening and search for differentially hybridised oligonucleotides by filtering on two types of volcano plots and dual fold-change analysis under a number of criteria.

Method	Filtering Criteria				Number of potentially differential hybridisation[d]	
VP	P-value[a]	MA-value	FCF_2	TH[bc]	Oligoprobes	Probe-Sets
VP1	< 0.05	≥ |0.75|	–	–	13694	10492
VP2	< 0.05	≥ |0.75|	≥ 1.5	–	7903	6722
VP3	< 0.05	≥ |0.75|	–	> 93.04	125	124
VP4	< 0.05	≥ |0.75|	≥ 1.5	> 93.04	10	10
TVP[e]	P-value[a]	FCD-value	FCF_2	FCP	Oligoprobes	Probe-Sets
TVP1	< 0.05	≥ |8.0|	–	–	1637	1563
TVP2	< 0.05	≥ |8.0|	≥ 1.5	–	59	59
TVP3	< 0.05	≥ |8.0|	–	> 2	50	50
TVP4	< 0.05	≥ |8.0|	≥ 1.5	> 2	8	8
DFC	FCP	FCF_2	TH[bc]		Oligoprobes	Probe-Sets
DFC1	≥ 2	≥ 1.5	–		3360	3132
DFC2	≥ 2	≥ 1.5	> 93.04		5	5

Abbreviations. VP: volcano plot, TVP: twin volcano plot, DFC: dual fold-change analysis, FCP: the cut-off of parent fold-change, FCF_2: the cut-off of F_2 fold-change, TH: the genomic DNA hybridisation intensity threshold, MA-value: binary average fold-change ratio, FCD-value: fold-change difference that is the square-root-transformation of the D-value.

[a]Benjamini-Hochberg adjusted P-values were calculated for multiple testing correction.

[b]The mask of multiple chips was applied. A technique where each signal is extracted from the minimal intensity of four gDNA chips in the single trait experiment to create a pseudo array that will be analysed under the ATM framework.

[c]Fukuyama-Sugeno's index was used to generate ATM-suggested gDNA hybridisation intensity threshold.

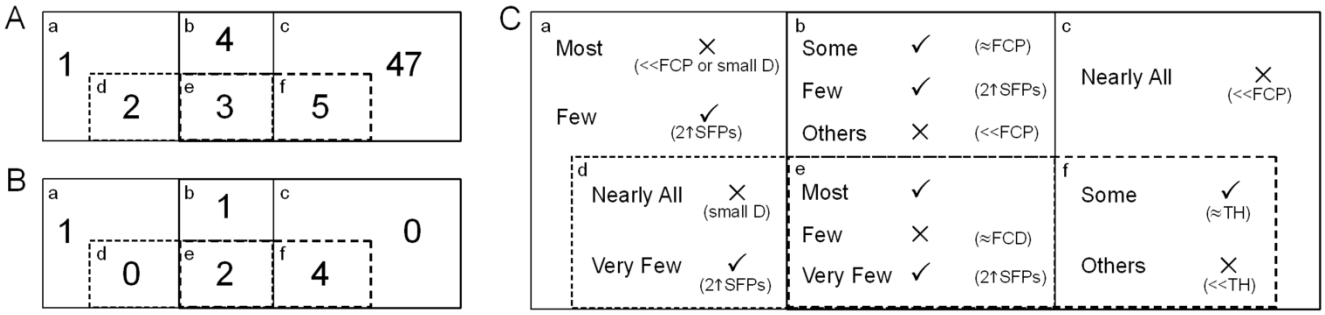
[d]SFPs were examined on the Perfect Match probe datasets in all cases.

[e]10% trimmed mean, $\gamma = 0.1$, of intensity difference was used.

Among the ten instances, VP4, TVP2, TVP4 and DFC2 were selected to acquire more dependable SFPs through Euler diagram analysis. Since TVP2 (*bcef*) and VP4 (*abde*) were proper supersets of

TVP4 (*ef*) and DFC2 (*de*) respectively, we could have a simplified version of the 4 unit diagram (Fig. 4). As seen in Table 2, DFC takes advantage of the hard cut-off values of FCP and genomic DNA hybridisation intensity and this nature has a limitation – it may cause possible oligonucleotides to be omitted where they detect repetitive elements within the genome of an investigated species. The set constructed by subtracting DFC2 from VP4 would have potential to overcome the limitation of DFC. So would the difference of TVP2 and TVP4. The intersection of four units, *e*, is a focus from which the most probable candidates would be found. In the example, 3 suspected oligoprobes were found in this intersection (Fig. 4A) but one of them was not considered as a potential SFP since its square root intensity difference was not much greater than the FCD cut-off (data not shown). An area where the overlap between VP4 and TVP2 excludes DFC2 is another focus. The elements of the area have possibilities that their parental fold-changes approach the cut-off value and signal intensities are not at the low end of the range. To take 258467_at_680_81 as an example, its parental fold-change was 1.96 (564/288), and hybridisation was clear and the ratio was very near to the cut-off of 2. There were 2 and 5 oligoprobes discovered in the sets of *d* and *f* (Fig. 4A), respectively, and both *d* and *f* were associated with FCP & FCF_2. Of the two possibilities for SFPs, the latter seemed more likely.

Figure 4. Euler Diagram Analysis. This was an example to show how potential SFPs can be selected by the POST and the DFC using Pigeons. The four-set diagram was established according toVP4 (*abde)*, DFC2 (*de*), TVP2 (*bcef*) and TVP4 (*ef*) illustrated in Table 2, where lowercase letters stand for the portions of the four filtering methods. (**A**) SFP Predecessors. Numbers in the partitions indicate the number of detected oligoprobes that can be recognised as potential SFPs. (**B**) Final Candidates. After careful selection and consideration portion by portion, potentially differentially hybridised oligonucleotides could be determined. They were *e*:264674_at_473_177, 257321_at_566_65; *b*:258467_at_680_81; *f*:244964_at_665_15, 255530_at_691_371, 257050_at_8_423 and 266293_at_656_319; *a*:265228_s_at_195_89. (**C**) Optimal strategy for potential SFP selection. Where ✓: candidates, ×: elimination, ≈FCP: the parental fold-change value is just a bit smaller than the cut-off, ≪FCP: the parental fold-change value is very much smaller than the cut-off, small D: tiny intensity difference, ≈FCD: the fold-change difference value is not much greater than the cut-off. ≪TH: poor hybridisation, ≈TH: the signal intensity is a little smaller than the value of gDNA hybridisation intensity threshold, 2↑SFPs: there are more than or equal to two potential SFPs found in a probe-set.



Although the discoveries of the former exceeded the ATM's suggested threshold and the cut-off of two fold-change parameters, they did not have a large intensity difference (data not shown) so should probably not be selected as candidates. On the other hand the partition *f* has a chance to find large

FCD-values with signal intensities slightly smaller than the gDNA hybridisation intensity threshold of the ATM suggestion. Out of the 5 filtered entities, there was only one having very poor hybridisation (42 vs. 93.04), and this was discarded. In a word, the partition built by deducting the intersection of the four units from TVP4 is able to complement another constraint of DFC – the hard cut-off value of gDNA hybridisation intensity. When it comes to the area where TVP2 excludes VP4 & TVP4, there were 47 candidates, the largest number in the Euler diagram, detected as statistically significant variable oligoprobes (Fig. 4A). But unfortunately, we did not consider any of these as potential SFPs. The reason is that nearly all elements of this set have a much smaller parental fold-change than the given cut-off. Similarly, most discovered probes in the portion where VP4 excludes TVP2 & DFC2 have either small intensity differences or small parental fold-change. In the analysis, there was one probe, 265228_s_at_195_89, belonging to this type of set and we regarded it as a candidate because of its strong hybridisation and reasonable parental ratio of FC (1822/962). The Euler diagram was then updated to show the situation of retained candidates in the units (Fig. 4B). Eventually, the meaningful selection enables us to produce a final list of potential SFPs for further validation *in vitro*. Through the small-size demonstration, an optimal strategy based on the Euler diagram for the selection of differentially hybridised oligonucleotides using POST and DFC can be summarised (Fig. 4C). Using this strategy, researchers could facilitate the organisation of the final list. Firstly, we suggest neglecting the subsets *c* and *d* and picking the elements of the intersection of four-set Euler diagram *e*. Next, the two buffers, *b* and *f*, need to be thoroughly examined as to whether there are any elements whose parental fold-change (for *b*) and signal intensities (for *f*) approximate to the predefined cut-off values, respectively, to find statistically significant variable oligoprobes. Finally, partition *a* should be checked to see if those signals which have strong hybridisation as well as a parental fold-change approaching the cut-off are there. In addition, we may have some opportunity to identify a probe-set having potentially differentially hybridised oligoprobes more than or equal to two in this partition. Ideally, a probe-set with the tendency of multiple SFPs ought to be detected in the intersection of TVP and VP if the trimmed mean percentage γ can be carefully chosen. In our example, γ=0.1 was used, implying the detection of two SFPs in the same set, and we did not discover any probe-sets with the observable property. Making the most of VP, TVP and DFC, it is believed that the capability for recognition of differentially hybridised oligonucleotides with respect to the phenotypic region in a non-model species could be increased.

4. Conclusions

Oligonucleotide microarrays have been verified as a powerful high-throughput technology to study plant genomics and transcriptomics. While most biochips are designed for model and major species investigation, there is limited availability of designed microarray platforms for the study of minor crop species that might currently be important food sources in some countries and have potential for future food production more widely. With the advent of the high density oligonucleotide arrays, Xspecies can be used to investigate the transcriptomes of underutilised plants. We have developed several computational algorithms and statistical methods to accompany this oligonucleotide probe-based cross-species platform for the analyses of oligoprobe selection/parsing and for finding potential SFP in minor crop species. These methods have been packaged in a computer program, named Pigeons, and focused around visualization and interactive studies of the datasets at the probe level. A number of case

studies and an illustration of the analysis of an underutilised crop dataset using Pigeons have also been performed to show the effectiveness and the usefulness of the proposed methods.

Acknowledgments

The authors would like to acknowledge Zoe Philips for hybridization of the Affymetrix Arrays.

Conflict of Interest

The authors declare no conflict of interest.

References and Notes

1. Wang, J. Computational biology of genome expression and regulation - a review of microarray bioinformatics. *J. Environ. Pathol. Toxicol. Oncol.* **2008**, *27*, 157-179.
2. Kumar, R.M. The widely used diagnostics "DNA microarrays" - a review. *American Journal of Infectious Diseases* **2009**, *5*, 214–225.
3. Hammond, J.P.; Broadley, M.R.; Craigon, D.J.; Higgins, J.; Emmerson, Z.F.; Townsend, H.J.; White, P.J.; May, S.T. Using genomic DNA-based probe-selection to improve the sensitivity of high-density oligonucleotide arrays when applied to heterologous species. *Plant Methods* **2005**, *1*, 10.
4. Hammond, J.P.; Bowen, H.C.; White, P.J.; Mills, V.; Pyke, K.A.; Baker, A.J.; Whiting, S.N.; May, S.T.; Broadley, M.R. A comparison of the Thlaspi caerulescens and Thlaspi arvense shoot transcriptomes. *New Phytologist* **2006**, *170*, 239-260.
5. Graham, N.S.; Broadley, M.R.; Hammond, J.P.; White, P.J.; May, S.T. Optimising the analysis of transcript data using high density oligonucleotide arrays and genomic DNA-based probe selection. *BMC Genomics* **2007**, *8*, 344.
6. Broadley, M.R.; White, P.J.; Hammond, J.P.; Graham, N.S.; Bowen, H.C.; Emmerson, Z.F.; Fray, R.G.; Iannetta, P.P.M.; McNicol, J.W.; May, S.T. Evidence of neutral transcriptome evolution in plants. *New Phytologist* **2008**, *180*, 587–593.
7. Davey, M.W.; Graham, N.S.; Vanholme, B.; Swennen, R.; May, S.T.; Keulemans, J. Heterologous oligonucleotide microarrays for transcriptomics in a non-model species; a proof-of-concept study of drought stress in Musa. *BMC Genomics* **2009**, *10*, 436.
8. Kreyszig, E. *Advanced Engineering Mathematics*, 10th ed.; John Wiley & Sons: New Jersey, USA, 2011; pp. 361–367, pp. 790–842.
9. Xu, R.; Wunsch II, D. Survey of Clustering Algorithms. *IEEE Transactions on Neural Networks* **2005**, *16*, 645-678.
10. Schena, M.; Shalon, D.; Heller, R.; Chai, A.; Brown, P.O.; Davis, R.W. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc. Natl Acad. Sci.* **1996**, *93*, 10614–10619.
11. Cui, X.; Churchill, G.A. Statistical tests for differential expression in cDNA microarray experiments. *Genome Biology* **2003**, *4*, 210.
12. Kooperberg, C.; Aragaki, A.; Strand, A.D.; Olson, J.M. Significance testing for small microarray experiments. *Stat. Med.* **2005**, *24*, 2281–2298.

13. Bezdek, J. *Pattern Recognition with Fuzzy Objective Function Algorithms*, 1st ed.; Plenum Press: New York, USA, 1981; pp. 15–42, pp. 95–154.
14. Jeffery, I.B.; Higgins, D.G.; Culhane, A.C. Comparison and evaluation of methods for generating differentially expressed gene lists from microarray data. *BMC Bioinformatics* **2006**, *7*, 359.
15. Dudoit, S.; Yang, Y.H.; Callow, M.J.; Speed, T.P. Statistical methods for identifying genes with differential expression in replicated cDNA microarray experiments. *Stat. Sin.* **2002**, *12*, 111-139.
16. Irizarry, R.A.; Hobbs, B.; Collin, F.; Beazer-Barclay, Y.D.; Antonellis, K.J.; Scherf, U.; Speed, T.P. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **2003**, *4*, 249–264.
17. Bolstad, B.M.; Irizarry, R.A.; Astrand, M.; Speed, T.P. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **2003**, *19*, 185–193.
18. Tukey, J.W.; McLaughlin, D.H. Less vulnerable confidence and significance procedures for location based on a single sample: Trimming/Winsorization 1. *Sankhya A* **1963**, *25*, 331-352.
19. Patel, K.R.; Mudholkar, G.S.; Fernando, J.L.I. Student's t approximations for three simple robust estimators. *Journal of the American Statistical Association* **1988**, *83*, 1203–1210.
20. Graham, N.S.; Clutterbuck, A.L.; James, N.; Lea, R.G.; Mobasheri, A.; Broadley, M.R.; May, S.T. Equine transcriptome quantification using human GeneChip arrays can be improved using genomic DNA hybridisation and probe selection *The Veterinary Journal* **2010**, *186*, 323-327.
21. Graham, N.S.; May, S.T.; Daniel, Z.C.T.R.; Emmerson, Z.F.; Brameld, J.M.; Parr, T. Use of the Affymetrix Human GeneChip array and genomic DNA hybridisation probe selection to study ovine transcriptomes. *animal* **2011**, *5*, 861-866.
22. Fukuyama, Y.; Sugeno, M. A new method of choosing the number of clusters for the fuzzy c-mean method. *Proc. 5th Fuzzy Syst. Symp.* **1989**, 247–250.
23. Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society B* **1995**, *57*, 289–300.