Fractionation and subfunctionalization following genome duplications: mechanisms that drive gene content and their consequences

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Abstract

A gene's duplication relaxes selection. Loss of duplicate, low-function DNA (fractionation) sometimes follows, mostly by deletion in plants, but mostly via the pseudogene pathway in fish and other clades with smaller population sizes. Subfunctionalization—the founding term of the Xfunctionalization lexicon—while not the general cause of differences in duplicate gene retention, becomes primary as the number of a gene's cis –regulatory sites increases. Balanced gene drive explains retention for the average gene. Both maintenance-of-balance and subfunctionalization *drive* gene content nonrandomly, and currently fall outside of our accepted Theory of Evolution. The "typical" mutation encountered by a gene duplicate is not a neutral loss-of-function; dominant mutations (Muller's lexicon; these are not neutral) abound, and confound Xfunctionalization terms like "neofunctionalization". Confusion of words may cause confusion of thought.

As with many plants, fish tetraploidies provide a higher throughput surrogate-genetic method to infer function from human and other vertebrate ENCODE-like regulatory sites.

Keywords. Whole genome duplication, fractionation, dominant mutation, genome dominance, Gene Balance Hypothesis, subfunctionalization, The Theory of Evolution.

No bullet points. Quote from Conclusion:

"Not only have studies on polyploid fractionation led to reconsiderations of fundamental evolutionary theory, but fractionation in polyploids permits higher-throughput comparative genomic experiments using ENCODE-like data yielding the logical precision expected of genetic analyses."

INTRDODUCTION AND BACKGROUND

We feature, with exceptions, results published from mid-2012 to mid-2015.

*Definitions: distinguishing fractionation and diploidization. "*Fractionation" was coined [1] to differentiate between two different processes that follow whole genome duplications (WGDs): fractionation (mutational loss-of-function of one or the other, but not both, of the newly duplicated genes) and diploidization (adaptations facilitating accurate meiotic segregation). The Salse lab[2] has found, in the grasses, that the phenomenon of genome dominance associated with ancient allopolyploids[3,4] not only influences the sub-genome equivalence of fractionation, but also influences which chromosomes are preferentially rearranged as a part of diploidization. Even so, fractionation is not diploidization. Figure 1 illustrates fractionation of genes, and also fractionation of conserved *cis*-acting elements.

Definitions: Clarifying "subfunctionalization," "neofunctionalization" and similar terms in the Xfunctionalization lexicon. The idea "subfunctionalization" (see Table 1, Row 4 and cartoon inset of Figure 1) was originally called "duplication, degeneration, complementation" by Force and coworkers [5] to explain why too many genes were retained as homeologous pairs after the boney fish tetraploidy. After subfunctionalization, it takes the pair to express the ancestral function at all the right times and places [5-7].

"Subfunctionalization" is one of the customized terms (the "Xfunctionalization" vocabulary [8]) describing the mutational fate of gene duplicates, given that mutants are (generally) neutral because, presumably, the wild-type gene of the pair covers the recessive phenotype [9]. Connant and Wolfe [10] accurately defined "subfunctionalized" and "neofunctionalized" gene homeologs containing a mutant by using examples. However, more generalized treatments were more complete, less accurate, and sometimes confusing from the perspective of a mutant's mechanism of action. For example, Innan and Kondrashov's lexicon [8] describes how either purifying or positive selection might treat each paired gene arrangement of "loss-of-function mutations". Table 1, Row 4, Column 5, compares cartoons of subfunctionalization with nonfunctionalization; "-" is neutral loss-of-function. When mutants are recessive, and neutral or nearly so, the Xfunctionalization terminology is useful.

Mutations precede selection. In 1932, H.J. Muller [11] categorized dominant mutants (Table 1, Column 2) based on how *morphological phenotypes* were affected in a background with alternative dosages of segments carrying the wild-type allele. Dominant mutants are not expected to be neutral . Muller's terminology can also be usefully applied at the level of RNA abundance and distribution: *expression phenotypes*. Deletion of a silencer *cis* site or obstructing looping of an enhancer onto its gene's proximal promoter are two ways to model a hypermorph (over-expression; Table 1, Row 4; Fig. 2A and 2D). Were there an associated hypermorph mutant phenotype, it would be dominant, not neutral. Applying the Xfunctionalization vocabulary to dominant mutants is problematical.

For example, it seems reasonable that a "neofunctionalization" requires a "neomorph" dominant mutant (Table 1, Row 8). Neomorphs (one type of dominant mutant) are something new, and are thus not responsive to any number of wild-type alleles. Eichenlaub and Ettwiller [12] studied the result of the massive (75%) fractionation of duplicates following the teleost fish lineage tetraploidy, and found rare neomorphs produced by a neofunctionalization-type fractionation. In these rare cases, one homeolog was deleted, leaving only a few fragments of exon *in situ*. These retained, conserved sequences had enhancer activity, but it was shown that the original exon DNA did not. Thus, this type of fractionation generated a new enhancer *for a neighboring gene*(s), a variation on the classical Lewis scheme[9] (duplication, repression of one gene which accumulates mutants, derepression, and a chance for a rare neomorph). Recent work [13] following expression of maize homeologs in vegetative leaf blades versus husk sheaths (these being different parts of leaves specialized for photosynthesis and kernel protection, respectively) reported that a remarkable 13% of the pairs were "regulatorily neofunctionalized" and that such pairs were necessarily expressed to different levels. Since dominant hypermorphs (Table 1, Row 4) are expected to be far, far more common than neomorphs, we think that the authors, problematically, used the word "neofunctionalization" in a way that does not demand that anything new has evolved, and called pairs with a dominant over-producer mutants "neofunctionalized". These dominant expression mutations are likely something expected, each like a deletion of an element with a negative function (like a silencer).

Fractionation in fish and plants. Teleost fish, with their lineage-specific tetraploidy, may mutate their *cis*-elements faster than non-polyploid control lineages [14]; we know of no other data on special polyploid mutational mechanisms. The fractionation mechanism is known for maize[15] and *Brassica rapa* [16] genes, and for *Brassica cis*-acting sites and G-box motifs [17]: deletion between short tandem repeats caused by intrachromatid recombination, *not* the pseudogene pathway. While pseudogenes do appear in specific regions of plant genomes [18,19], these broken reading frames were not found at fractionated loci in those plants studied , and plant pseudogenes are not scattered about euchromatin as they are in humans [20]. Many plant WGDs happened 10-80 million years ago[21]. The salmonoid-lineage tetraploidy seen within the rainbow trout genome[22] is the first characterized vertebrate ancient polyploid comparable in time to those in plants. [It should be noted that the trout lineage tetraploidy is thought to be *auto*tetraploid while maize and *Brassica* lineages—those plants studied for fractionation mechanism-- are both *allo*tetraploid.] Trout homeologs have a modal Ks (calculated synonymous nucleotide substitution rate) of 20%, comparable to *Brassica rapa* and maize homeologs, which have a modal Ks of approximately 15%. However, in contrast to these plant genomes, trout fractionation generated many pseudogenes at about the same frequency as totally or partially deleted genes. Assuming that this one autotetraploid reflects "fish", and the two allopolyploids reflect "plants", one simple explanation is that the avidity of the mutational mechanisms underlying purifying selection have adapted to fit effective population sizes, and that, because of pollen, is very large for plants[23] (and this may affect the way plants exhibit "binding site turnover" [24] and see this citation's annotation).

EVOLUTIONARY CONSEQUENCES OF FRACTIONATION

Fractionation is biased because of genome dominance exhibited in ancient allotetraploid genomes. For most ancient plant tetraploidies studied [25], the subgenomes differ in number of ancestral genes surviving fractionation [26-28]. Those sequenced genomes that do display biased fractionation also display genome dominance, where genes on the subgenome that is most intact also tend to express to higher mRNA levels [3], as first documented in maize [29]. This phenomenon is easily visible in two-gene RNAseq FPKM plots of maize homeologs, using datasets from many different cells/tissues/organs/organ components or inductive conditions (Fig. 2). *Gene dominance* is when the genes on one subgenome in a tetraploid tend to express to higher levels than do the gene on the homeologous subgenome; exceptions abound. (Gene dominance, see dotted lines of Fig. 2, when accumulated for all homeologs, constitutes the argument for the trend of *genome dominance*.) Recent analyses on several genomes with polyploidies generalized the link between biased fractionation and genome dominance for plants; it also established a link between unbiased gene fractionation and genome equivalence, as exhibited by the most recent tetraploidy in the banana lineage [3]. Garsmeur, Schnable and coworkers hypothesized, but did not prove, that the difference was allopolyploidy versus autopolyploidy. The Wendel laboratory found that a cotton polyploid occurring 60 million years ago still displays genome dominance [4]. While the Freeling-Wang collaboration on *Brassica* found siRNA coverage—coverage of transposons near genes-- to preferentially mark the not-dominant subgenome with little regard for homeolog expression ratio [30], the Wendel laboratory's data on cotton similarly implicated siRNA, but did not find transposon involvement [4]. Our working hypothesis: the mechanism by which siRNAs lead to the down regulation of nearby genes, as suggested by Hollister and Gaut [31], is "spreading " RNA-dependent DNA methylation and its reinforcing marks, as recently reviewed [32]. Such position effects have been used to hypothesize function for bulk junk DNA and to solve, hypothetically, the C-value paradox [33].

Genes in different GO categories show differing resistances to fractionation following duplication. Reviews document that genes tend to be retained as pairs following a WGD if they encode transcription factors, ribosomal proteins, proteasome core proteins, components of interactive machines or networks, regulatory proteins, signal transducers and similar [34-36] or are associated with many conserved noncoding sequences (CNSs) [37]. Highly expressed genes also tend to be retained, but this is more the case in *Paramecium* than in plants [38]. In Arabidopsis, genes in GO categories retained as tandem duplications at a high frequency are retained as post-WGD pairs at a low frequency, and *vice versa* [36]; this inverse relationship suggested strongly that the *general* reason for gene fractionation resistance was *not* subfunctionalization. This inverse relationship was predicted by the Gene Balance Hypothesis [39,40]. Data from other eukaryotes supports The Gene Balance Hypothesis (reviews[34,41-43]). Gene content of the recently sequenced genomes of trout [22] and *Brassica rapa* [44] are reported to conform to gene-balance expectations. Genes encoding transcription factors, especially "response to" functions, tend to be CNS-rich [45], indicating an abundance of conserved, potentially *cis*-regulatory information. There are at least two explanations for transcription factor gene retention post-WGD: 1) their products participate in protein-protein-DNA complexes [37] *and 2) the genes themselves present long "promoter" targets for subfunctionalization*. Of the 1224 *B. rapa* homeolog pairs with exactly two CNSs, only 9.2% are subfunctionalized at the DNA sequence level, but this increases to a high of 87% for those doublets with 21-61 CNSs [46]. The 5' region of one of these cis-complicated gene doublets is in the inset of Figure 2. Chettoor and coworkers [47] found that genes expressed in maize pollen were significantly resistant to fractionation, perhaps because of increased purifying selection on haploid gametophytes (preferentially applicable to outcrossing species). Data from human tandem duplications indicates that balance in the absence of subfunctionalization may be an important mechanism for retention [48].

Conversely, Duarte and coworkers [49] found that some plant gene families were preferentially fractionated down to singletons. de Smet and coworkers [50] found that many ancestral genes in 20 diverse plants remained mostly single copy (about 2000) or always single copy (a few hundred) even after several rounds of tetraploidies. Overrepresented categories of singleton genes are in DNA metabolism, replication, recombination and DNA repair; one of de Smet and coworkers suggestions was that singletons avoid dominant (like antimorphic) mutations that might disrupt wild type gene function. (Our "dominant mutation" suggestion: cellular processes requiring RNA-DNA or DNA-DNA "loops" [51] with a heteroduplex component might be selected to avoid mismatches). Post-polyploidy changes in gene content in the small genome of the bladderwort, *Utricularia gibba*, also favor singletons [52]; the authors suggest, but do not prove, that the fractionation mechanism is particularly avid in this C-value-decreasing lineage.

Balanced Gene Drive and Subfunctionalization both drive evolution in nonrandom directions. The word "drive" is used here rigorously, as in "meiotic drive" [53,54]. Each WGD *drives* genes with interactive products into the "population"[36] and subfunctionalization *drives* genes with many cis-sites into the "retained" category as well. WGDs—gross sorts of mutations-- cause these drives. The direction of these drives is toward regulatory complexity and redundancy. The rise in morphological complexity (but not other complexities) in green plants has been explained based on balanced gene drive and duplicate gene networks [55], but this hypothesis has not been tested. We hope this bit of mutationist (not selectionist) theory is inoffensive. Note that there is no desire to "drive" to any particular place; there is no "adaptionist paradigm" here. Goldschmidt would probably have called a WGD a "systemic mutation", and argued that the behavior of different sorts of mutations-- not only recombination, selection and population size--, must have a place in a useful theory of evolution. Goldschmidt's 1952 essay is a must-read [56]. Our reigning theory, called the "Modern Evolutionary Synthesis" of the late 1940s, (T. Dobzahnski and several others) disrespects mutation as an evolutionary force.

Fractionation drives last for tens of millions of years, but perhaps not forever. Schnable and coworkers [57] using plant data, and Gout and Lynch [58] using *Paramecium* and yeast data, support similar conclusions: as homeologs increasingly express themselves to different levels, eventually the less expressed homeolog will be lost; this mitigates *balanced gene drive. Subfunctionalization drive* to accumulate genes with complex promoters may be more difficult to mitigate.

Muller's Lexicon [11]				Innan and Kondrashov's [8] Lexicon	
Dominant/ Recessive	Mutant allele (term)	Molecular behavior (example)	Phenotype selection sees (example)	2 essential *cis* sites on duplicate genes	term
D	Wild-type	Specifies product	Wild-type, duplication relaxes selection	+ + + +	"afunctionalization"
R	Both homeologous *cis* elements knockout	Zero product from either gene in target cell	Assume negative, "death"	+ - + -	No term
R	Single knockout, deletion	Zero product from one mutant gene of pair	Possibly none; mutants could be neutral because selection relaxed	+ + + -	Nonfunctionalization
R	Two knockout, deletions	Two *cis* knockouts in each pair	Possibly none, as above	+ + - - - + + -	Nonfunctionalization arrangement Subfunctionalization arrangement
D	Hypermorph	(Deletion of a suppressor); over-producer. (Blocks looping).	"Triplo-insufficient" and negative OR none OR looks like a gain of function but is not.	+ +^{UP} + +	Nonfunctionalization (Nothing "new" here, just "more")
D	Hypomorph	Too little product; Under-producer. (Blocks looping.)	"Haplo-insufficient" and negative, OR none.	+ +^{DOWN} + +	Nonfunctionalization (nothing new here)
D	Antimorph	Product stops function in *trans*	(Antisense RNA, misfolded protein gums up works), Likely negative.	+ + + + m	No term. Could be something new here.
D	Neomorph	New DNA info; (suspect transposons)	Gain-of-function, negative, cis or coding; potential for positive selection	+ + M + +	Neofunctionalization (definitely something new here)
D	Knockout plus hypermorph (an example)	1 gene of the pair with two *cis* mutants in it	Like hypermorph	- +^{UP} + +	Nonfunctionalization, but "molecular subfunctionalization".
D	Mutant in *trans*	Both homeologs up/down regulated	Possible neomorph in a gene regulating the homeologs being studied	+ + + +	No term. Not likely to be two *cis* mutants

Table 1. Muller's classes of mutants on the same page as Innan and Kondrashov' Xfunctionalization terms for mutant arrangements in a gene duplicate with two essential 5' cis-acting sites. + is a wild-type site; - is a site loss-of-function. The arrows in the antimorph row (Row 7) indicate that the product (m) from the mutant gene down-regulates itself and its homeolog (in *trans*). "M" in Row 8 is new information, like a transposon, inserted into the promoter. It is probably best to use the molecular (e.g. "over-producer") rather than Muller's (e.g. "hypermorphic") term when there is no morphological/physiological mutant phenotype. To understand the behavior of any one pair of homeologs (Fig. 2) requires at least one outgroup control and is often an intellectual challenge that cannot be approached using the Xfunctionalization lexicon.

USING FRACTIONATION AS A GENETICAL TOOL TO ACHIEVE PRECISION IN COMPARATIVE GENOMIC EXPERIMENTS

Fractionation analyses can bring inferred, and sometimes proved, function to otherwise pure associations among ENCODE [59] -like features (e.g. DNase Hypersensitive Sites with footprints, DHSs, [60], or DNA protein binding sites via ChIP-seq). Occupied chromatin does not equal function [61-64]. If an ENCODE-like feature existed in the Arabidopsis segment of Figure 2—say a DHS with one protected motif footprint -- and this footprint was fractionated *along with a specific expression character* in a homeologous pair, then that ENCODE-like signature of function is now inferred to have actually functioned as part of a gene.

CNSs, because they are unexpectedly conserved, correlate with past function, but not necessarily in *cis* on the nearest gene. The upper panel of Figure 1 demonstrates how a fractionation pattern (here in a *Brassica*) sometimes allows researchers to infer which gene is the target of any particular CNS activity. For example, since the 5 CNSs located around *At GeneX* (Fig. 1) are deleted in *Brassica* when an ortholog is deleted, they are inferred to act as part of *GeneX*. There are dozens of CNSs spread between Arabidopsis genes *Y* and *Z* in Figure 1. The fractionation pattern in this *Brassica* suggests that none of them act in *GeneY* since its ortholog has been deleted in this *Brassica* and the CNSs remain; *GeneZ* is inferred to include all 47 CNSs. To strengthen specific inferences, experiments on gene fractionation patterns in additional *Brassica* and related radish species – there are several sequenced genomes and all carry the same hexaploidy—can test expectations.

The outcomes of fractionation can be used as part of an analytical method to predict CNS function (‘fractionation mutagenesis’) [20]. Use of this technique requires—at minimum-- a sequenced polyploid with a sequenced outgroup that is not duplicated, each with RNAseq data from many comparable, specific biological endpoints. In plants, only inbred B73 maize (an ancient tetraploid) with a sorghum outgroup (not tetraploid) fits these criteria at this time. Figure 2 shows two-homeolog FPKM plots (from www.qTeller.com) where each point records both FPKMs from one individual RNAseq experiment (like the point for “microspore biological replicate 1); the FPKMs for “comparable” sorghum control endpoints are also indicated. For example, in Panel A, RNA levels in the microspore are off-the-line. Examination of the sorghum microspore data indicates that the maize gene on the x-axis is an over-producer (i.e., a hypermorphic mutant, as opposed to the maize gene on the y axis being an expression knock-down). Our observations indicate that, depending on the biological endpoint, over-producer mutations are approximately as common as under-producer/knockouts. The data in the other three panels, and the legends, support conclusions that are similarly genetic-like in their precision. After proofing FPKM data as reads aligned to the annotated chromosomal segment, examination of the actual DNA sequences resulting from fractionation mutagenesis can deliver candidate “enhancers” or “silencers” for further study.

CONCLUSIONS If there were a tetraploid mammal, it would be the star of the human ENCODE project. The fish tetraploidies could be used intelligently to analyze deeply conserved human ENCODE signatures-of-function.

Not only have studies on polyploid fractionation led to reconsiderations of fundamental evolutionary theory, but fractionation in polyploids permits higher-throughput comparative genomic experiments using ENCODE-like data yielding the logical precision expected of genetic analyses.

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TABLE FOOTNOTES AND FIGURE LEGENDS

Table 1. Muller's classes of mutants on the same page as Innan and Kondrashov' Xfunctionalization terms for mutant arrangements in a gene duplicate with two essential 5' cis-acting sites. + is a wild-type site; - is a site loss-of-function. The arrows in the antimorph row (Row 7) indicate that the product (m) from the mutant gene down-regulates itself and its homeolog (*in trans*). "**M**" in Row 8 is new information, like a transposon, inserted into the promoter. It is probably best to use the molecular (e.g. "over-producer") rather than Muller's (e.g. "hypermorphic") term when there is no morphological/physiological mutant phenotype. To understand the behavior of any one pair of homeologs (Fig. 2) requires an outgroup control and is often an intellectual challenge that cannot be approached using the Xfunctionalization lexicon.

Figure 1. The upper panel is a GEvo graphic (at www.genomevolution.org/coge/) using an eight gene segment of Arabidopsis (*At*) chromosome 4 as query in a blastn sequence comparison to two of its orthologous segments in *Brassica rapa* var. Chiifu (*Br*), the "LF" "dominant" segment and an MF "not dominant" segment. *At-Br*-LF blast HSPs are orange; *At-Br*-MF blast HSPs are brown. The purple rectangles are a compilation of three laboratories' Arabidopsis conserved noncoding sequences, called "VHS-merged" CNSs [51]. "No" means "No fractionation". The inset is a blowup of the indicated 5' region of a crucifer *At GeneZ* with 47 *At-Aethionema* CNSs. "-" indicates deletion, not point mutation[17], of a "+" CNS. (We must assume that, when a gene is deleted, its CNSs are deleted with it.)

Figure 2. Four example categories of mutations recognized during the practice of fractionation mutagenesis in maize plotting RNA levels (FPKM rendered and plotted at www.qTeller.com) of both maize homeologs (ancient tetraploid), with outgroup sorghum (not tetraploid) ortholog RNA-level data embedded in each panel when it exists.. A point is one experiment from the Small Reads Archive; sometimes labels have been condensed, but experiment may be regenerated at qTeller.com. The sorghum expression data is essential to understand the mutants. Slope x/y is *gene dominance*. Ovals enclose focal biologically similar or replicated data points. **A.** *GRMZM2G702426* expresses off-the-line in microspores (haploid male cells in the tetrad) because it is most likely a dominant microspore hypermorph. **B.** Subfunctionalization to extreme cell-within-tissue-component specificity**.** Since there is no sorghum data, it is impossible to know which gene has altered it expression specifically in the adaxial epidermis of the plastochron 7 (ca. 0.7 cm long) leaf primordium, but not in the epidermises of adjacent pre-sheath or pre-blade organ components; RNAseq reads from [65]. **C.** Since both homeologs are vastly over-expressed in pollen expression as compared to the sorghum, we infer a *trans* regulator. **D.** Apparent quantitative subfunctionalization of pollen and microspore expression. Given the sorghum data, this subfunctionalization is expression only, since it is specified by *two mutations in the one gene GRMZM2G134866* comprising an over-production (i.e. dominant hypermorph) in pollen and probably a knockdown of expression in microspores.

FIGURES

Figure 1.

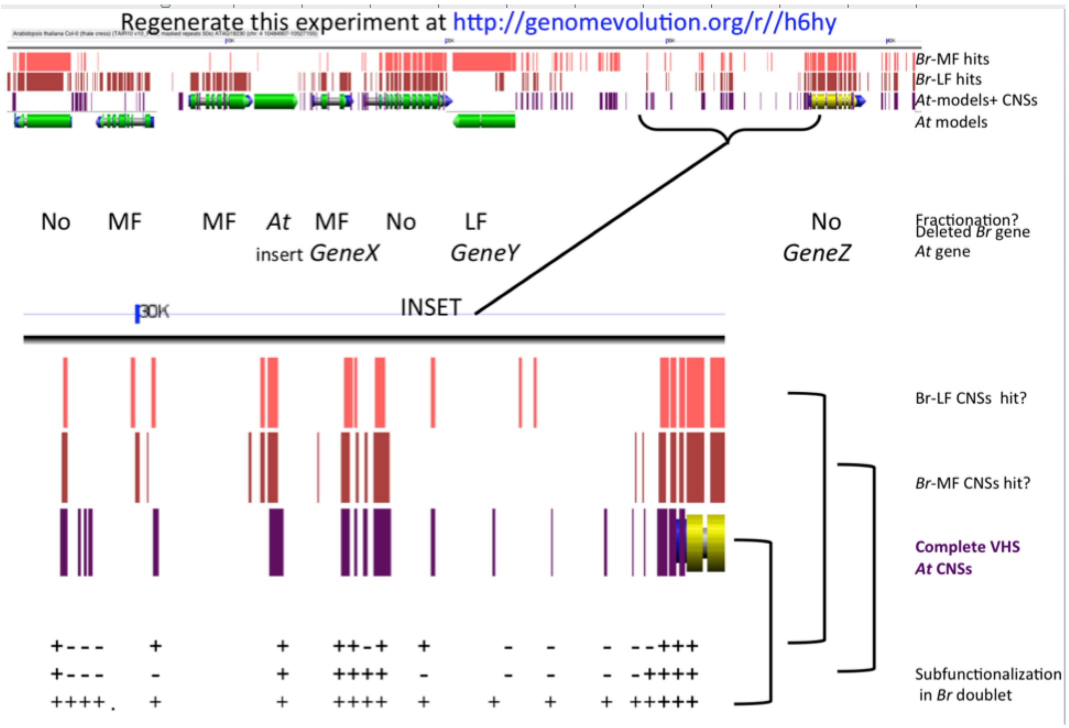
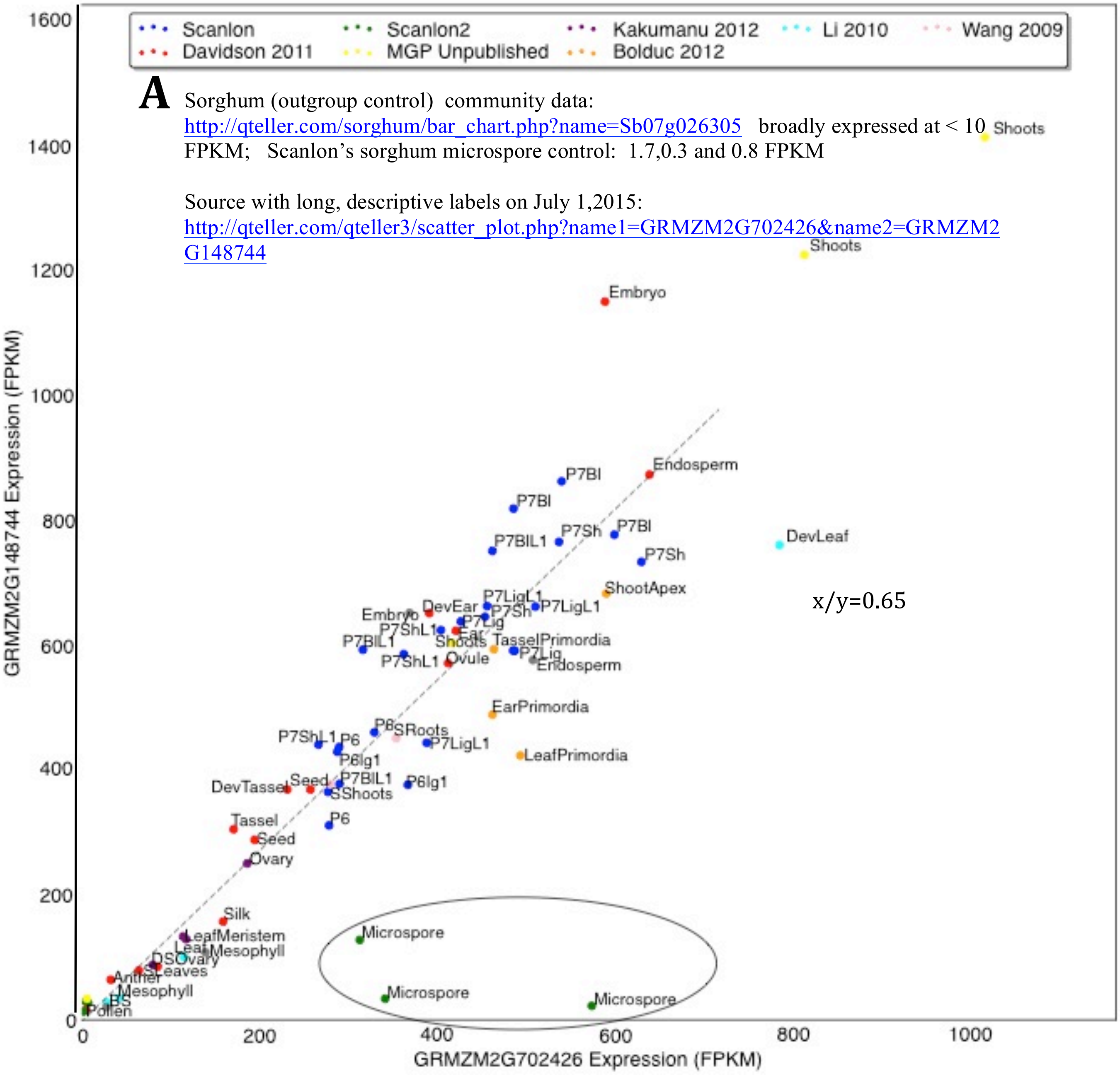


Figure 2 (original Word docs)

Fig.2 A-D on four Word pages. These graphics have the highest resolution.



Sorghum (outgroup control) community data: http://qteller.com/sorghum/bar_chart.php?name=Sb07g026305 broadly expressed at < 10 FPKM; Scanlon's sorghum microspore control: 1.7,0.3 and 0.8 FPKM

Source with long, descriptive labels on July 1,2015: http://qteller.com/qteller3/scatter_plot.php?name1=GRMZM2G702426&name2=GRMZM2G148744

x/y=0.65

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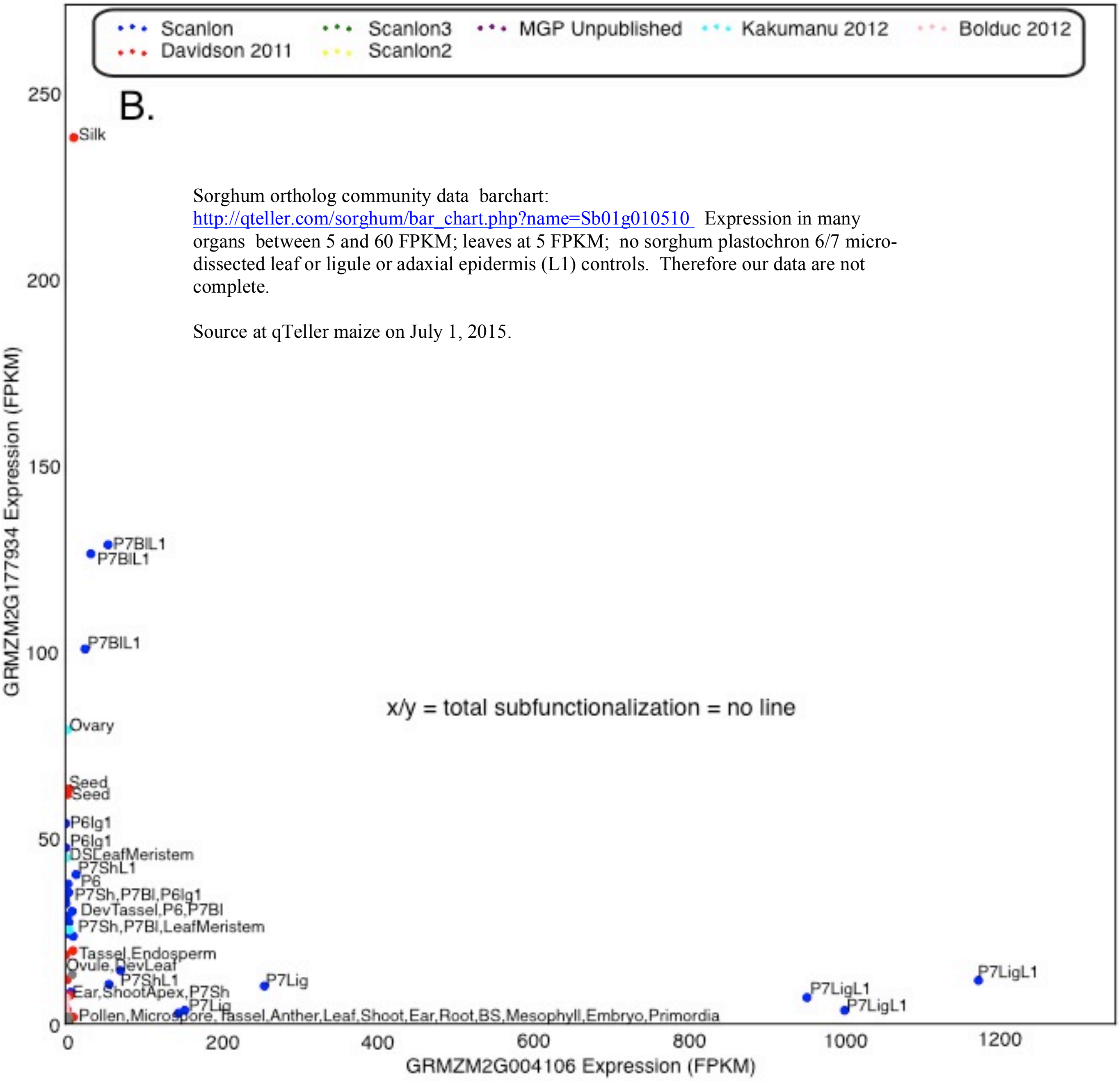
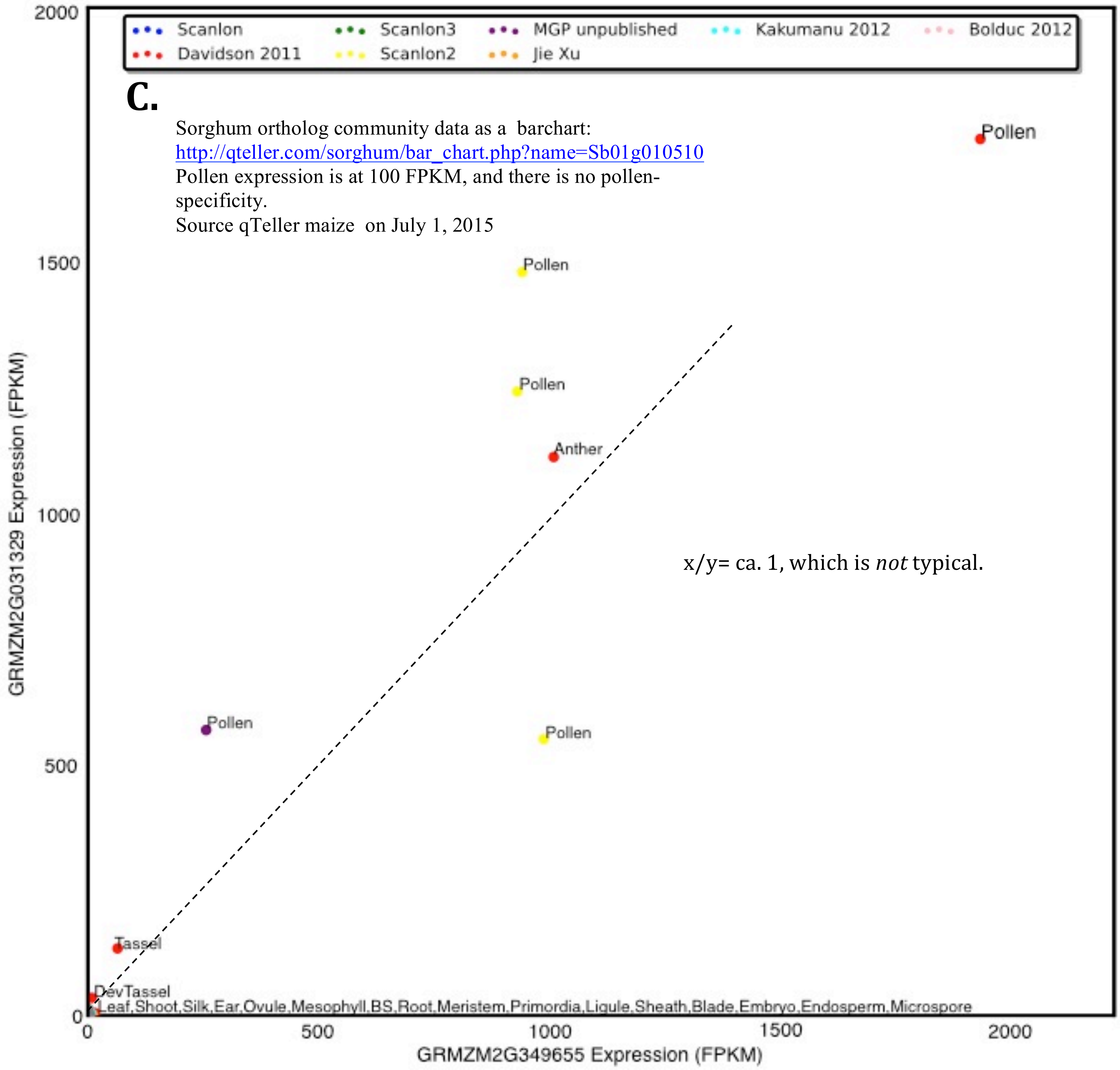
2B



Sorghum ortholog community data barchart: http://qteller.com/sorghum/bar_chart.php?name=Sb01g010510 Expression in many organs between 5 and 60 FPKM; leaves at 5 FPKM; no sorghum plastochron 6/7 micro-dissected leaf or ligule or adaxial epidermis (L1) controls. Therefore our data are not complete.

Source at qTeller maize on July 1, 2015.

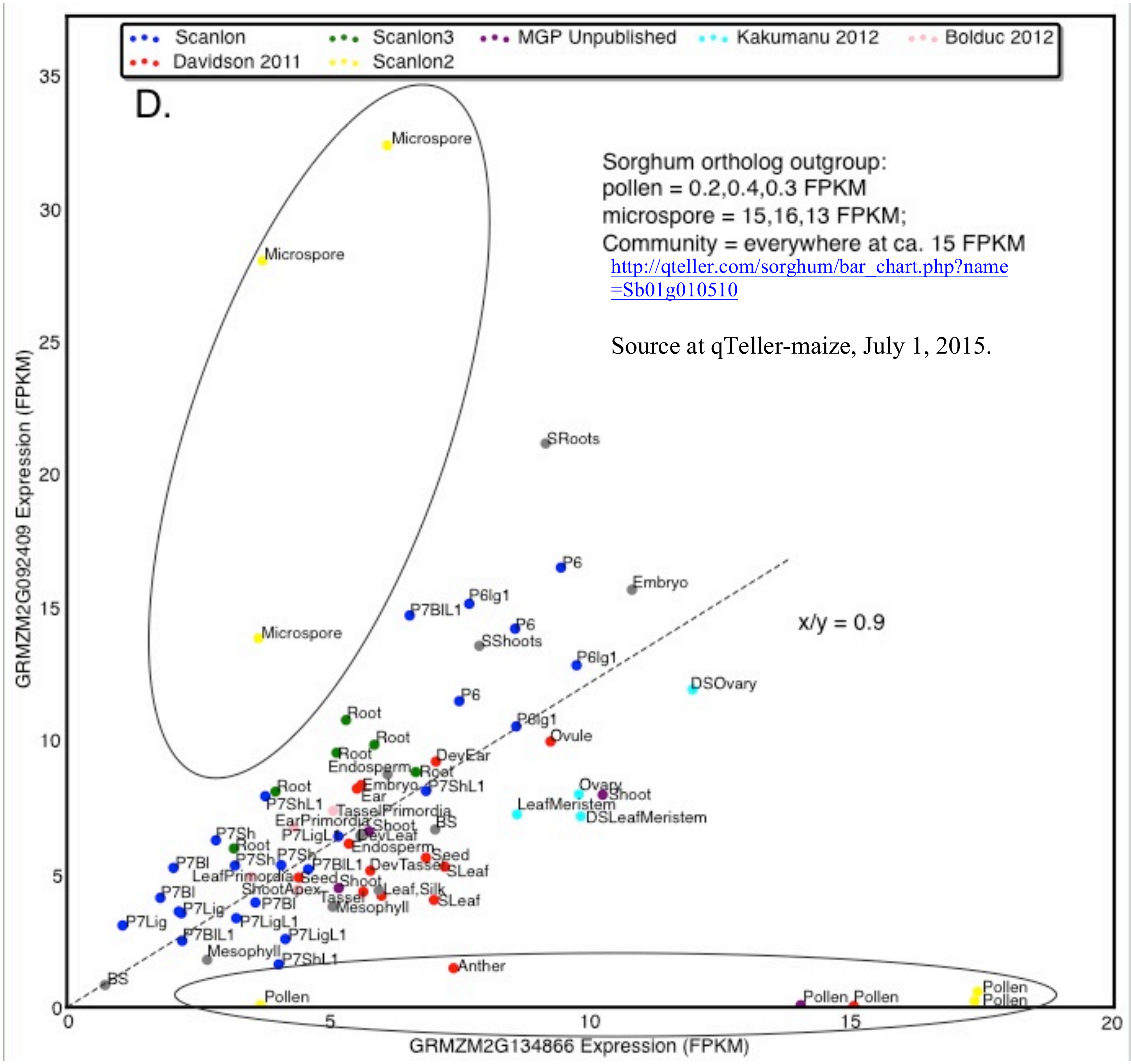
2C



Sorghum ortholog community data as a barchart:
http://qteller.com/sorghum/bar_chart.php?name=Sb01g010510
Pollen expression is at 100 FPKM, and there is no pollen-specificity.
Source qTeller maize on July 1, 2015

x/y= ca. 1, which is *not* typical.

2D



Sorghum ortholog outgroup:
pollen = 0.2,0.4,0.3 FPKM
microspore = 15,16,13 FPKM;
Community = everywhere at ca. 15 FPKM
http://qteller.com/sorghum/bar_chart.php?name=Sb01g010510

Source at qTeller-maize, July 1, 2015.

Stand alone Annotated Reference List for COGD 4.1 MF, JF MJS There are 65 refs.

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