# Whole genome phylogeny for 21 *Drosophila* species using predicted 2b-RAD fragments

Type IIB restriction endonucleases are site-specific endonucleases that cut both strands of double-stranded DNA upstream and downstream of their recognition sequences. These restriction enzymes have recognition sequences that are generally interrupted and range from 5-7 bases long. They produce DNA fragments which are uniformly small, ranging from 21-33 base pairs in length (without cohesive ends). The fragments are generated from throughout the entire length of a genomic DNA providing an excellent fractional representation of the genome. In this study we simulated restriction enzyme digestions on 21 sequenced genomes of various *Drosophila* species using the predicted targets of 16 Type IIB restriction enzymes to effectively produce a large and arbitrary selection of loci from these genomes. The fragments were then used to compare organisms and to calculate the distance between genomes in pair-wise combination by counting the number of shared fragments between the two genomes. Phylogenetic trees were then generated for each enzyme using this distance measure and the consensus was calculated. The consensus tree obtained agrees well with the currently accepted tree for the *Drosophila* species. We conclude that multi-locus sub-genomic representation combined with next generation sequencing, especially for individuals and species without previous genome characterization, can accelerate studies of comparative genomics and the building of accurate phylogenetic trees.

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## 1 Introduction

13 Evolutionary relationships of species derived by comparing single orthologous genes or groups of

14 genes can be negatively affected by potential horizontal gene transfers, incomplete lineage-

15 sorting, introgression, and the unrecognized comparison of paralogous genes (Delsuc et al. 2005).

16 However, with the advent of the genomic era, it is now possible for researchers to use the

17 complete genomes of fully sequenced organisms for building trees. Though such trees offer

18 robustness for analysis, it becomes impractical to use traditional methods for constructing large

19 scale alignments and for generating trees from these alignments, mainly because of their large

20 size and their highly heterogeneous nature. As a result, there are now sophisticated methods that

21 don't rely on alignment and are optimized for large scale data. These methods generally use

22 vector representation of genes (Qi et al. 2004; Stuart et al. 2002) or features such as gene content

23 (Huson & Steel 2004; Snel et al. 1999; Tekaia et al. 1999), gene order (Bourque & Pevzner 2002;

24 Korbel et al. 2002), intron positions (Roy & Gilbert 2005), or protein domain structure (Lin &

25 Gerstein 2000; Yang et al. 2005).

26 Despite a strong recent interest in the various large-scale non-alignment methods, they are often

27 viewed as somewhat less rigorous and less reliable. In addition, even with the dramatic decrease

28 in the cost of genome sequencing, it is still not attractive to sequence the genomes of those

29 organisms that have little economical value, especially if their genomes are extremely large. On

30 the other hand, the possibility of obtaining a large and representative set of fragments, instead of

31 the whole genome sequence, can be economically feasible even for the lesser known species and

32 can provide a valuable alternative for many types of genomic scale studies, including

33 phylogenomics.

34 Recently, several approaches have been developed to represent the genome by randomly

35 sampling the entire genome. These approaches give a good reduced representation of the genome

36 and are based on restriction sites on the genome combined with the next generation sequencing

37 methods. Some popular methods include Complexity Reduction of Polymorphic Sequences

38 (CRoPS) (van Orsouw et al. 2007); restriction site-associated DNA sequencing (RAD-seq) (Baird

39 et al. 2008a; Etter et al. 2011); Genotyping by Sequencing method (GBS); double-digest RAD-

40 seq (Peterson et al. 2012), and 2bRAD (Wang et al. 2012). All these methods provides good sub

41 samples from homologous locations within genomes and are widely used to study population

42 genetics (Baxter et al. 2011; Hohenlohe et al. 2010). These methods have the potential to uncover

43 detailed information about a wealth of genomic markers. Complex interactions among markers

44   can also be extracted at the population level ([Baird et al. 2008b](#); [Davey & Blaxter 2010](#)).

45   Recently, these fragments have also been used for evolutionary studies ([Emerson et al. 2010](#);

46   [Rubin et al. 2012](#); [Yi & Jin 2013](#)).

47   A novel class of enzymes, known as Type IIB restriction endonucleases ([Roberts et al. 2003b](#)),

48   are site-specific endonucleases that cut both strands of double-stranded DNA upstream and

49   downstream of their recognition sequences. These restriction enzymes have recognition

50   sequences that are generally interrupted and range from 5-7 bases long. They produce DNA

51   fragments which are of uniform length, ranging from 21-33 base pairs in length (without cohesive

52   ends) ([Roberts et al. 2003a](#)). The fragments are generated from throughout the entire length of a

53   genomic DNA providing an excellent fractional representation of the genome. This method of

54   generating fragments using Type IIB enzymes is termed 2bRAD ([Wang et al. 2012](#)) and these

55   fragments have been used for various purposes including population studies, digital karyotyping

56   ([Stebbins 1950](#)), for pathogen identification by computational subtraction ([Tengs et al. 2004](#)) and

57   genomic profiling to identify and quantitatively analyze genomic DNAs ([Dunn et al. 2002](#)). In

58   this study, we show that these fragments can be used for efficient phylogenetic study for

59   determining evolutionary relationships between distinct species. We have tested this method *in*

60   *silico* and shown that 13 different types of IIB restriction enzymes can be used to accurately

61   reconstruct the phylogeny of a diverse set of 21 *Drosophila* species that are currently available.

62   **2    Materials and Methods**

63   2.1    Obtaining datasets

64   Whole genome, nucleotide sequences for the 21 *Drosophila* species were downloaded from the

65   FlyBase ([McQuilton et al. 2012](#)), NCBI databases and from Princeton University website([Rebeiz](#)

66   [et al. 2009](#)) on July 10, 2010.

67   2.2    Simulated restriction digestion

68   The PERL program "Phyper" was used to simulate restriction digestion for all 16 Type IIB

69   endonuclease enzymes and for processing the obtained fragments. This program generated a

70   representative list of unique fragments *i.e.,* single-copy fragments (most abundant) and fragments

71   that are present as multiple identical copies (less frequent). The former fragments are most likely

72   to belong to divergent fragment families, within a given genome that display one or a few

73   mutations relative to each other and were identified and removed from the analysis.  The

74   representative list of fragments were generated for each genome, for each enzyme separately.

75 2.3    Fragment comparisons

76  The representative lists of fragments were then used with another PERL program "Phyppa" for

77  comparative analyses. This program compares each fragment of a genome with every fragment of

78  another genome in order to find identical fragments and similar fragments (fragments with up to

79  6 mismatches for ensuring more than 70 % similarity among sequences). A total of 210 such

80  comparisons were done in order to generate the full list of shared fragments (identical fragments

81  and similar fragments) for every pair of genomes (both PERL scripts are available upon request).

82  Analyses was performed on a standard laptop with a quad core processor (1.73 GHz Intel Core

83  i7) and with 6 GB RAM. For each enzyme, the scripts required about 6 hours to finish for both

84  fragment generation and comparison between all genomes.

85 2.4    Distance calculations

86  The number of shared fragments between a pair of genomes was then used to calculate the

87  evolutionary distance by calculating the ratio of shared fragment to the total fragments and

88  converting them to negative natural log (Equation 1). Conversion to negative natural log was

89  essential to ensure that the distances computed were always positive.

90  Equation

$$Distance = -\ln\left(\frac{Identical\ fragments + Similiar\ fragments}{Total\ fragments\ of\ both\ species}\right)$$

91 2.5    Building trees

92  Distance measures for all the pairwise comparisons for a particular enzyme were used to build

93  trees using the *neighbor* program from the Phylip ([Felsenstein 2005](#)) package. A consensus tree

94  was them produced by combining trees for all the enzymes with the *consensus* program from

95  Phylip. The flowchart for the entire process is given in Figure. 1

96 **3    Results and Discussion**

97 3.1    Datasets

98  The full nucleotide sequences for 21 *Drosophila* species downloaded from various sources are

99  listed in Table 1. The genome size ranged from 137.82 mb for *D. simulans* to 235.52 mb for *D.*

100 *willistoni*. *D. willistoni* had the lowest GC content of all with 37.89% and *D. pseudoobscura* had

101 the highest GC content (45.43%).

102 3.2 Type IIB restriction enzymes

103 The 16 Type IIB restriction endonucleases that could be used for simulating the restriction

104 digestion of *Drosophila* genomes along with their recognition sites, average distance between the

105 restriction sites assuming random distribution of nucleotides and without any compositional bias,

106 and the size of fragment (blunt) that the enzymes leaves behind are given in Table 2 (Tengs et al.

107 2004). Unlike traditional Type II enzymes, Type IIB enzymes cleave on both sides of the

108 recognition sequence (about 7-15 bases upstream and downstream, depending on enzyme)

109 generating a fragment of uniform length. Also, the recognition site is usually split into two parts

110 by some fixed number of random bases. They normally leave 2-3 base overhangs on the

111 generated fragment.

112 3.3 Fragment analyses

113 The numbers of representative fragments obtained from each genome for each enzyme are listed

114 in Table 3. The most frequent cutting enzymes such as *BslFI* had generally higher numbers of

115 fragments within all genomes compared to other enzymes. Also, *D. pseudoobscura* and *D.*

116 *persimilis* had relatively higher numbers of fragments compared to other genomes with most of

117 the enzymes. Following fragment extraction, the original genomic sequences downloaded from

118 various source databases were represented as a collection of fragments of uniform length. For

119 each genome a total of 16 fragment sets were generated by using 16 different type IIB enzymes.

120 The number of fragments generated by each genome was not closely related to the size of their

121 genomes but they were related to the GC content. Most of the enzymes used in the analysis

122 recognized a GC rich recognition site which is reflected in the number of fragments generated

123 with GC rich genomes. The genomes that were GC rich such as *D. pseudoobscura* and *D.*

124 *persimilis* had higher numbers of fragments compared to other genomes. Similarly the genomes

125 that had lower GC content such as *D. willistoni* and *D. grimshawi* generated fewer fragments.

126 Overall, the number of fragments obtained for each species were within the range of expected

127 fragments based on their genome size and estimated distance between restriction cut sites

128 (assuming random sequence without GC content bias). Most enzymes predicted to be frequent

129 cutters generated large number of fragments like *BslFI*. Predicted rare cutters like *PsrI, PpiI, AloI*

130 and *CspCI* generated fewer fragments than other enzymes.

131 3.4 Distance Matrices and Phylogenetic Trees

132 A comparison of fragments between genomes provided a list of fragments that were shared by

133 those genomes. Closely related organisms are expected to share higher numbers of similar

134    fragments (including identical fragments) compared to other distantly related genomes. Similar

135    fragments are defined as those with 6 or fewer mismatches. Since the average length of fragments

136    generated from various enzymes was around 27 bases, allowing 6 bases mis-match ensured at

137    least 75 % similarity among the sequences. The fragments being compared between 2 genomes

138    ranged from 21 bp to 33 bp long (average size of 27 bp). The identical fragments between the 2

139    genomes are most likely to represent homologous or even orthologous sections of the genomes.

140    Even for a fragment length of 21 bp (smallest fragment size produced by these enzymes), the

141    probability that a particular 21 bp sequence exists one or more times in a genome of 150 Mb is

142    0.00341 %. The pair-wise distance matrices constructed using the similar fragments detected by

143    each enzyme were used to estimate phylogenetic trees (Figure 2). The individual NJ trees

144    obtained for each enzyme were largely consistent with the currently accepted relationships among

145    the various *Drosophila* groups and subgroups, as was the single consensus tree obtained (Figure

146    3). Per cent support values were calculated based on number of enzymes supporting the particular

147    branch.

## 4    Conclusions

149    The 21 species of *Drosophila* used here included the subgenus *Sophophora* and the subgenus

150    *Drosophila*. The *Sophophora* group was represented by *melanogaster*, *obscura* and *willistoni* and

151    the *Drosophila* group was represented by *virilis*, *repleta* and *mojavensis*. Out of the 12 subgroups

152    within the *melanogaster* group, 9 subgroups *viz., ananassae, montium, melanogaster, suzukii,*

153    *takahashii, ficusphila, elegans, rhopaloa* and *eugracilis* were represented by 15 species. Of these,

154    only 2 subgroups had multiple members within our data set, but both displayed a monophyletic

155    arrangement within the final tree shown in Figure 2. The placement of the 12 well-studied

156    *Drosophila* species *viz., D. simulans, D. sechellia, D. melanaogaster, D. erecta, D. ananassae, D.*

157    *yakuba, D. pseudoobscura, D. persimilis, D. willistoni, D. mojavensis, D. virilis and D.*

158    *grimshawi* within our tree corresponds exactly to the currently accepted phylogeny (Clark et al.

159    2007; Hahn et al. 2007; Haubold & Pfaffelhuber 2012; Stark et al. 2007).

160    Overall, the topology of our 21 species tree agrees precisely with those presented by Van der

161    Linde and Houle, (van der Linde et al. 2010),  Haubold and Pfaffelhuber (Haubold &

162    Pfaffelhuber 2012) and Yang et al. 2012 (Yang et al. 2012) and all the branches were completely

163    resolved.   The subgenus *Sophophora* was clearly distinguished into old world clades

164    *melanogaster/obscura* and neo world clade *willistoni* in our tree (van der Linde & Houle 2008).

165    The largest group *melanogaster,* had multiple subgroups *viz., melanogaster, montium, ananassae*

166 and oriental subgroup cluster (*eugaracilis*, *suzukii*, *takahashii*, *elegans*, *rhopaloa*, *ficusphila*).

167 Many previous studies have failed to completely resolve the nodes within the oriental subgroup

168 cluster (Da Lage et al. 2007; Toda 1991). In our tree, *ananassae* group formed the earliest branch

169 in the *melanogaster* group followed by *montium* subgroup with strong branch support values.

170 Most of the earlier studies confirmed this topology (Da Lage et al. 2007; Kopp 2006;

171 Prud'homme et al. 2006) except for two studies that placed them together as a sister clade from

172 rest of the subgroups (Schawaroch 2002) or reversed the order of branching (Yang et al. 2004).

173 Both these studies had poor branch support. The oriental subgroups cluster formed three sub

174 clades. The first sub-clade included *elegans* and *rhopaloa* with *ficusphila* as the sister sub-group,

175 the second sub-clade included *suzukii* and *takahashii* and the third sub-clade included the

176 *eugracilis* sub-group. The placements of these sub-clades were controversial among the literature

177 surveyed and was attributed to the explosive radiation of these oriental groups (van der Linde &

178 Houle 2008). The *eugracilis* clade consisting of *D. eugracilis* is most inconsistently placed clade

179 and it is either placed as sister species of *melanogaster* sub group, as in our tree (Haubold &

180 Pfaffelhuber 2012; Pelandakis & Solignac 1993; van der Linde et al. 2010) or as sister species of

181 the sub clade formed by *suzukii* and *takahashii* (Yang et al. 2004) or as sister species of *elegans*

182 and *rhopaloa* within the *elegans - rhopaloa - ficusphila* clade (Yang et al. 2012). The placements

183 of the other two clades, *suzukii - takahashii* and *elegans - rhopaloa - ficusphila* within the

184 *melanogaster* group in our tree is in agreement with other published studies (Kopp 2006; Kopp &

185 True 2002). The sub-clade formed by *suzukii* and *takahashii* is well supported by most studies

186 including ours with the strong branch support (Da Lage et al. 2007; Kopp & True 2002;

187 Schawaroch 2002; Yang et al. 2004). Most studies have confirmed that the *rhopaloa* subgroup is

188 the sister group of the *elegans* subgroup but the *ficusphila* sub group is considered to be

189 polytomic branching clade in the *melanogaster* group (van der Linde & Houle 2008). However,

190 in our tree *ficusphila* sub group is presented as the sister species of *rhopaloa - elegans* subgroups,

191 albeit with low branch support. Within the *Drosophila* subgenus, all three groups (*virilis*, *repleta*

192 and *grimshawi*) exhibited a topology frequently observed in other studies (van der Linde & Houle

193 2008).

194 A variety of sub-genomic sampling methods have been used previously for population studies

195 and are especially effective on non-model organisms, but are rarely used for generating

196 phylogenies for a diverse set of distinct species. We show here that multi-locus data obtained

197 from short sub-genomic fragment sets, essentially 2b-RAD, provides good phylogenetic signal

198  and produces a well resolved and well-supported species phylogeny. The wide adoption of

199  various RAD-like methods is due to the fact that deep sequencing of the fragments produced can

200  be easily accomplished following two simple steps: adapter ligation, and then PCR.  These

201  methods are applicable to any organism irrespective of its genome size. The 2b-RAD approach to

202  fragment generation and characterization in particular is simple, quick and cost effective (Wang

203  et al. 2012). This method also shares some similarity with the recently described, alignment free

204  multi-locus "co-phylog" method (Yi & Jin 2013).  Both use a large number of short homologous

205  fragments and, consequently, both can be profitably applied to short sequence reads derived via

206  next generation sequencing, even prior to assembly.  However, the co-phylog method is distinct

207  in that it makes use of standard alignment algorithms applied to each locus to generate estimates

208  of relatedness for building phylogenies.  Effective application of the co-phylog method generally

209  requires that the genomes being compared be closely related, and this would be expected to be

210  true for our method as well, since effective matching of homologous short fragments in either

211  case requires a significant degree of local sequence similarity.  Despite this expected limitation,

212  we note that the *Drosophila* species compared herein are relatively diverse, spanning

213  approximately 40-50 million years of evolution.

## 5    References

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# Figure 1

Workflow of the entire process of generating phylogeny from the Type IIB fragments.

Collecting whole genome nucleotide sequence data from public databases.

*Phyper*: Extracts fragments and generate a representative list of fragments for each genome, for every enzyme.

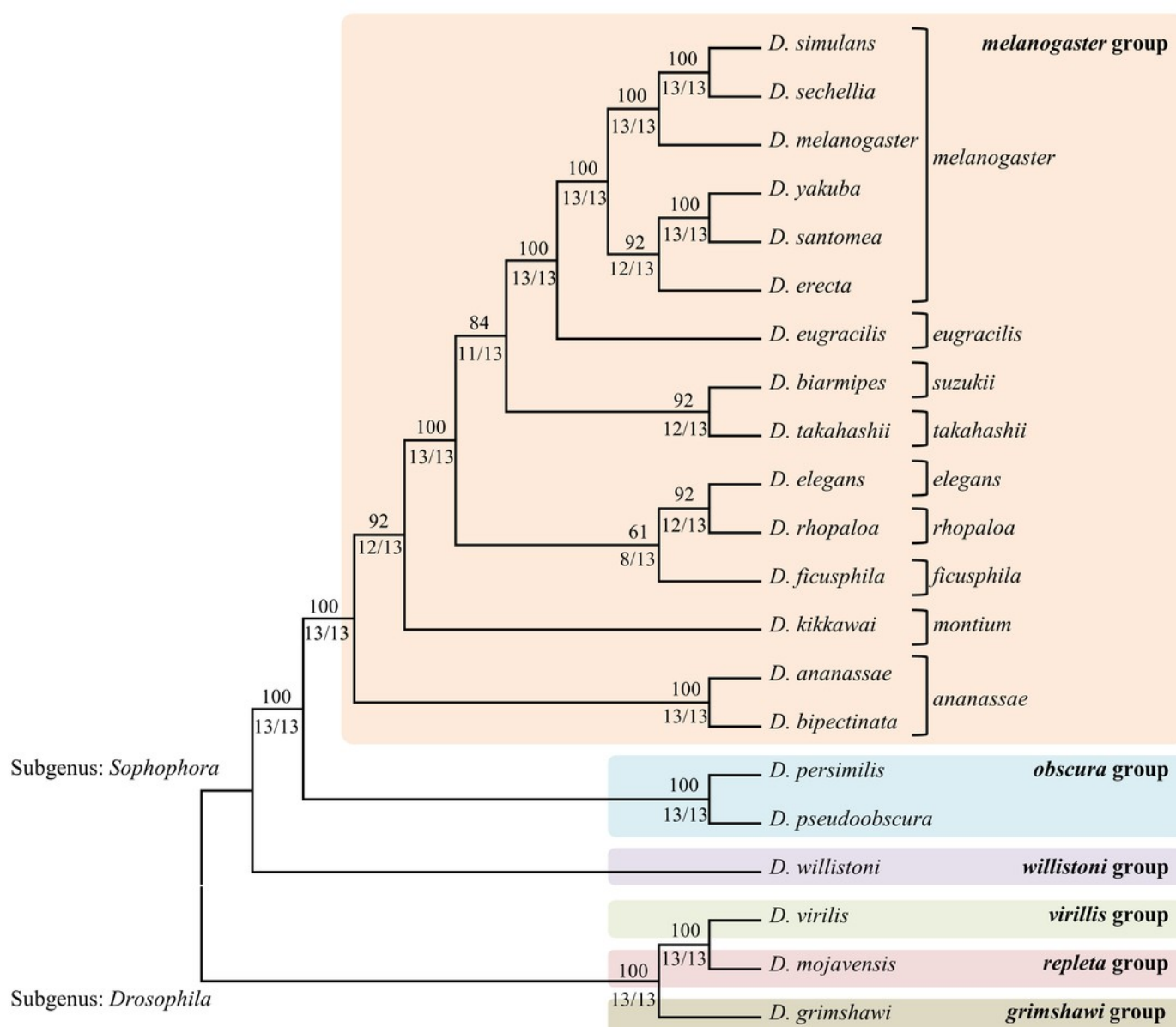
*Phyppa*: Pairwise comparison of each of the 21 genomes with other genomes to calculate number of shared fragments between them.

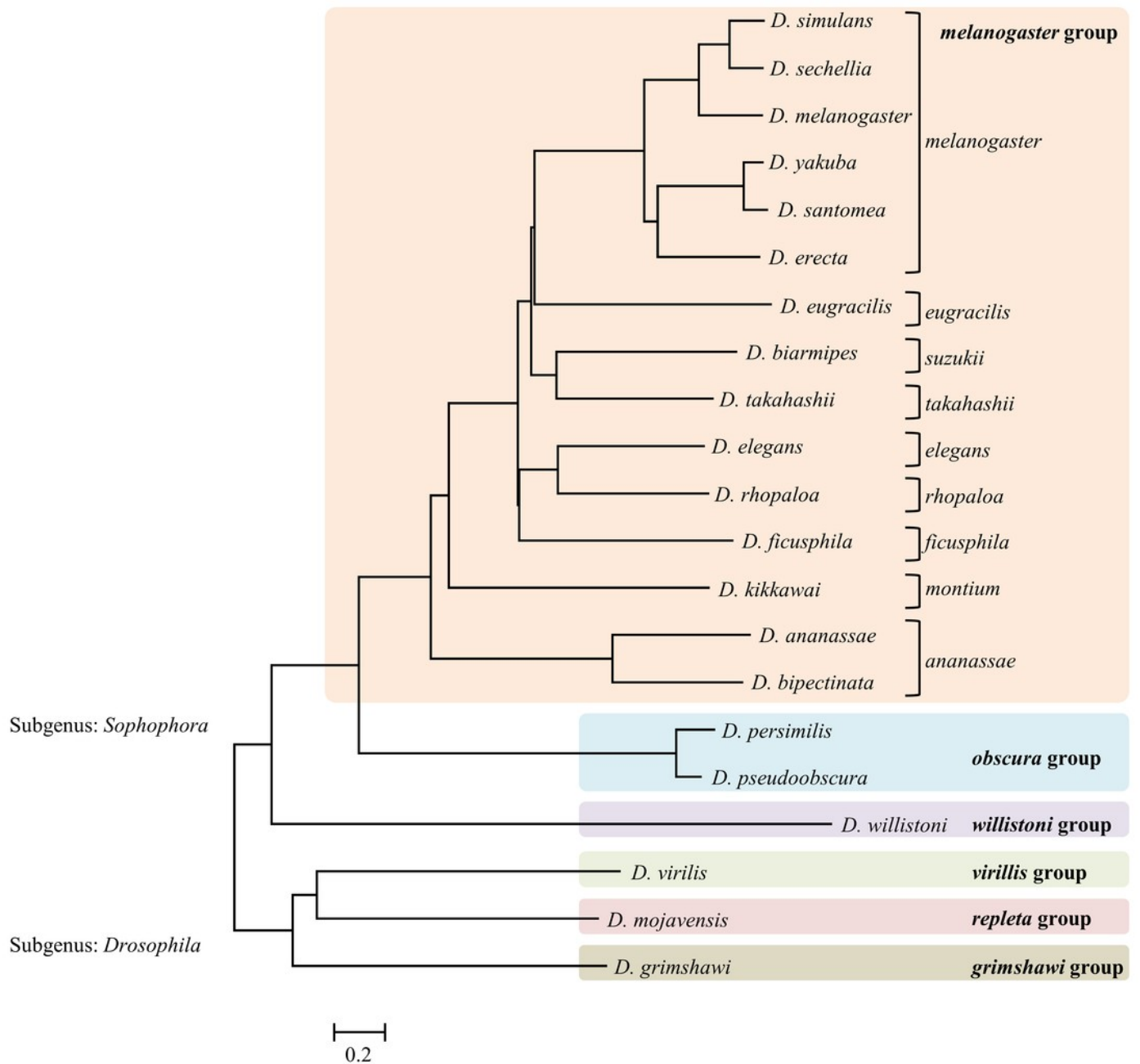
Calculate pairwise distance using
$$- ln \left( \frac{Shared\ fagments}{Total\ fragments} \right)$$

Phylogenetic tree using *neighbor* program from the PHYLIP package

# Figure 2

The consensus phylogenetic tree obtained by combining the trees obtained for each of the 13 enzymes.

The phylogenetic tree for each enzyme was calculated by extracting the corresponding fragments and then counting the number of shared fragment between every pair of species. The upper branch support values represent the percentage agreement over 13 enzymes and the bottom values indicate number of enzymes out of total 13 enzymes supporting the branch.

# Figure 3

Single enzyme tree (*Alo*I enzyme) showing the branch length.

Various Drosophila species and source databases used for the analysis. The GC % for each genome was calculated using infoseq from the EMBOSS package.

| Genome | GC % | Size | Source |
| --- | --- | --- | --- |
| *D. ananassae* | 42.56 | 230.99 mb | FlyBase |
| *D. biarmipes* | 41.82 | 168.58 mb | NCBI |
| *D. bipectinata* | 41.62 | 166.39 mb | NCBI |
| *D. elegans* | 40.31 | 170.51 mb | NCBI |
| *D. erecta* | 42.65 | 152.71 mb | FlyBase |
| *D. eugracilis* | 40.90 | 156.31 mb | NCBI |
| *D. ficusphila* | 41.93 | 151.04 mb | NCBI |
| *D. grimshawi* | 38.84 | 200.46 mb | FlyBase |
| *D. kikkawai* | 41.38 | 163.57 mb | NCBI |
| *D. melanogaster* | 42.05 | 168.73 mb | FlyBase |
| *D. mojavensis* | 40.22 | 193.82 mb | FlyBase |
| *D. persimilis* | 45.29 | 188.37 mb | FlyBase |
| *D. pseudoobscura* | 45.43 | 152.73 mb | FlyBase |
| *D. rhopaloa* | 40.07 | 193.90 mb | NCBI |
| *D. santomea* | 38.52 | 165.75 mb | Princeton University |
| *D. sechellia* | 42.53 | 166.57 mb | FlyBase |
| *D. simulans* | 43.06 | 137.82 mb | FlyBase |
| *D. takahashii* | 40.01 | 181.00 mb | NCBI |
| *D. virrilis* | 40.80 | 206.02 mb | FlyBase |
| *D. willistoni* | 37.89 | 235.51 mb | FlyBase |
| *D. yakuba* | 42.43 | 165.69 mb | FlyBase |

List of enzymes used for the fragment generation from the 21 Drosophila species.

Frequency indicates estimated distance between cut sites given a random sequence with all the 4 bases in equal probability and length refers to blunt tag length.

| Enzyme | Recognition sequence | Frequency | Length |
|--------|---------------------|-----------|--------|
| *AlfI* | GCANNNNNNTGC | 4096 | 32 |
| *AloI* | GAACNNNNNNTCC | 8192 | 27 |
| *BaeI* | ACNNNNGTAYC | 4096 | 28 |
| *BcgI* | CGANNNNNNTGC | 2048 | 32 |
| *BplI* | GAGNNNNNCTC | 4096 | 27 |
| *BsaXI* | ACNNNNNCTCC | 2048 | 27 |
| *BslFI* | GGGAC | 512 | 21 |
| *Bsp24I* | GACNNNNNNTGG | 2048 | 27 |
| *CspCI* | CAANNNNNGTGG | 8192 | 33 |
| *FaII* | AAGNNNNNCTT | 4096 | 27 |
| *HaeIV* | GAYNNNNNRTC | 1024 | 27 |
| *PpiI* | GAACNNNNNCTC | 8192 | 27 |
| *PsrI* | GAACNNNNNNTAC | 8192 | 27 |

Total number of fragments generated using 13 different Type IIB restriction enzymes for each of the 21 *Drosophila* genomes.

| Genomes | AlfI | AloI | BaeI | BcgI | BplI | BsaXI | BslFI | Bsp24I | CspCI | FalI | HaeIV | PpiI | PsrI |
|---------|------|------|------|------|------|-------|-------|--------|-------|------|-------|------|------|
| D. ananassae | 34804 | 11421 | 6151 | 51646 | 21457 | 52433 | 101183 | 46042 | 16405 | 38109 | 74174 | 11193 | 8344 |
| D. biarmipes | 41242 | 12667 | 6875 | 63518 | 22752 | 51248 | 109404 | 44554 | 18178 | 41284 | 75291 | 12177 | 10210 |
| D. bipectinata | 35642 | 10893 | 6616 | 51208 | 20363 | 50001 | 98937 | 45563 | 17131 | 39286 | 73197 | 10545 | 8622 |
| D. elegans | 43207 | 11314 | 6068 | 59905 | 18764 | 45496 | 93763 | 43259 | 18466 | 41866 | 75238 | 11027 | 9753 |
| D. erecta | 42781 | 10517 | 5914 | 60434 | 18119 | 43684 | 85735 | 40020 | 17793 | 31931 | 66412 | 9979 | 8677 |
| D. eugracilis | 36455 | 10170 | 5699 | 51988 | 18236 | 43177 | 86365 | 42020 | 17568 | 40795 | 72398 | 9682 | 8335 |
| D. ficusphila | 38374 | 11698 | 5338 | 60448 | 20161 | 47056 | 89928 | 39223 | 17489 | 37380 | 69222 | 11070 | 8868 |
| D. grimshawi | 49667 | 5891 | 5212 | 61420 | 17341 | 30379 | 58175 | 35658 | 16642 | 34409 | 64560 | 8062 | 6977 |
| D. kikkawai | 39192 | 10361 | 5516 | 54698 | 21908 | 50258 | 99784 | 44066 | 16846 | 40965 | 68593 | 10765 | 8126 |
| D. melanogaster | 39711 | 9908 | 6037 | 59203 | 16840 | 41168 | 81877 | 39221 | 17651 | 31350 | 68204 | 9243 | 8303 |
| D. mojavensis | 54782 | 6294 | 5234 | 64186 | 21048 | 33289 | 60708 | 36674 | 14774 | 33071 | 65210 | 9090 | 8012 |
| D. persimilis | 43327 | 10706 | 7567 | 59923 | 25287 | 53206 | 113002 | 48862 | 16329 | 31779 | 76473 | 12267 | 8940 |
| D. pseudoobscura | 43650 | 10461 | 7466 | 60237 | 25174 | 53269 | 111423 | 48990 | 16358 | 31417 | 74808 | 12175 | 8774 |
| D. rhopaloa | 36920 | 10920 | 6177 | 56203 | 18139 | 44894 | 93524 | 41357 | 17133 | 40153 | 76711 | 10442 | 9247 |
| D. santomea | 40344 | 9877 | 5957 | 56771 | 17044 | 41850 | 80010 | 38107 | 17037 | 32142 | 67070 | 9414 | 8378 |
| D. sechellia | 39876 | 10371 | 5808 | 59204 | 17430 | 42659 | 83936 | 39380 | 17276 | 31541 | 68359 | 9792 | 8289 |
| D. simulans | 38549 | 9815 | 5547 | 56820 | 16777 | 40735 | 79826 | 37436 | 16666 | 30304 | 64321 | 9148 | 7773 |
| D. takahashii | 37489 | 11463 | 5431 | 58887 | 19189 | 45240 | 91825 | 39992 | 26269 | 37277 | 74002 | 10801 | 8987 |
| D. virrilis | 58785 | 6943 | 5774 | 64912 | 18097 | 31951 | 66710 | 38679 | 15733 | 37692 | 65275 | 9290 | 8551 |
| D. willistoni | 34033 | 7083 | 6177 | 43299 | 15103 | 35578 | 70085 | 39996 | 17240 | 42202 | 77102 | 7941 | 9626 |
| D. yakuba | 42202 | 10300 | 6165 | 59442 | 17885 | 43748 | 83095 | 39920 | 18007 | 33024 | 69632 | 9887 | 8765 |