

1                   **Title: Dengue viruses cluster antigenically but not as discrete serotypes**

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50 **Abstract:** The four genetically divergent dengue virus (DENV) types are traditionally classified  
51 as serotypes. Antigenic and genetic differences among the DENV types influence disease  
52 outcome, vaccine-induced protection, epidemic magnitude, and viral evolution. We  
53 characterized antigenic diversity in the DENV types by antigenic maps constructed from  
54 neutralizing antibody titers obtained from African green monkeys and after human vaccination  
55 and natural infections. Genetically, geographically, and temporally, diverse DENV isolates  
56 clustered loosely by type, but we found many are as similar antigenically to a virus of a different  
57 type as to some viruses of the same type. Primary infection antisera did not neutralize all viruses  
58 of the same DENV type any better than other types did up to two years after infection and did  
59 not show improved neutralization to homologous type isolates. That the canonical DENV types  
60 are not antigenically homogenous has implications for vaccination and research on the dynamics  
61 of immunity, disease, and the evolution of DENV.

62 **Main text:** Dengue virus (DENV) infects up to 390 million people each year, and of the 96  
63 million individuals who develop an acute systemic illness, approximately 500,000 experience  
64 potentially life-threatening complications, including hemorrhage and shock (1, 2). The four  
65 genetic DENV types have long been thought to exist as four serotypes, and the antigenic  
66 differences between the types are believed to have a key role in the severity of disease, epidemic  
67 magnitude, viral evolution, and design of vaccines (3–5).

68 The description of DENV types as serotypes originated with the observation that the  
69 human immune response following primary DENV infection fully protected against challenge  
70 with viruses of the homologous type but only partially, and transiently, protected against  
71 challenge by viruses of a heterologous type (6). This finding was supported by *in vitro*  
72 neutralization experiments in which each DENV type was on average better neutralized by  
73 homologous than heterologous DENV infection antisera (7). The immune response immediately  
74 after a primary DENV infection varied from individual to individual, but generally was  
75 characterized by high levels of neutralizing antibody titers to multiple DENV types. The  
76 neutralizing response was observed to become more DENV type-specific over time (8). It was  
77 later shown that antibodies to a heterologous DENV type could enhance infection *in vivo* and  
78 were associated with increased risk of severe disease in nature (9, 10). Although antigenic  
79 variability was observed within DENV types from the earliest studies, this variation is generally  
80 considered to be substantially less than the differences between types, and not thought to modify  
81 type-specific protection (11, 12). Together, the DENV types clearly form an antigenic subgroup  
82 within the *Flaviviridae* (13, 14). Analyses of envelope (E) proteins, and later full genomes,  
83 showed that the four types are as genetically divergent among themselves as sequences assigned  
84 to different viruses within the genus *Flavivirus* (15). These deep evolutionary divergences

85 between DENV types were evident in the phylogenetic tree of the genetically diverse E-gene  
86 sequences of the viruses we investigated here (Fig. 1A; fig. S1; and table S1) (16). Similarly, a  
87 map of amino acid differences between the E proteins revealed four compact, segregated types  
88 (Fig. 1B and fig. S2), as the number of amino acid substitutions between heterologous types far  
89 exceeded the maximum difference within a type.

90         However, investigations that rely on the classification of DENV into serotypes do not  
91 fully explain clinical and epidemiological phenomena. Despite this, antigenic properties are still  
92 thought to play a critical role in the biology of DENV infections. One hypothesis is that  
93 antigenic differences are critical, but that categorization by serotype alone is too coarse a  
94 measure. For example, differences in epidemic magnitude might be determined not only by the  
95 serotype but also by the antigenic differences between the particular infecting viruses that  
96 populations experience during sequential epidemics. Antigenic variation within and among the  
97 DENV types has also been hypothesized, in addition to intrinsic viral fitness and other factors, to  
98 explain phenomena including extinction and replacement of previously successful lineages and  
99 variation in disease outcome caused by genetically similar viruses (17–19). Here, we empirically  
100 test the antigenic relationships among a panel of diverse DENV isolates and re-examine the  
101 serotype concept.

102         Antigenic differences among viruses are caused by amino acid differences that lead to  
103 structural changes on viral proteins that modify antibody binding. The structural effect of such  
104 amino acid substitutions is difficult to predict from genetic sequences alone. In some instances  
105 substitutions have no antigenic effect, sometimes single substitutions cause substantial antigenic  
106 change, and other times it takes multiple substitutions (20, 21). Thus today, antigenic differences  
107 must be determined by phenotype, including by an antibody neutralization assay (13). Most

108 often, viruses are measured against multiple sera to form a table of neutralization data from  
109 which antigenic relationships are inferred (22). However, such inferences are notoriously  
110 difficult to make, and this has hindered the reliable systematic antigenic characterization of  
111 DENV. The difficulties are caused by random error, the use of diverse methods among  
112 laboratories, and the intrinsic variability among immune sera due to differences in hosts and  
113 infection histories (23, 24). Moreover, neutralization data often contain apparent contradictions  
114 that are difficult to interpret, such as higher-than-homologous titers and sera that similarly  
115 neutralize multiple DENV types.

116         Previous antigenic analyses of DENV have addressed such challenges by using  
117 monoclonal antibodies, averaging responses of many individuals, or excluding sera with unusual  
118 patterns of reactivity. Despite careful work, these approaches have not produced a unified  
119 framework for understanding patterns across large neutralization data sets. Antigenic  
120 cartography is a method that positions viruses and antisera as points in a map, such that the  
121 distance between each virus and antiserum is derived from the corresponding neutralization titer  
122 in the tabular data. This method exploits variation in host responses to better triangulate the map,  
123 reduces the effect of some measurement errors because each virus is measured against multiple  
124 antisera (and vice versa), and has been shown to accurately interpret apparent contradictions in  
125 the data (25).

126         We formed the Dengue Antigenic Cartography Consortium, an open collaboration of  
127 international research laboratories, to establish empirically how DENV types relate to one  
128 another antigenically. Thirty-six African green monkeys (*Chlorocebus sabaenus*, hereafter NHP)  
129 were experimentally inoculated with diverse DENV isolates, and their sera were tested for  
130 neutralizing antibody potency against the genetically (all known genotypes), temporally (1944-

131 2012), and geographically (20 countries) diverse panel of DENV isolates shown in Fig. 1 (table  
132 S1). Serum samples were taken three months post-inoculation, and titrations were conducted  
133 using an immunofocus reduction neutralization test on mosquito cells (*C6/36, Aedes albopictus*)  
134 (tables S2-S7 and fig. S3) (16, 26). A conventional interpretation of the raw antibody  
135 neutralization titers was consistent with previous observations, both for DENV and for other  
136 flaviviruses: antisera were generally able to neutralize viruses of the infecting type better than  
137 heterologous types.

138 The cartographic analyses fit these data with low error and were internally consistent  
139 (figs. S4, S6, and S7). Only 1% of map distances differed by more than four-fold from the  
140 measured titer (table S8). The positions of viruses and antisera were robust to different methods  
141 of calculating neutralization titers and to the exclusion of outliers (figs. S5, S8-S12 and table  
142 S10). Maps made with random subsets of the data set could predict excluded titers within two-  
143 fold error ( $r=0.90$  for the relation between all measured and predicted titers) (table S9).

144 Our analyses showed that the DENV isolates in our panel did group according to current  
145 serotype classification (Fig. 2), and the majority of viruses neighboring any given virus are of the  
146 same DENV type. However, many of the viruses were positioned as close to a virus of another  
147 DENV type as to some viruses of their own type, and the distance within and between types was  
148 comparable. Similarly, while neutralizing antisera responses clustered closely to viruses of the  
149 homologous type, almost all were at least as close to a heterologous-type isolate (table S11, table  
150 S12).

151 To examine these findings in detail, we evaluated whether the observed antigenic  
152 diversity of the virus types was also observed with human antisera and over time, and whether  
153 the neutralizing responses of individual antisera became increasingly type-specific over time.

154 We titrated human antisera derived from vaccination with a live-attenuated chimeric  
155 DENV vaccine against the genetically diverse DENV panel. Individuals lacking detectable  
156 neutralizing antibodies against DENV or other flaviviruses were each inoculated with one  
157 monovalent component of the National Institutes of Health DENV vaccine (n=40 in total, 10 per  
158 DENV type). Antisera drawn 42 days post-injection were titrated against the DENV panel  
159 (n=36) using the neutralization test on mosquito cells. The resulting antigenic map is consistent  
160 with the NHP map in that the distance between DENV types was equivalent to the spread within  
161 type, and the overall orientation of DENV1-4 was the same (Fig. 3A).

162 We measured the antigenic relationships among the DENV panel as recognized by  
163 antisera drawn from naturally-infected individuals, who had neutralizing responses  
164 representative of the cohort study from which they were selected. Serum samples drawn from 20  
165 Nicaraguan children in the year following their first DENV infection were titrated, using the  
166 neutralization test on mosquito cells, against 14 viruses that captured the breadth of variation  
167 seen in the DENV panel in Fig. 2. Again, the antigenic distances among the DENV types were  
168 similar to those observed with NHP and human vaccine antisera, although the DENV4 cluster  
169 was positioned adjacent to DENV1 and DENV2 (Fig. 3B).

170 We also analyzed neutralization data from other studies that had used antisera from  
171 monovalent vaccine recipients and naturally infected human travelers, as well as different  
172 neutralization assays (22, 27, 28). Again, the antisera from these studies also recognized the  
173 antigenic relationships among the DENV isolates similarly to the three-month NHP antisera  
174 (figs. S23-S25).

175 The early antibody response is assumed to broadly neutralize all DENV types, but over  
176 time cross-type neutralization is thought to be lost so that the antibody response remaining in the



177 months to years after infection only potentially neutralizes isolates of the infecting type (8, 29, 30).  
178 We compared how antisera taken at various time points after infection recognize antigenic  
179 relationships among the DENV panel. The human antisera used to make the antigenic maps  
180 described above were taken at various times following infection, ranging from 42 days for the  
181 monovalent vaccine antisera to more than one year for the natural infection antisera. We also  
182 made an antigenic map of a published neutralization data set of 44 DENV isolates titrated with  
183 one-year post-inoculation monkey antisera and found a similar range of antigenic variants among  
184 the four DENV types (fig. S26) (12). Thus, in maps made with early (one month) as well as late  
185 convalescent (three months to one year) antisera, the antigenic relationships among diverse  
186 DENV isolates were similar to those observed with three-month NHP antisera.

187 We tested if the patterns of antigenic recognition of the antisera from serially sampled  
188 individuals changed with time. We titrated antisera from the experimentally inoculated NHPs  
189 one month (n=36) and five months (n=16) post-infection against the DENV panel. As expected,  
190 the magnitude of the neutralizing titers generally dropped between one, three, and five months  
191 (table S14). However, viruses on the one and five-month antigenic maps showed the same  
192 orientation of types as the three-month antisera. At one month after infection, 55%, and at five  
193 months after infection, 41% of the viruses, respectively, clustered as closely to a virus in a  
194 heterologous type as to some viruses of the same type (Fig. 4A and B; table S11; table S13; and  
195 table S15). The antigenic relationships among isolates were conserved across time-points (fig.  
196 S13). We thus found that the antigenic relationships among the isolates in the DENV panel  
197 were recognized similarly by early and late convalescent antisera from the same individuals.

198 We measured changes in neutralizing type-specificity for each NHP by comparing the  
199 antiserum positions in the one, three, and five-month antigenic maps. The antiserum positions

200 shifted (on average, greater than four-fold) between one month and three months, consistent with  
201 the period of somatic hypermutation and selection for affinity matured B cells (Fig. 4A and fig.  
202 S14). However, few antisera showed improved neutralization of the infecting DENV type  
203 relative to heterologous types between one and three months. The antiserum positions changed  
204 minimally between three months and five months, despite a significant decline in the magnitude  
205 of titers over that period, in some cases below the assay limit of detection (Fig. 4B and table  
206 S14). Thus, we did not observe a systematic shift toward increasing neutralizing specificity to  
207 viruses of the infecting type nor decreasing specificity toward heterotypic viruses (fig. S15 and  
208 fig. S21).

209         Published studies of neutralizing responses in the first year after experimental inoculation  
210 also reported stability of neutralization specificity. In one study, the ratio between homologous  
211 and heterologous neutralizing titers for 16 Rhesus monkeys between 4-13 months after  
212 experimental inoculation was remarkably consistent. NHPs that were initially type-specific  
213 remained so, while those that exhibited early cross-type titers maintained titers to those types to  
214 the end of the study period (fig. S28) (31). A second study following the neutralizing responses  
215 of *Aotus nancymae* monkeys for 1-4 months to DENV1 and DENV2 isolates showed similarly  
216 stable neutralization specificity to the infecting type and heterologous types (fig. S29) (32).

217         We further analyzed the neutralizing responses in the natural human infection data set to  
218 look at the type-specificity of antisera obtained during the first two years after infection. The  
219 antisera in the map in Fig. 3B ranged in neutralizing type-specificity, with 55% of antisera  
220 responses clustering as closely to a heterologous isolate as some homologous isolates. For each  
221 individual, the serum position in Fig. 3B, made with titrations conducted on mosquito cells,  
222 closely corresponded to the serum position in the map made with titrations using human cells

223 expressing the DENV attachment factor, DC-SIGN (Fig. 3B and fig. S16). The position of the  
224 DENV4 cluster was between DENV1 and DENV2 on both maps (Fig. 3B and fig. S16). We  
225 compared the antibody titrations after one and two years for each individual, and found that all  
226 maintained the pattern of neutralization, including cross-neutralization, observed in the first year  
227 after infection (fig. S17 and S18). Thus, neutralizing antibody responses in natural human  
228 DENV infections did not show a trend toward increasing type-specificity even two years after  
229 infection.

230         Type-specific and cross-reactive neutralizing antibodies are thought to target distinct viral  
231 structures, and thus potentially may produce different antigenic maps (33). We therefore tested  
232 whether cross-reactive neutralizing antisera recognized different antigenic relationships among  
233 the DENV panel than type-specific neutralizing responses, using the serum positions of the  
234 monovalent vaccine map (Fig. 3A). Despite the fact that all ten individuals for each DENV type  
235 were inoculated with the same vaccine component, the antisera responses to the isolates varied.  
236 Collectively, the antisera provided a coherent description of antigenic patterns among the isolates  
237 (fig. S19). The relationships among the DENV panel changed minimally between maps made  
238 with only the most central, cross-reactive 20 antisera or only the most peripheral, type-specific  
239 20 antisera (fig. S20 and fig. S22). Thus, the DENV type-specific and cross-reactive neutralizing  
240 responses recognized the same antigenic relationships among the DENV panel.

241         The antigenic characterization of any pathogen relies on the biological relevance of the  
242 assay used to generate the data. Both recent and historical studies have found significant  
243 associations between pre-infection neutralization titers and DENV viremia or infection outcome  
244 (34–37); however, other studies have been inconclusive (38, 39). Thus, the identification of  
245 immune correlates of protection including, but not exclusively, potentially neutralizing antibodies,

246 is an active area of research for DENV (40–42). Notably, the antigenic patterns in our data are  
247 similar to those in antigenic maps we made of DENV antibody neutralization data from other  
248 published studies using different cell lines, virus preparations, methods for detecting infected  
249 cells, and plaque or immunofocus reduction end-points (figs. S23-S27) (12, 19, 22, 27, 28). We  
250 also found that the human antisera from natural infections titrated on mosquito cells showed  
251 similar neutralization profiles to those titrated on human cells (fig. S16 and S18). The antigenic  
252 variation we observed is thus not limited to the assay or samples that we used.

253         While overall, prior immunity to a heterologous DENV type still remains the strongest  
254 risk factor for disease, there is evidence that neutralizing responses to the particular DENV  
255 lineages circulating in a population modifies the magnitude and severity of epidemics caused by  
256 subsequent infecting lineages (17, 18). In one study, cross-type neutralization provided by prior  
257 DENV1 immunity correlated with a mild epidemic caused by one lineage of DENV2, but  
258 showed no neutralization of other DENV2 lineages that in immunologically similar populations  
259 caused severe epidemics (fig. S27) (19). These, and our, studies highlight the importance of  
260 studying the specific relationship between antigenic distances as measured with neutralizing  
261 antibody titers and protection. The approach described here, in combination with global  
262 surveillance of the genetic, antigenic, and clinical features of DENVs as well as further detailed  
263 studies of natural infection and vaccination-derived protection, has the potential to inform  
264 whether vaccination protects against circulating isolates as well as recognize gaps in vaccine-  
265 induced protection should they emerge over time.

266         The antigenic analyses shown here using one, three, and five-month NHP antisera,  
267 human monovalent vaccine antisera, late-convalescent human natural infection antisera, and  
268 published neutralization data show that the DENV types do not fall into order as distinct

269 serotypes. We have found that while DENV isolates are usually located closer to other viruses  
270 of the same type, some viruses, both modern and historical, have greater antigenic resemblance  
271 to viruses of a different type than to some viruses of the same type. We find that primary  
272 infection neutralizing antibody titers, although they drop in magnitude, do not systematically  
273 become more type-specific in the year after primary infection. As expected, individuals infected  
274 with the same or different antigens have variable patterns of neutralization, but cross-neutralizing  
275 responses consistently recognize the same antigenic relationships within the DENV panel as do  
276 the neutralizing responses that are most type-specific. These findings shift our understanding of  
277 the antigenic properties of DENV, enable more detailed study of the antigenic determinants of  
278 clinical severity, epidemic magnitude, and DENV evolution, and provide additional methods for  
279 the selection of future vaccine strains and global surveillance of the antigenic dynamics of  
280 dengue viruses.

281 **Figure legends**

282 **Fig. 1.** Genetic analyses of the DENV panel (n=47). **(A)** Phylogenetic tree showing the  
283 evolutionary relationships of DENV E gene sequences. Sequences were aligned with MAFFT,  
284 and a maximum likelihood tree (ML) was estimated using a general time reversible model,  
285 accounting for both among site rate variation and invariant sites (GTR+G<sub>4</sub>+I). Bootstrap support  
286 values of at least 75% are shown. **(B)** Amino acid map of dengue E protein sequences (493-495  
287 amino acids in length). The total amino acid differences between pairs of E sequences  
288 correspond to distances between points on the geometric display.

289 **Fig. 2.** Antigenic map of the DENV panel (n=46) titrated against three-month post-infection  
290 African green monkey antisera (n=36). Each unit of antigenic distance (length of one grid-  
291 square side, measured in any direction) is equivalent to a two-fold dilution in the neutralization  
292 assay. Each antiserum (open shape) and virus (closed shape) is colored according to the  
293 infecting genetic type (*I6*). The size and shape of each point is the confidence area of its  
294 position.

295 **Fig. 3.** Human primary infection antigenic maps. **(A)** Antisera from individuals inoculated with  
296 each monovalent component of the NIH live vaccine (10 per group) were drawn 42 days post-  
297 infection and titrated against 36 viruses in the DENV panel. **(B)** Antisera from 20 Nicaraguan  
298 children drawn in the year after their first DENV infections were titrated against an antigenically  
299 diverse subset of the DENV panel (n=14).

300 **Fig. 4.** Antigenic maps of the DENV panel made with antisera drawn from NHPs one and five  
301 months post-infection. **(A)** An antigenic map of 47 DENV isolates titrated against 36 NHP  
302 antisera drawn one month post-infection. Colored arrows (DENV1=yellow, DENV2=blue,

303 DENV3=green, DENV4=red) show the change in antiserum positions between one and three  
304 months. The black arrows show the average shift in serum position for each DENV type. The  
305 star denotes the antigenic center for each DENV type. **(B)** An antigenic map of 37 DENV  
306 isolates titrated against 16 NHP antisera drawn five months post-infection. Arrows point from  
307 positions of antisera at three months to the corresponding five-month positions.

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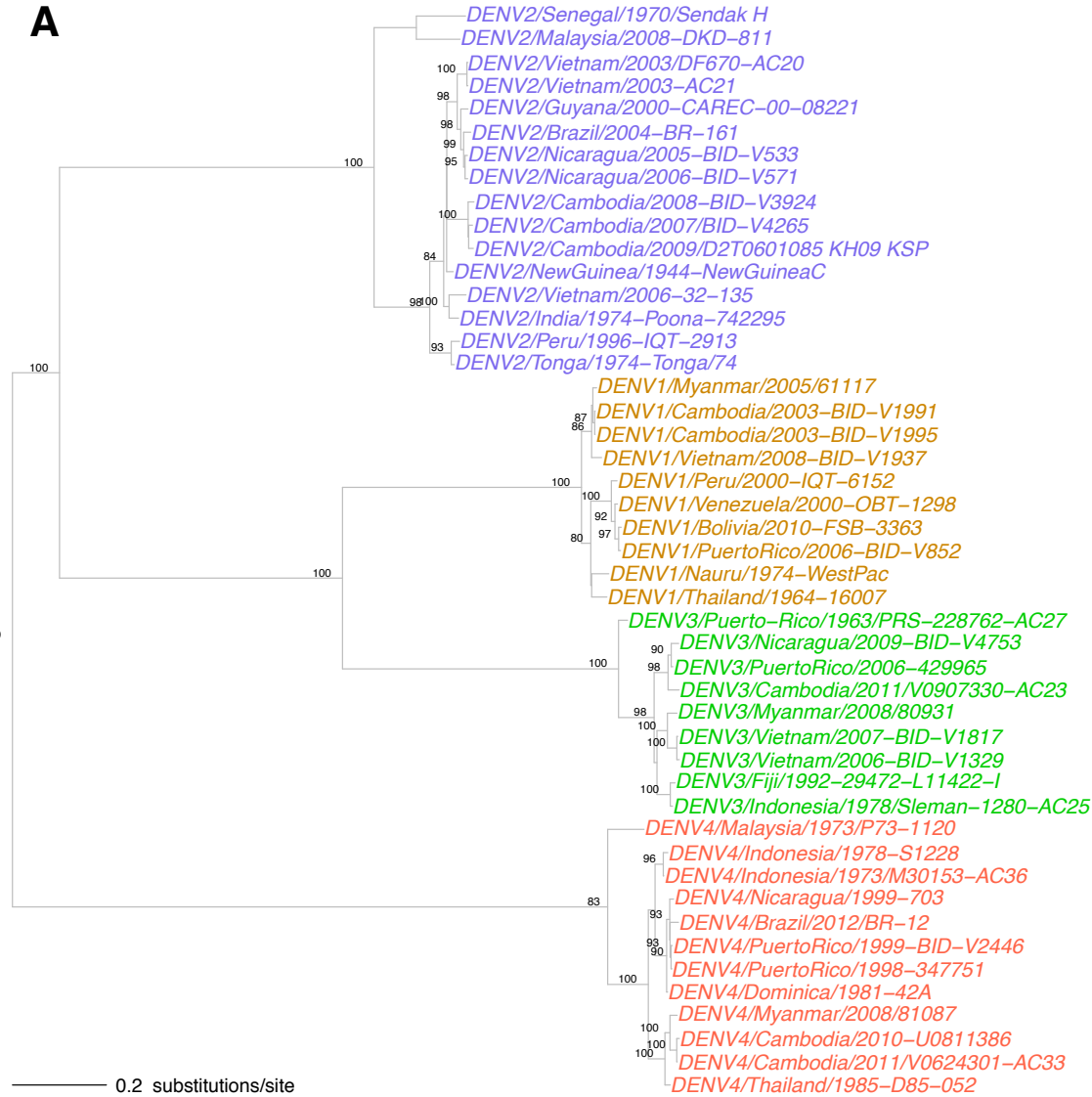
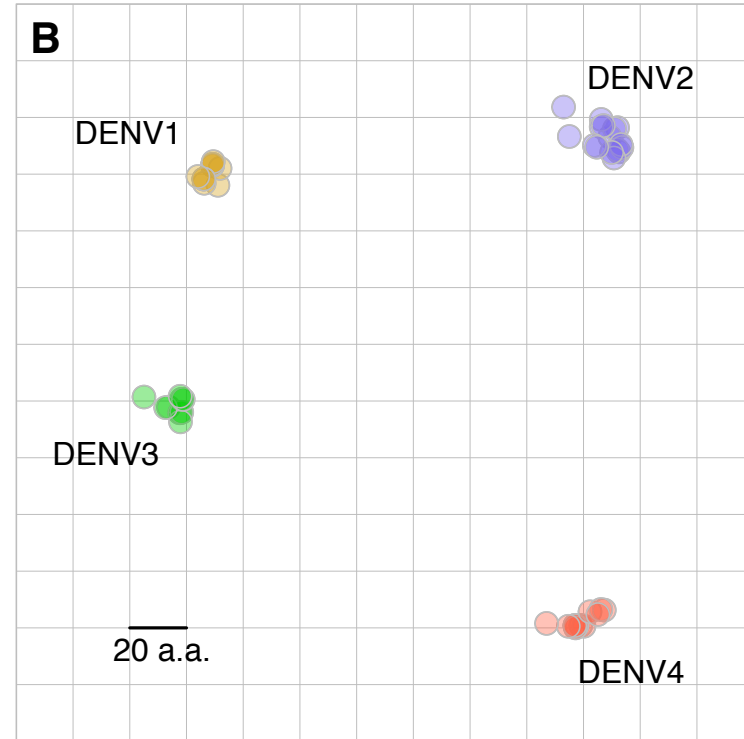
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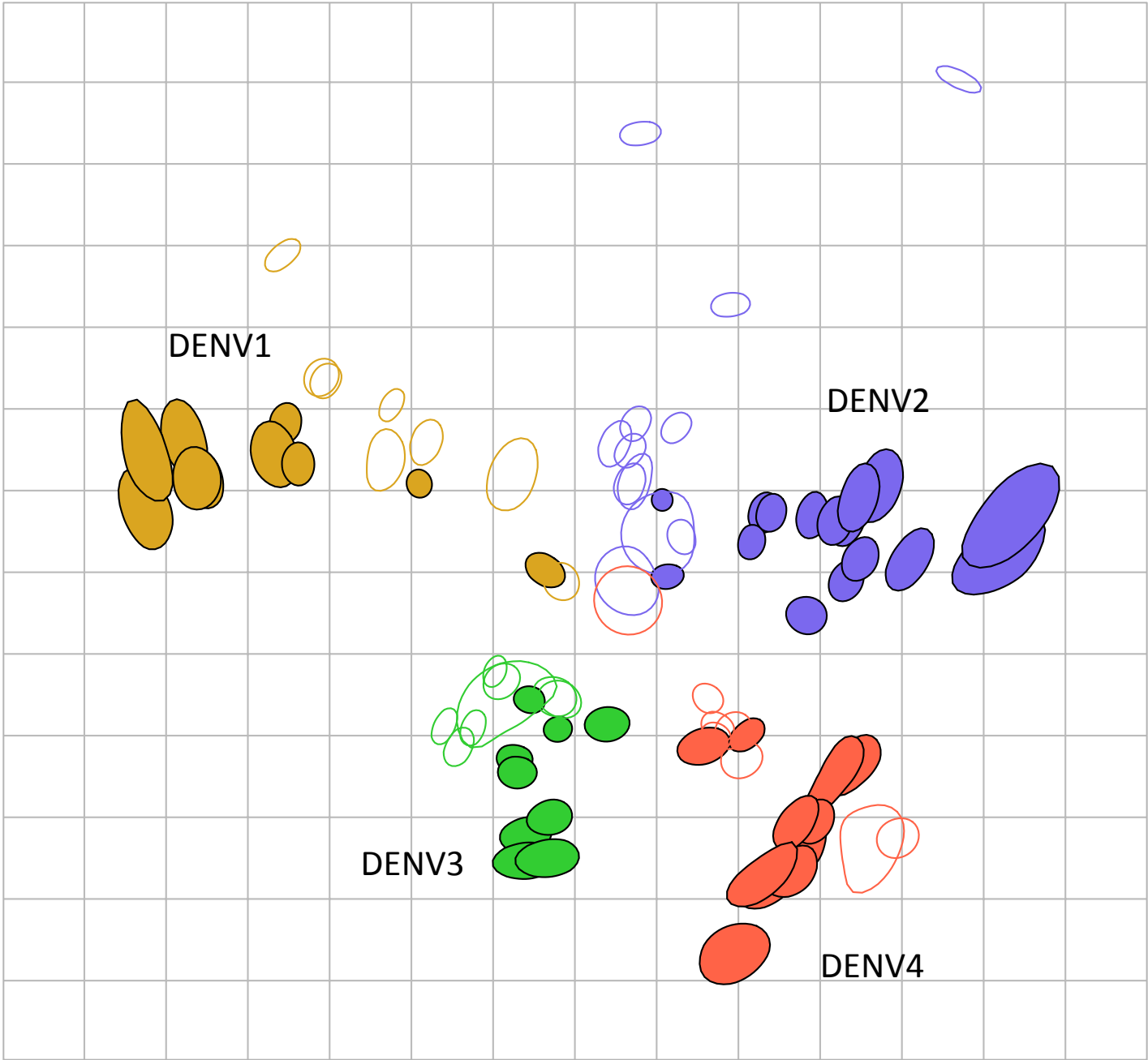
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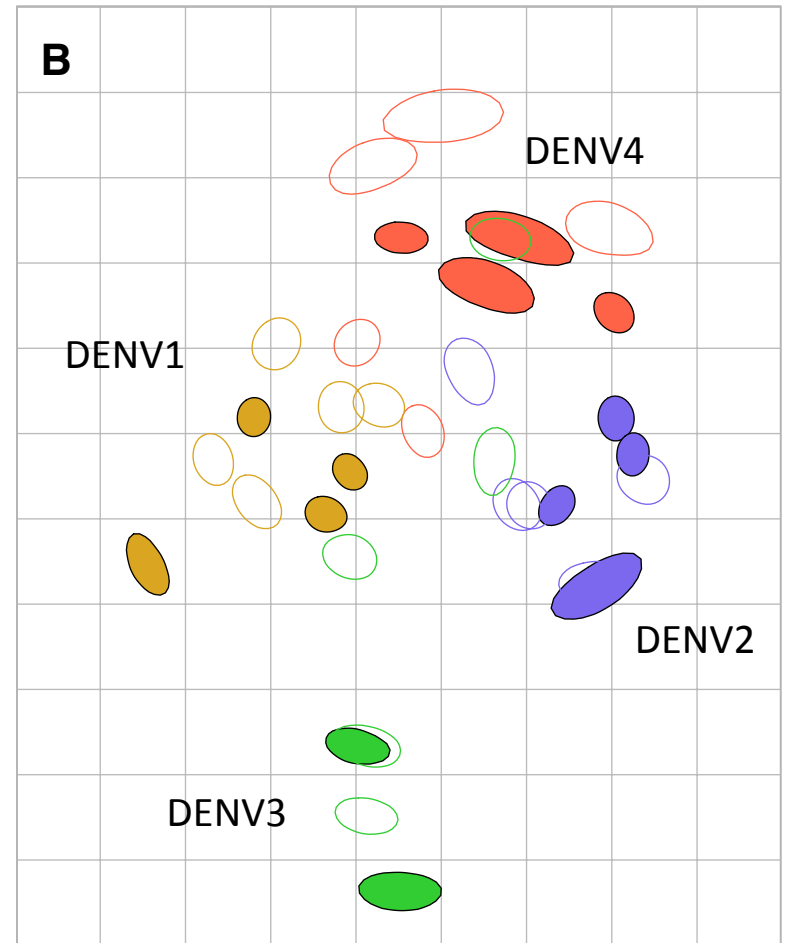
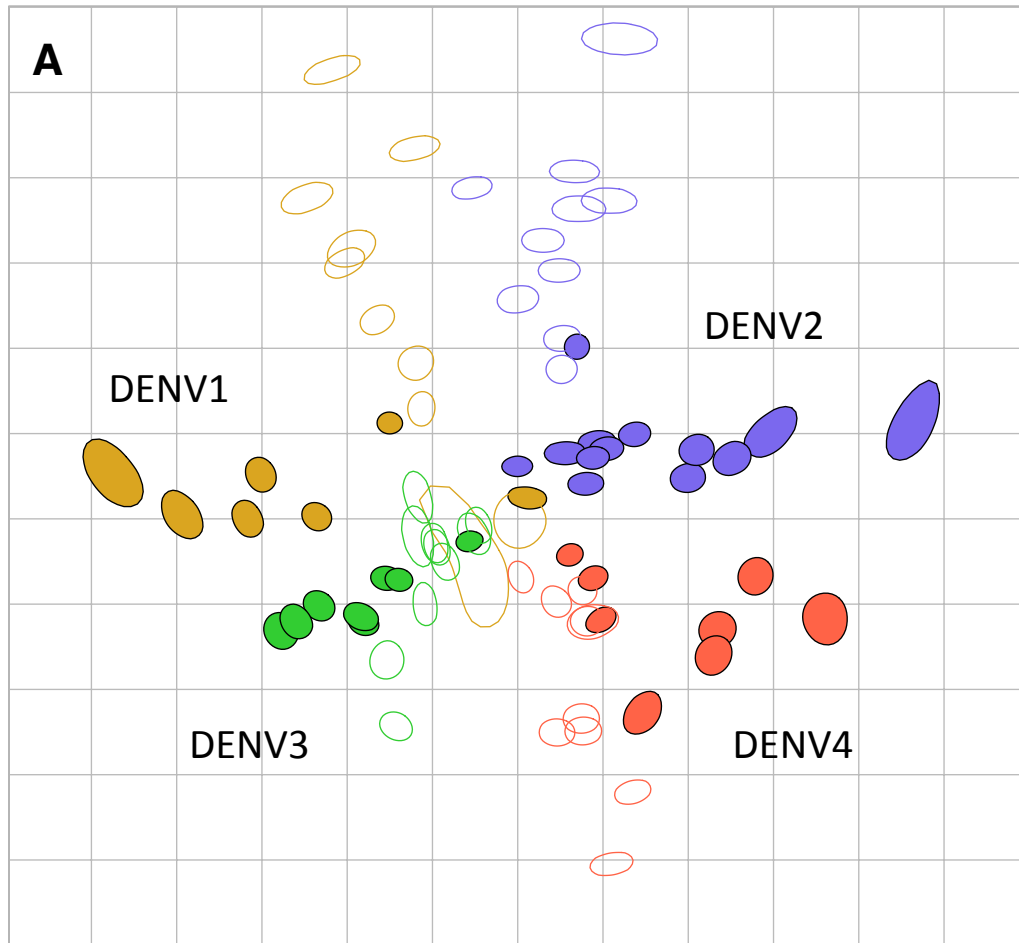
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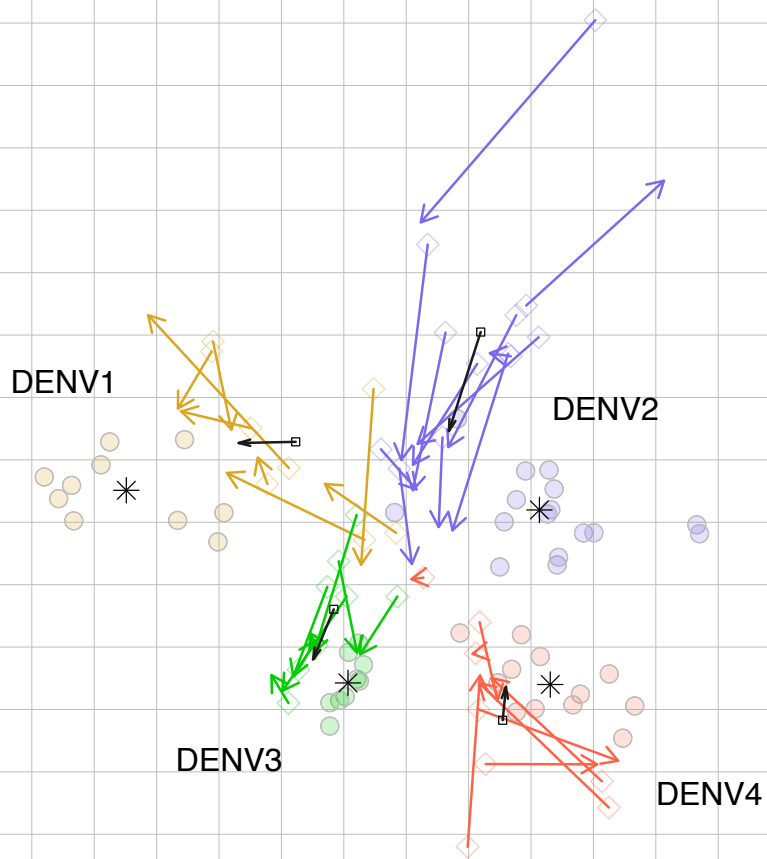
507	<b>Supplementary Materials:</b>
508	
509	Materials and Methods
510	Figs. S1-S29
511	Tables S1-S15
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513	References (43-66)

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