# An objective criterion to evaluate sequence-similarity networks helps in dividing the protein family sequence space

**Short title:** An objective criterion to evaluate sequence-similarity networks

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## Abstract

The deluge of genomic data raises various challenges for computational protein annotation. The definition of superfamilies, based on conserved folds, or of families, showing more recent homology signatures, allow a first categorization of the sequence space. However, for precise functional annotation or the identification of the unexplored parts within a family, a division into subfamilies is essential. As curators of an expert database, the Carbohydrate Active Enzymes database (CAZy), we began, more than 15 years ago, to manually define subfamilies based on phylogeny reconstruction. However, facing the increasing amount of sequence and functional data, we required more scalable and reproducible methods. The recently popularized sequence similarity networks (SSNs), allows to cope with very large families and computation of many subfamily schemes. Still, the choice of the optimal SSN subfamily scheme only relies on expert knowledge so far, without any data-driven guidance from within the network. In this study, we therefore decided to investigate several network properties to determine a criterion which can be used by curators to evaluate the quality of subfamily assignments. The performance of the closeness centrality criterion, a network property to indicate the connectedness within the network, shows high similarity to the decisions of expert curators from eight distinct protein families. Closeness centrality also suggests that in some cases multiple levels of subfamilies could be possible, depending on the granularity of the research question, while it indicates when no subfamily emerged in some family evolution. We finally used closeness centrality to create subfamilies in four families of the CAZy database, providing a finer functional annotation and highlighting subfamilies without biochemically characterized members for potential future discoveries.

## Author Summary

Proteins perform a lot of functions within living cells. To determining their broad function, we group similar amino-acid sequences into families as their shared ancestry argue for shared functionality. That's what we do in the CAZy database, which covers >300 Cazbohydrate-Active enZyme families nowadays. However, we need to divide families into subfamilies to provide finer readibility into (meta)genomes and guide biochemists towards unexplored regions of the sequence space. We recently used the popularized Sequence Similarity Networks (SSN) to delineate subfamilies in the large GH16 family, but had to entirely rely on expert knowledge to evaluate and take the final decision until now, which is not scalable, not eough automated and less reproducible. To accelerate the construction of protein subfamilies from sequence similarity networks, we present here an investigation of different network properties, to use as indicators for optimal subfamily divisions. The closeness centrality criterion performed best on artificial data, and recapitulates the decisions of expert curators. We used this criterion to divide four more CAZy families into subfamilies, showed that for others no subfamilies exist. We are therefore able to create new protein subfamilies faster and with more reliability.

67 **Keywords**

68 CAZy, carbohydrate active enzymes, sequence-based protein classification, protein subfamilies,
69 SSN, sequence similarity network

## Introduction

The amount of newly produced genomic data increases exponentially. This leads to various challenges in how to treat this data, including how to store and analyze it. Another of these challenges is the functional annotation of such data. Ideally, functional annotation is obtained after the experimental demonstration of protein activity, but due to the deluge of data this is impossible (1,2). Computational annotation is performed instead in the vast majority of cases. Many methods are available to annotate genomes (2), and most rely on the assignment of sequences to groups of homologs. This is followed by the transfer of the annotation of experimentally characterized proteins to non-characterized group members. In this context, several levels of granularity can be considered from superfamilies to subfamilies. Superfamilies group many remote homologs that display a similar fold but can largely vary in their molecular functions/specificities, only allowing to have a very general annotation but for many proteins (3). In contrast, subfamilies tend to group very few but closely-related sequences, offering a more reliable/precise annotation but only when at least one member has been biochemically characterized. For the delineation of subfamilies, sequence similarity networks (SSNs) have become increasingly popular (4–6). SSNs consist in running pairwise comparisons (typically BLASTp) between all family members, and select all matching pairs satisfying a given criterion (typically an e-value cutoff, sometimes with coverage requirements) to draw a network in which the connected components would be subfamilies. The main advantage of SSNs compared to phylogeny is their ability to cope with large families for which the alignment computation and results are problematic. Their main limitation is that (i) they require the exploration of the many possible cutoffs and (ii) the critical step for the determination of the optimal solution fully relies on human expertise. While expert knowledge is arguably one of the very principles of science, it lacks the scalability required to deal with nowaday's amount of data and the reproducibility for sharing scientific advances. For experts, SSN tools do not necessarily produce human-friendly visualization of the results, making the selection of a suitable network, out of all possible SSNs, a real challenge. As curators of a specialist resource, the Carbohydrate-Active enZyme (CAZy) database, one of our objectives is to enhance the predictive power of our protein annotation, since our community relies on our annotation and expert knowledge to build hypothesis leading to future discoveries (7). Due to the large amount of CAZy families (currently >300) and the existence of very large and multifunctional families, there is an urgent need to make the subfamily investigations more performant, as well as reproducible. We therefore decided to investigate currently available methods and indicators from which the optimal SSN can easily and reproducibly be derived by expert curators. We investigated various clustering methods, dimensionality reduction techniques and various network properties, which indicate grouping and spread of nodes within the network. We applied these methods to artificial datasets, as well as to the recently published SSN of glycoside hydrolase family GH16 (8) and to various families from the structure function linkage database (9). We show that the *closeness centrality* criterion is a fast and relevant indicator for optimal subfamily delineations. It allows the curator to identify a few possibly optimal networks out of often more than a hundred produced in SSN computations. We furthermore show that in some cases multiple

levels of subfamilies could be possible, whereas, in others, no subfamilies exist. After determining this as the appropriate method, we generated and analyzed the networks of nine CAZy families, accelerating our efforts in subfamily creation.

## Results

Our goal was to identify an objective criterion to determine optimal SSN e-value cutoffs, given that our assumption is that an optimal cutoff should maximize the number of connections within the subnetworks/subfamilies, without having any connections towards the other subnetworks/subfamilies. We initially investigated various network properties on artificial data and identified the *closeness centrality* as the best suited. This criterion was used to reanalyze already published data based on real families of protein sequences, showing overall good agreement. We further compared SSN results to clustering and dimensionality reduction (see supplementary results), and confirmed the better suitability of the SSNs. We therefore applied this criterion to guide the analysis of subfamilies for several CAZy families, glycoside hydrolase family GH19, GH51, GH45, GH54, GH55, GH68 and GH130 and the polysaccharide lyase family PL26.

### Closeness centrality is the best indicator on artificial data

Artificial data was generated (see Methods) to simulate a protein sequence family composed of at least one, or two, subfamilies. In real data, such family would appear as a large network at high e-value thresholds, as all members display a detectable similarity to all others, either directly or by transitivity. With the decrease of the e-value cutoff, edges successively disappear leading to the disconnection of single-nodes or subnetworks which should correspond to subfamilies (Figure 1).

Figure 1: Illustration of closeness centrality behaviour on artificial data.Closeness values were averaged over all nodes using two perfect subnetworks of size 50, which sequentially get connected by edges. As it can be seen in A and B, each perfect subnetwork separately reaches a closeness centrality of 1, as well as the combined network C. In all three cases no new edges have been added yet. This value increases with each added edge, slighthy with one or two edges (D and E), to intermediate values (e.g. F, 200 edges), up again to a maximum value if all edges are connected at G. The critical point is between C and D. While only one component is present in D, it gets evaluated a lot lower than C, despite having more edges.

Our artificial data thus consisted in *network collections*. Each *collection* is designed to simulate the SSNs of a family for increasing e-value thresholds, one *network* corresponding to one threshold and containing more edges than the previous and less than the next. Hence all networks in the *collection* are constructed upon two identical subnetworks but could be distinguished by the number of edges between the subnetworks (interconnections but no

151 intraconnections), from one to all possible connections. The different *collections* should
152 simulate families with distinct evolutionary properties, from one (the main network without
153 any subfamilies) to many subfamilies, and correspond to the combination of different types of
154 subnetworks (from fully-connected to single-node subnetworks).

155 We investigated the behavior of several classical network properties like degree centrality,
156 betweenness centrality and closeness centrality on the *artificial* data. The ideal property should
157 reach its global optimum for a network, which maximizes the connections within its
158 subnetworks, and shows no connections to other subnetworks. In our artificial setting, it should
159 indicate as local optima both the networks consisting of (i) all connections between the two
160 subnetworks and (ii) the two disconnected subnetworks. The desired behavior was obtained
161 from *closeness centrality* (with *wf_improved* parameter set to False) (Figure 1). Especially this is
162 interesting to note the partially logarithmic behavior of closeness centrality for the lowest
163 number of connections, which thus scores extremely low. *Betweenness centrality* (and its
164 variations, listed in the methods) showed a similar behavior in some network combinations, but
165 was discarded after investigating its behavior in combination with single-node networks.
166 Indeed, *Betweeness centrality* weights singles as perfect networks, which is not a desired
167 behavior, since this would favor strongly disconnected networks. A network consisting out of
168 only singles would be evaluated to be as good as a perfect network, which is not what we were
169 looking for. All other criteria behaved either similarly or identical to one of these (one similar to
170 closeness centrality, four similar to betweenness, where betweenness centrality source, load
171 centrality and betweenness behave identical), were monotonous in their behavior (e.g. two
172 perfect networks with a single connection was evaluated better than a single perfect network;
173 five in total), or not quantitative enough (e.g. asynchronous label propagation indicating one or
174 two subnetworks; three criteria). The remaining four other criteria were (i) the eigenvector
175 centrality and the clustering coefficient, where the former showed parabola-like behavior and
176 the latter an inverted parabola behavior, therefore scoring better with less and more edges
177 than with a medium number of edges, which is the opposite of the desired behavior; (ii) edge
178 load centrality and global reaching centrality, which both partially showed a bimodal
179 distribution. This means that with these both algorithms either both less and more edges would
180 be favored or disfavored. An overview over all behaviors on two networks with a size of 50 can
181 be seen in supplementary Figure S3.

182 Based on artificial data results, we therefore opted to use *closeness centrality (*with
183 wf_improved set to False), applied to each subnetwork, as criterion for further evaluation.

184 ***Closeness centrality* supports findings by expert curators**

185 To confirm the relevance of closeness centrality, it was applied to determine the optimal
186 cutoffs in published SSN analysis by several independent research groups, based on real protein
187 family datasets, to compare with the cutoff determined by expert curators. We created the
188 SSNs of one Interpro family, three SFLD families, and two CAZy glycoside hydrolase families.

189 We first compared the closeness centrality performance to an analysis that used the popular
190 SSN tool EFI-EST (5). We selected one among its most recent citations in PubMed (September

191 2020), and with easily reproducible methods (10). In this publication about the domain
192 IPR037434, Travis *et al.* identified three relevant cutoffs including $10^{-55}$ corresponding to 5
193 subfamilies with more than 3 members. We generated SSNs from $10^{-5}$ to $10^{-100}$ (steps of $10^{-5}$),
194 on which *closeness centrality* computations show a local optimum for an e-value of $10^{-60}$
195 (difference possibly influenced by the increase in the database size; 228 sequences in Travis et
196 al., 376 in this investigation), which corresponds to 10 families with more than 3 members (51
197 subfamilies total). A consensus probably resides between those two.

198 For SFLD family 159 of tautomerases (11), the authors concluded first on an e-value of $10^{-11}$ for
199 subfamily assignment, but due to the great diversity in their sequences also further investigated
200 an e-value of $10^{-18}$ to define 18 subfamilies. The closeness centrality computations show local
201 optima at e-values of $10^{-9}$ and $10^{-18}$, the latter displaying 15 subfamilies with >100 members.
202 However, an absolute maximum was obtained at $10^{-34}$, although this would result in 12 more
203 subfamilies (with >100 members), and more than 2000 smaller networks. For SFLD subfamily 19
204 of glutathione transferases (12), two cutoffs were identified by the authors, $10^{-13}$ and $10^{-25}$, the
205 second level allowing more detailed inspections. The closeness centrality computations for this
206 family peak at $10^{-14}$ and $10^{-29}$, with an absolute maximum at $10^{-44}$. For SFLD family 122 of
207 nitroreductases, the published cutoff of $10^{-18}$ (13) also corroborates with the first local
208 optimum of closeness centrality computations at $10^{-19}$. The global optimum was still not
209 reached at $10^{-69}$, where we stopped our computations.

210 For our own recent SSN analysis of family GH16 (8), an e-value of $10^{-55}$ was published (SSNs
211 generated in increments of $10^{-5}$), while closeness centrality revealed a peak at $10^{-50}$. Similarly,
212 for GH130 (14), the authors determined the separation at $10^{-70}$, corresponding to 15 relevant
213 subfamilies (>20 members). Closeness centrality reaches a peak at $10^{-75}$, resulting in 16 such
214 relevant subfamilies. The subfamilies in CAZy are created according to the numbering of (14),
215 with the omission of families purely derived from metagenomic data (UC4 and UC6) replaced by
216 additional subfamily we discovered (GH130_10 and GH130_12).

217

218 ***Closeness centrality* accurately points to suitable novel subfamily schemes**

219 Closeness centrality was therefore applied to analyze SSNs generated for several CAZy families
220 for which no subfamily scheme was designed yet. The family selection was based on potential
221 interest for on-going projects, as well as on the diversity and total number of functional
222 annotations available. Functional annotations are essential indicators to decide which scheme
223 will be the most relevant and durable, among the several optimal options suggested by
224 closeness centrality.

225 We first investigated the small GH55 family (1052 sequences after fragment filtering). This
226 family also display low functional diversity as all characterized members breakdown b-1,3-
227 glucans (mainly tested on laminarin), with the activity only differing by the mode of action: exo
228 (EC 3.2.1.58), endo (EC 3.2.1.39), or undetermined (appearing in CAZy as laminarin-degrading
229 enzymes, EC 3.2.1.-). Many characterized enzymes were described by Bianchetti and

230 collaborators (15) who hypothesized that most members should be exo-acting. In their analysis
231 of 177 GH55 proteins, the reconstructed phylogeny suggests three clades: a fungal, a bacterial
232 and a divergent (bacterial) clade. The closeness centrality values for the generated SSNs
233 indicated a local optimum at $10^{-12}$ and the global optimum, close to maximum, at $10^{-29}$ (Figure
234 2). At $10^{-12}$, two subfamilies emerged, one corresponding to the bacterial clade, the second
235 gathering the fungal and "divergent" clades previously described. At $10^{-29}$, the fungal and
236 divergent subfamilies split, resulting in a nearly perfect closeness centrality value. The two
237 distinct modes of action did not segregate among the three subfamilies, given both are
238 retrieved in the bacterial and in the fungal subfamilies, while the divergent subfamily does not
239 include any characterized representative. As a consequence, GH55 will be divided into
240 subfamilies 1 to 3, by ascending size order, which correspond respectively to the bacterial,
241 fungal and divergent subfamilies.

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244 Figure 2: Closeness value and corresponding networks of the GH55 SSN analysis. A) At $10^{-5}$ the
245 network is still a single network, and subnetworks become visible. The closeness value is in a
246 medium range. B) The closeness value reaches its lowest point at $10^{-11}$. The two subnetworks
247 within GH55 are only sparsely connected. C) At $10^{-12}$ the two subnetworks are disconnected,
248 and the closeness value increases. D) The closeness value reaches another low at $10^{-28}$, and two
249 other subnetworks are clearly visible, only sparsely connected with a single edge. A single
250 disconnected node has emerged. E) At $10^{-29}$ three nearly perfect subnetworks are visible, and
251 the closeness value is nearly perfect with a value of 0.99.

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255 **Multiple levels of subfamilies are possible**

256 We investigated another small CAZy family, GH68 (1510 sequences after filtering). Three
257 enzymatic activities were reported in this transglycosidase family (i.e. able to not only
258 breakdown but also assemble glycans depending on the concentration of the substrate and
259 product of the reaction): invertase (b-fructofuranosidase; EC 3.2.1.26), inulosucrase
260 (sucrose:fructan 1-fructosyltransferase; EC 2.4.1.9) and levansucrase (sucrose:fructan 6-
261 fructosyltransferase; EC 2.4.1.10). In this family, closeness computations never reached the
262 initial maximum of the fully-connected network ($10^{-5}$). However, several local optima could be
263 considered notably at $10^{-51}$, $10^{-124}$ and $10^{-154}$ corresponding to closeness centrality values of
264 0.93, 0.95 and 0.96 (Figure 3 C, G and H). At $10^{-51}$, the family would be divided into two
265 subfamilies of similar size. At $10^{-124}$, both former subfamilies get split into two (all having more
266 than 150 members) while only one subfamily with >20 members emerge (plus 17 tiny groups
267 including 5 singletons). At $10^{-154}$, two more large groups emerge from the former four families.

At the functional level, at $10^{-51}$, the first subfamily gathers EC activities 2.4.1.9 and 2.4.1.10, whereas the second displays 2.4.1.9, 2.4.1.10 and 3.2.1.26 (including one protein with dual activities 2.4.1.9 and 2.4.1.10, as well as one with dual 2.4.1.10 and 3.2.1.26). At $10^{-124}$, each of the five main subfamilies include a characterized member, but most activities are shared these subfamilies, for example with 2.4.1.10 found in subfamilies 1 to 4; 2.4.1.9 in subfamilies 2, 4 and 5; and 3.2.1.26 in subfamilies 2, 3 and 5. At $10^{-154}$, 9 of the 19 subfamilies include a characterized member, five of which display a single activity. However, this activity is not unique but shared (see supplementary table 2), given the split is mostly driven by the taxonomy, as at $10^{-124}$. Therefore, GH68 will be divided into two subfamilies following $10^{-51}$ threshold, based on the absence of functional information gain, and the increasing number of unclassified sequences at lower optimal thresholds (none at $10^{-51}$ versus 74 and 96 at $10^{-124}$ and $10^{-154}$ resp., that is >6%).

Figure 3: Closeness value and corresponding networks of the GH68 SSN analysis. Unlike in the GH55 analysis, the best network is less well determinable. A) At $10^{-5}$ the network is one solid component, and no subnetworks are visible. B) at $10^{-50}$ the closeness value is lowest, and two sparsely connected networks are visible. C) At $10^{-51}$ these two networks are disconnected, and the closeness value increases. D) to H) show the closeness values and networks at $10^{-111}$, $10^{-113}$, $10^{-123}$, $10^{-124}$ and $10^{-154}$ respectively. D and F show undesirable connections with sparely connected subnetworks, whereas E, G and H are alternative desirable network configurations which could be used to determine subfamilies.

We then analyzed the larger (>10,000 sequences after filtering) GH51 family. This family displays so far four different EC numbers on a total of 83 characterized enzymes, 76 sharing the same a-L-arabinofuranosidase activity (EC 3.2.1.55), five with an endo-b-1,4-glucanase activity (EC 3.2.1.4) and two bifunctional enzymes: an a-L-arabinofuranosidase/xylan 1,4-b-xylosidase (EC 3.2.1.55 and EC 3.2.1.37) and an endo-b1,4-glucanase/endo-b-1,4-xylanase (EC 3.2.1.4 and EC 3.2.1.8). The *closeness centrality* criterion showed again multiple possible optimal SSNs, at $10^{-58}$, $10^{-91}$ and $10^{-147}$, corresponding to closeness values of 0.71, 0.67 and 0.79 that are lower than previously analyzed families (Figure 4 A, C and D)). At $10^{-58}$, the network divides into two large subnetworks (5712 and 3960 sequences) and one small network (114 sequences), hereafter referred as to subfamilies 1 to 3, and 55 smaller subnetworks (<100 sequences). Both subfamilies 1 and 2 contain enzymes with EC 3.2.1.55 activity, and subfamily 2 also contained the bifunctional enzyme EC 3.2.1.55 and 3.2.1.37. Proteins in subfamily 2 often display a small conserved domain of unknown function (annotated in the Gene3D superfamily 2.60.120.260) in the N-terminal region, unlike the enzymes in subfamily 1, further supporting the split between these groups. Subfamily 3 contains all five characterized enzymes with the EC number 3.2.1.4,

307 including the bifunctional enzyme with EC 3.2.1.4/3.2.1.8. Most enzymes in subfamily 3 also
308 contain a CBM domain from families known for binding cellulose (CBM2, CBM11, CBM30),
309 which are not present in subfamilies 1 and 2. A small subfamily (55 sequences) also showed a
310 different domain architecture as most members contain a CBM66 module, and some are fused
311 to a GH127 enzyme. These features are not seen in any of the other subfamilies.

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314 Figure 4: Closeness value and corresponding networks of the GH51 SSN analysis. As with GH68,
315 multiple configurations could be of interest. A) The network is still only a single component. B)
316 at $10^{-57}$, two main groups are sparsely connected, and the closeness value reaches its lowest
317 point. C) At $10^{-58}$ these networks are disconnected, and the closeness value increases. D) $10^{-147}$
318 represents the highest peak after $10^{-58}$, and many more possible subfamilies are visible. All
319 represented networks have been scaled down to 25% of their nodes, to make visualization
320 possible. The network of A) was scaled down to 10%.

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323 At $10^{-91}$, subfamily 1 and 3 split into two subfamilies, both containing characterized enzymes
324 with similar activity/EC numbers. The split of subfamily 3 seems driven by the phylogeny, and
325 resulted in 139 unclassified sequences as result of the split of subfamily 1. As a consequence,
326 based on e-value range and functional specialization, 12 subfamilies of GH51 were build
327 according to the $10^{-58}$ cutoff discussed above, including the three main subfamilies and nine
328 small subfamilies among the 55 (having more than ten members; total 216 sequences) without
329 functional evidence so far.

330 **Iterative SSNs might be necessary**

331 The investigation of family GH45 initially resulted in 2467 modules sequences after fragment
332 filtering. This only corresponded to a reduced fraction (77%) of the 3208 proteins annotated as
333 GH45 and in-depth analysis revealed that the GH45 HMM did not allow to extract complete
334 modules in many cases. Such situation might result from extreme sequence diversity within a
335 family, for which a single model will necessarily be highly degenerated on the most variable
336 regions. An initial SSN was thus built on the 2467 proteins that passed the filter, and the
337 closeness criterion used to determine a first optimal split at $10^{-7}$ allowing the generation of
338 two subgroups. Two HMMs were built (one for each subgroup) and used to successfully extract
339 3200 more complete modules (passing the filter) among the 3208 GH45 sequences. Therefore,
340 after one iteration, a second SSN was built, and analyzed with the closeness criterion.

341 There are three known activities in family GH45, namely endoglucanase (EC 3.2.1.4),
342 xyloglucan-specific endo-β-1,4-glucanase (EC 3.2.1.151) and endo-β-1,4-mannanase (EC
343 3.2.1.78). Notably 13 enzymes display dual 3.2.1.4/3.2.178 activities, one enzyme dual
344 3.2.1.4/3.2.1.151, and another enzyme dual 3.2.1.78+3.2.1.151. A previous analysis identified 3

345 subgroups for this family (16). Closeness centrality identified, after the initial $10^{-6}$ cutoff,
346 another a single additional optimum at $10^{-26}$ (Figure 5). At this cutoff, four major groups (>100
347 sequences; one with approx. 2000, two of ~440-510 and one of ~240) were identified,
348 corresponding to the previously identified three groups (16) and an additional group. This new
349 group (~440 members) consists nearly exclusively of Basidiomycetes sequences, which are
350 globally more distant from other GH45 sequences, and have no characterized member yet. In
351 the previous publication by (16) this group has not been seen, due to the small number of
352 available Basidiomycetes sequences (only one sequence present in the previous analysis;
353 XP_761686 in group C). This group did not appear in the first SSN, since the initial HMM did not
354 detect a domain in all of its sequences. The sequences caught in the second SSN round were
355 not limited to this group but also distributed over other small groups, therefore showing that
356 the iterative SSN expanded multiple groups. No separation of EC numbers could be seen, with
357 the largest group containing all three EC numbers, and the three smaller groups containing only
358 the EC 3.2.1.4. Even at low e-values, e.g. $10^{-111}$, no full separation of the three activities could
359 be obtained.

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362 Figure 5: Closeness value and corresponding networks of the second GH45 SSN analysis. A) At
363 $10^{-5}$ the network is still one component, although sparsely connected. B) At $10^{-6}$ the two
364 subnetworks are disconnected and the closeness value increases. C) Another subnetwork has
365 emerged, and two more are visible. D) At $10^{-26}$ the closeness value reaches its absolute
366 maximum at 0.95 and four major subnetworks are visible.

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369 **Functional versus structural separation into subfamilies**

370 Family GH19 was investigated, following a recent publication about its putative subfamilies
371 (17). We obtained 11,290 sequences after filtering, with in total two different activities,
372 chitinase (EC 3.2.1.14) and lysozyme (EC 3.2.1.17) across 97 characterized enzymes. Closeness
373 centrality identified multiple possible optima for subfamily separation (Figure 6). The first
374 optimum at $10^{-26}$ (closeness of 0.57) splits the family into two main subgroups which display a
375 single and specific activity, as well as 5 smaller subfamilies (>=10 sequences) and 0.9%
376 unclassified sequences. Some lower e-value cutoffs lead to higher values of closeness centrality
377 ($10^{-58}$ reaches 0.58; $10^{-76}$ reaches the global optimum with 0.77) and would theoretically result
378 in a better subfamily separation. It however leads to much more subfamilies (62 and 88 total
379 with more than 10 members resp.), much more unclassified sequences (7% and 13% resp.),
380 while no further separation of functions was observed, since known functions are already
381 optimally split at $10^{-26}$. As discussed in (17), deeper subfamily splits result in a separation of
382 structural features, and not of functions. We therefore decided to split GH19 into only two
383 subfamilies.

Figure 6: Closeness value and corresponding networks of the GH19 SSN analysis. As with GH68 And GH51, multiple configurations could be of interest. A) The network is still mostly a single component. B) At 10E-26 two main networks are visible. C+D) 10E-58 and 10E-76 are other optima, showing a vast amount of subnetworks. All networks have been scaled down to 10% for visualization purposes.

**Not all protein families have subfamilies**

While we were able to detect subfamilies for several families, some did not seem to display subfamilies based on the available data, such as families PL26 and GH54. For PL26 we obtained 1657 sequences after filtering, with only one characterized enzyme (EC 4.2.2.24). The closeness centrality value started perfectly at 1 for an e-value threshold of $10^{-5}$ and decrease very slowly up to 0.95 before a local optimum (closeness back to 1) at $10^{-46}$, where one small group of 21 sequences emerged (Figure 7). This group did not contain any known activities, and consisted out of 20 sequences from the phylum Bacteroidetes and one of the phylum Balneolaetoa. At the highest e-value, $10^{-179}$, the PL26 network would still consist out of one main subnetwork of 1632 nodes, the small subnetwork of 21 nodes that appeared at $10^{-46}$, two linked sequences and two singles.

Figure 7: Closeness value and corresponding networks of the PL26 SSN analysis. A) The small red network is split off the main blue network B) Final network as depicted below the graph, at an evalue of $10^{-179}$.

For GH54 we obtained 1594 sequences after filtering, including 20 sequences with known activities (19 with α-L-arabinofuranosidase, EC 3.2.1.55; one with dual α-L-arabinofuranosidase and β-xylosidase, EC 3.2.1.37 activities). In this case, closeness centrality also starts at 1 for the highest e-value cutoffs, stays stable until $10^{-67}$ (closeness centrality of 0.99) and slowly decreases until $10^{-131}$ (closeness of 0.70) before raising back to a local optimum at $10^{-139}$ (closeness of 0.92; Figure 8). At this lowest cutoff, the largest subnetwork contains 1376 out of 1594 sequences, while the 216 remaining sequences spread over 25 smaller subnetworks (maximum size 86; only four with more than ten sequences). These subgroups follow

taxonomic splits, and no functional division could be seen, since all characterized enzymes were contained in the main network.

We therefore concluded that based on the available data, and given the absence of characterized members among emerging small groups, no subfamily should be created for both PL26 and GH54.

Figure 8: Closeness value and corresponding networks of the GH54 SSN analysis. A) The network is a single component, no subnetworks are visible. B) The closeness value changes slightly, and two disconnected nodes emerge. C) The closeness value reaches its first local minimum. While subgroups are visible, they are all still connected. D) The closeness value reaches its only peak. The main network still contains 86% of all sequences, and the smaller networks all follow phylogeny. E) At the maximum evalue of $10^{-179}$ various smaller networks emerge, yet no sensible bigger subnetworks are visible.

## Discussion

The goal of this work was to identify an objective and performant criterion to facilitate the exploration of SSNs by expert curators during an analysis of subfamily delineation. We demonstrated on artificial data that the *closeness* centrality criterion displays encouraging behavior, given that it favors perfect subnetworks, and penalizes sparsely connected networks, which is exactly what is desired for protein subfamilies translated into a SSN. We showed that the suggested optimal values are extremely close to those manually chosen by expert curators in previously published analyses, who sometimes identified several possible thresholds/subdivisions based on phylogenetic levels. In most cases, closeness centrality also identified several optima. In the analyzed families, optima of lowest e-values reflected the taxonomic separation of the sequences and likely strict orthologous groups, while more functionally-relevant splits were obtained at higher thresholds. This likely reflect the evolution of proteins with ancient duplication leading to neofunctionalization bringing fitness to various surviving species, while taxonomic groups are accentuated by species survival/extinction, and sometimes by a bias in genome sequencing. The existence of several optima still raises the question of the limitation of defining a single level of “subfamily”. A more general framework with flexible number of sub-, subsub-, or more levels of classification might be relevant to have. This would allow (i) a better coverage of the sequence space, as many sequences cannot be assigned to a subfamily at lower thresholds; and (ii) finer functional prediction, associated to the diversity of the functions in the subdivision, hence broader at higher thresholds. In some cases, this might also depend on the scope of the database. For the specialized GH19 database (17), the authors chose to annotate multiple levels of subfamily, corresponding to first

457 functional and later structural differences. The CAZy database is primarily interested in the
458 functional differences, and we therefore decided that one subfamily-level annotation is
459 sufficient, although this might depend on the families or enzyme classes. Therefore, we only
460 implement one subfamily hierarchy level, as do many other specialized databases like MEROPS
461 (18), ESTHER (19), or SulfAtlas (20). The subfamily division is however not applicable to all
462 families in a systematic/automatic manner, as it requires in-depth analysis to ensure long-term
463 stability of the proposed classification, the availability of enough characterized members and
464 human decision when several optima are found. This highlights the need to support such expert
465 databases, which are dedicated to mine the functional annotation of (meta)genomes in the
466 literature, as such effort can no longer be supported by universal databases.

467 For GH55, we found an agreement to prior analysis (15). Bianchetti et al. Identified, in a large-
468 scale screening of GH55, three major subfamilies. Two of these families contained functional
469 enzymes, and were divided into a fungal and a bacterial family, with the third family containing
470 non-functional bacterial proteins. These three families correspond to the three groups
471 identified by our SSN investigation, although we did not further subdivide based on phylogeny,
472 as Bianchetti et al. did (15). In this case we can not only show that SSNs are able to delineate
473 functionally different enzymes, in some cases they also might be able to delineate non-
474 functional enzymes, as seen by the identification of a subfamily where no functions could be
475 found even after testing. This potentially allows to save time in future endeavors by ruling out
476 known non-functional proteins.

477 Our analyses also globally agreed with prior investigations of families GH68 (21), GH51 (22) and
478 GH45 (16). In case of GH68, this family would separate into two subfamilies and does not result
479 into any unclassified sequences. A possible further in-depth classification, as also suggested by
480 Velazquez-Hernandez, could be considered in the future, in case new functions are discovered
481 in this more distant and small groups.

482 For GH51, three main subfamilies, corresponding to the three main clades identified in (22),
483 will be created, as well as nine smaller ones. The two biggest subfamilies, representing 96% of
484 the data, are distinguished by a structural feature in the sequences. One protein in one of the
485 subfamilies contains a minor side function. Further biochemical characterization is necessary to
486 see if this is a property of the whole subfamily and if it might be a distinguishing factor between
487 the both major subfamilies. The third major subfamily represents a distinct function, whereas
488 the fourth is of unknown function, but is characterized by the presence of a carbohydrate
489 binding domain, indicating considerable differences within this family.

490 The GH45 case differs from the previous cases as none of the subfamilies demonstrated
491 separate biochemical functions. Yet four subfamilies will be implemented, since this family
492 seems to be very diverse, and the initial HMM for the detection of GH45 did not allow a full
493 detection of module sequences. The subfamily HMMs yield an improved performance and
494 better detection, making their implementation therefore necessary.

495 For some families, we were not able to identify any sensible subgrouping. We hypothesized
496 that two or more groups could exist in GH54, because most enzymes in GH54 contained only

497 one function, but one enzyme from *Trichoderma koningii* has been deemed to have an dual
498 activity (additional β-xylosidase activity) (23). The SSN analysis indicated that no such major
499 subgroups exist though. While reviewing the literature, it appears that this β-xylosidase activity
500 is only a minor side-activity, which also was reported for other enzymes in this group before
501 (24,25). We therefore conclude that no subgroups exist in GH54, and that potentially all
502 members might have a dual activity.

503 Family PL26 was included in our analysis, since it is the largest PL family, for which no
504 subfamilies were previously created (26). After the creation of the SSN, we were unable to
505 detect any major subfamilies. Given that only one protein has been characterized so far, it
506 might be that this family truly does not contain any more functions or subfamilies.

507 A limitation of the *closeness centrality* is that it is independent of the amount of subnetworks
508 and of their size. Two perfect subnetworks of the size 50 will give the same outcome as 50
509 perfect subnetworks of size 2. An option would be to penalize subnetworks below a certain
510 size, typically below the required family size, by downgrading their values e.g. assigning a value
511 of 0 as for singlets. While this might be necessary in case many different networks are built at
512 the same time, in most circumstances a manual evaluation by the curator with the amount of
513 networks in mind should suffice.

514 An unexpected limitation which we encountered is the usage of the e-value for the generation
515 of SSNs. Most researchers decrement the e-value to generate their SSNs (4,5). The e-value is a
516 floating point value, where reaching 0.0 is considered to be the most significant value,
517 indicating a very good match, most often resulting in perfect matches. The e-value is dependent
518 on database size and query length though. In case of too large families, or very long protein
519 domains (e.g. PL26s average is 867 AA), the e-value will often result in 0 (see supplementary
520 Figure S3, with an overview of e-values per percentage identity), due to the limit of $10^{-308}$ for a
521 64-bit floating point value with double precision (IEEE 754 standard for floating point
522 arithmetic, (27) ; the equivalent limit for 32-bit is $10^{-38}$). Distribution of e-values can often be
523 seen with a maximum of $10^{-160}$ or $10^{-180}$, with more significant values being 0 for long domains.
524 For these long domains this can lead to many hits, which identity percent can reach fairly
525 low/limit values between 20-30%, and yet lead to an e-value of 0. In case of PL26, e-values of 0
526 were obtained at worst with a sequence identity of 32%. Bitscores (or a combination of identity
527 percent and coverage control), should rather be used instead of e-values in case of unusually
528 long sequences.

529 We also tried to evaluate a proposed mechanism for network filtering from the field of
530 neuroscience (28), but this proved to be computationally infeasible, with runtimes of more than
531 3 days for comparatively small networks from our collection. We furthermore hoped to aid our
532 annotation efforts with well-known algorithms for grouping data points, like clustering or PCA,
533 despite them not being favored in earlier research (29). It turned out that even for rather
534 simple group combinations with rather little sequences, like two or four groups in GH68, these
535 algorithms are already severely challenged (see supplementary results). At this point it needs to
536 be evaluated if the results from the other techniques like PCA are correct, and the results based

on *closeness centrality* might be incorrect. A manual inspection with Cytoscape (30) proofed to be efficient and simple for the small SSNs, and confirmed that the results obtained by the evaluation of *closeness centrality* are indeed correct. Overall, these results from the other techniques make our exploration of other criteria even more worthwhile. We hope that the *closeness centrality* metric will be used during other research projects in this field, to combine expert knowledge with reproducible technical measures.

## Materials and Methods

### Generation of artificial data

Artificial data was generated with the Python networkX 2.4/5 library (31).

We first used the network generators to create different *subnetworks* (to be connected during the second step): (i) two perfect networks (all possible edges between all nodes exist) of size 50 and 200, using the *nx.complete_graph* function; six random networks of size 200 with 300, 500, 1000, 2000, 3000 and 4000 edges (representing from 1.5% to 20.1% of a complete graph) using the *nx.gnm_random_graph* function; and two networks consisting out of 50 or 200 single nodes, or singletons.

Secondly, we generated network *collections*. A *collection* consists in multiple *networks* build upon the same initial pair of *subnetworks* (described above; see Table 1). A *collection* starts with a first *network* simply consisting in the two disconnected *subnetworks*. The collection is completed by additional *networks* obtained by randomly connecting the nodes of one *subnetwork* to the other with an increasing number of edges, from one to the maximum possible (see Supplementary Figure S1A). This means that a combination between a *subnetwork* of size 50 and another of size 200 would result in a *collection* that contains 10,001 *networks*, one without connection and then from a single connection between a random node of each *subnetwork*, up to the connection all 50 nodes of the first *subnetwork* to the 200 nodes of the second *subnetwork*. In case of two perfect *subnetworks*, the lastly created *network* in the *collection* would also be a perfect network.

In the specific case of a single-node *subnetwork* combined to a random or perfect *subnetwork*, two additional strategies of connections were performed to better explore the space of topologies, while ending in the same number of *networks* in the *collection*: 1) all single nodes (initially one and lately all) were first randomly connected to nodes of the other *subnetwork*. Once all connected, a second edge was added to each of these nodes, and this was repeated until all connections were made (see Supplementary Figure S1B); 2) a random node of the single-node *subnetwork* was first connected to all nodes the other *subnetwork* (initially to one and lately to all), and only afterwards a second single node was connected in the same way, until all connections were made (see Supplementary Figure S1C).

Not all possible *subnetwork* pairs were used to generate *networks/collections*, as only the following cases were considered: perfect network of size 50 randomly connected to all possible

575 *subnetworks* (single node of size 1, 50 and 200; perfect subnetworks of size 3, 50 and 200,
576 random networks of size 50 with 250 and 500 edges, and random networks of size 200 with 500
577 to 4000 edges) and single node networks (size 50 and 200) connected to random networks of
578 size 200 with 500 to 4000 edges with the three connection strategies. An overview is given in
579 supplementary table 1.

580 **Network properties**

581 Before the execution of any computation, the networks were converted to an undirected
582 graph. Afterwards, various network properties were calculated, using networkX, and
583 investigated for local and global minima and maxima. These 19 properties include the number
584 of components, edges, s-metric, average degree centrality, average closeness centrality (with
585 the parameter *wf_improved* - allowing to cope with disconnected components - either True and
586 False), average betweenness centrality, average degree, average clustering, eigenvector
587 centrality, betweenness centrality source, edge betweenness centrality, load centrality, edge
588 load centrality, subgraph centrality, harmonic centrality, global reaching centrality, the amount
589 of local bridges, the shortest path and asyn_lpa_communities (32,33). For the *artificial* data, all
590 these properties were calculated, while CAZy families GH16 (8), GH19 (17), GH45, GH51, GH54,
591 GH55, GH68, GH130 (14) and PL26, as well as different families of the Structure Function
592 Linkage Database (SFLD) (3), SFLD families 19, 122 and 159 and Interpro IPR037434, were
593 investigated with the closeness criterion only (see results).

594 **Generation of SSNs**

595 Sequence similarities on SFLD and CAZy families were computed with Blastp v2.5 (34). Standard
596 parameters were used, besides the parameter *max_target_seqs*, which was scaled to the size of
597 the family/target database. The Blastp pairwise hits, based on e-value cutoff, were transformed
598 into a digraph with the python library networkX v2.4/2.5 (31), while sequences without any hit
599 were added as single nodes. We generated SSNs for each e-value cutoff, from $10^{-5}$ up to $10^{-160}$,
600 by step of $10^{-1}$. Exceptions to these start- and end-points were made when either the network
601 did not form any more subnetworks at high cutoffs, or when the network disintegrated into too
602 many subnetworks (typically >1000) at low cutoffs.

603 All networks were visualized with Cytoscape version 3.7.2 (30). Graphs showing the evolution of
604 the network properties were generated with Matplotlib (35-37).

605 GNU parallel version 20161222 has been used during this and other steps of this study (38).

606

607 **EFI-EST**

608 The EFI-EST website (39) was used to reproduce the published IPR037434 family analysis,
609 similarly to (10). An initial e-value cutoff of $10^{-5}$ was used, and subnetworks were produced
610 based on this network by decreasing the e-value cutoff by steps of 10-5 until the maximum
611 selectable e-value of $10^{-100}$. Steps of $10^{-1}$ were prohibitive in an online-tool without the

612 possibility of automation, and edge values were not included into EFI-EST SSNs during our first
613 tests.

614 For reading xgmml files, networkxgmml v0.1.6. was used
615 (https://pypi.org/project/networkxgmml/).

616

617 **Extraction of domain sequences**

618 SFLD sequence data for families 19 of glutathione transferase (12), family 159 of tautomerase
619 (11), and family 122 of nitroreductase (13) were downloaded from
620 http://sfld.rbvi.ucsf.edu/archive/django/superfamily/index.html (9). For these families the full
621 protein sequences were used.

622 Data for glycoside hydrolase for families GH16, GH19, GH51, GH54, GH55, GH68, GH130 and
623 polysaccharide lyase family PL26 were directly extracted from the CAZy database (40), and
624 were further supplemented with data by collaboration partners. Domain sequences were
625 extracted from the full proteins using in-house pipeline based on the results of *hmmscan*
626 function in HMMer3.3 (41) as follows. HMM searches using the in-house CAZy HMM profiles
627 were performed and only matches with an e-value <1-4 were further processed. The sequence
628 and HMM coordinates were extracted in an attempt to reconstruct a domain sequence from
629 fragments created by the local detection of HMM searches. Two consecutive fragments were
630 assembled only in the given conditions: (i) sequence start coordinates must be separated by
631 >20 amino-acids, as well as sequence end coordinates; (ii) sequence end of the first fragment
632 should not be separated from the start of the second fragment by >200 amino acids; (iii) HMM
633 coordinates must not overlap (end of the first vs start of the second) by more than 30 amino
634 acids. Any domain sequence, initially selected or reassembled, shorter than half of the HMM
635 length was discarded. A manual inspection was performed for sequences longer or shorter than
636 the average sequence length +/- three standard deviations, to discard gene models issues. In all
637 cases >95% of the original input sequences passed the screening, except for GH45 (see Results
638 section). For the construction of the second SSN for GH45, domains were extracted as
639 described but using two GH45 HMMs. In case both HMMs identified a domain in the same
640 protein, the longer domain was used.

641

642 **Minimized version for visualization**

643 Since many of the generated networks were too large to be visualized in Cytoscape, and
644 preliminary clustering seemed to warp network structure in some cases, networks for
645 visualization were generated differently. A custom and modified version of uniform edge
646 sampling with graph induction was implemented (42), to generate representative smaller
647 graphs from the full graph.

To retain network structure, subgraphs were generated per connected component. Components smaller than a chosen node amount (in most cases 10) were retained as they were. Larger components were sampled down to a specified percentage (in most cases 25 or 50%; exact values specified per figure), with respect to the minimum amount of nodes of small components. The subsampled graphs, initially empty, were completed by random edges (Python *random.choice* function) and their nodes sequentially until the minimum amount of nodes was reached. At last, all edges connecting the sampled nodes in the initial component were added as well (42). In case a component was split by the random subsampling, reconnection was realized as follows. Iterating over all split sub-components, the shortest path algorithm (as implemented in networkX) was used to determine the shortest path between these selected nodes based on the initial component, and all necessary edges were iteratively added. The computation was stopped when the component was again fully connected.

## Data availability

New subfamilies are published on the CAZy website, www.cazy.org.

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## Conflict of interest

The authors declare that there are no competing interests associated with the manuscript.

## Author contributions

Conceptualization: BVHH

Data curation: BVHH, NT

Formal analysis: BVHH

Funding acquisition: NT

Investigation: BVHH

Methodology: BVHH, NT

Project administration: NT

Resources: NT

Software: BVHH

Supervision: NT

Validation: BVHH

Visualization: BVHH

Writing – Original draft preparation: BVHH, NT

Writing- Review and editing: BVHH, NT

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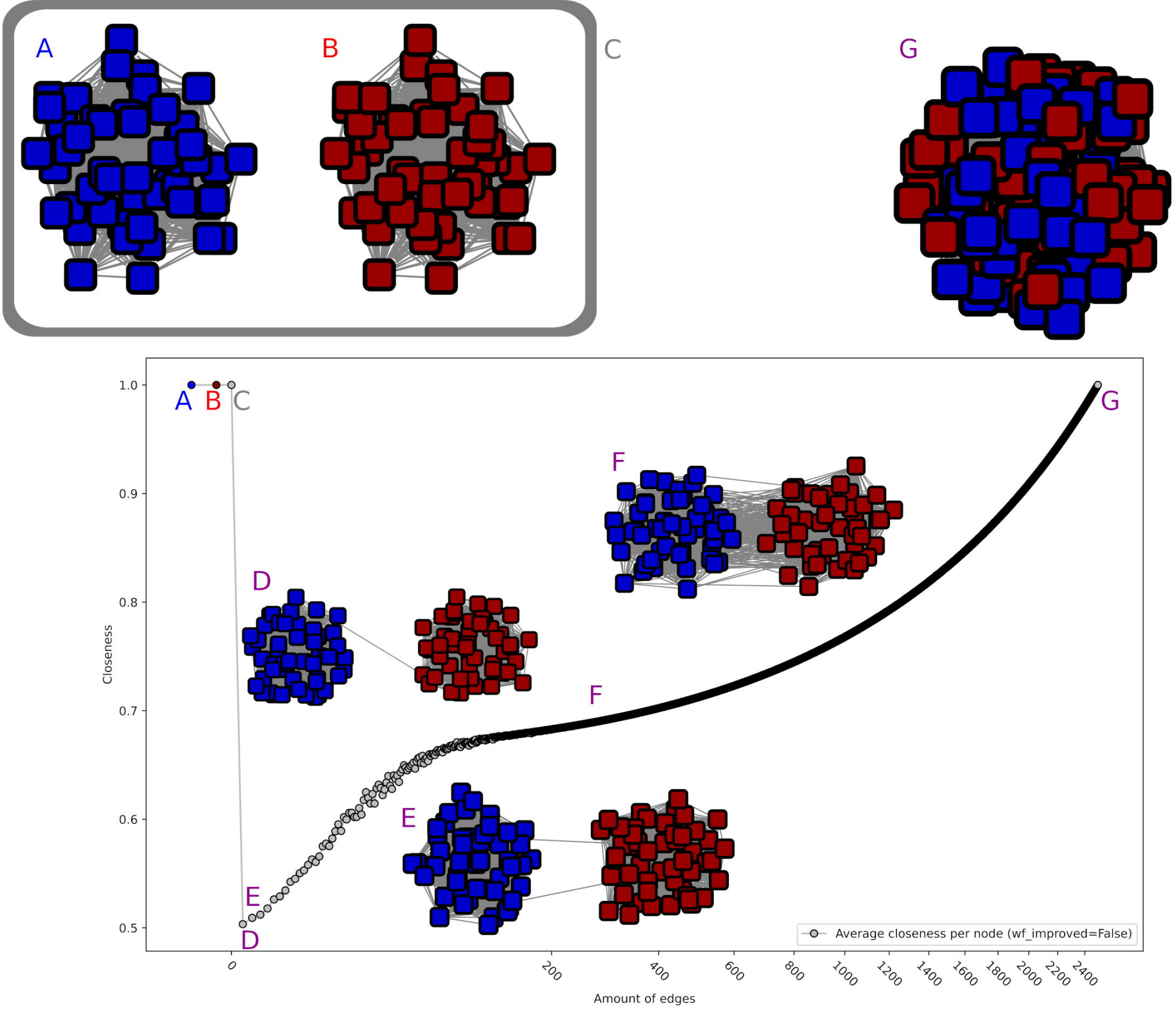
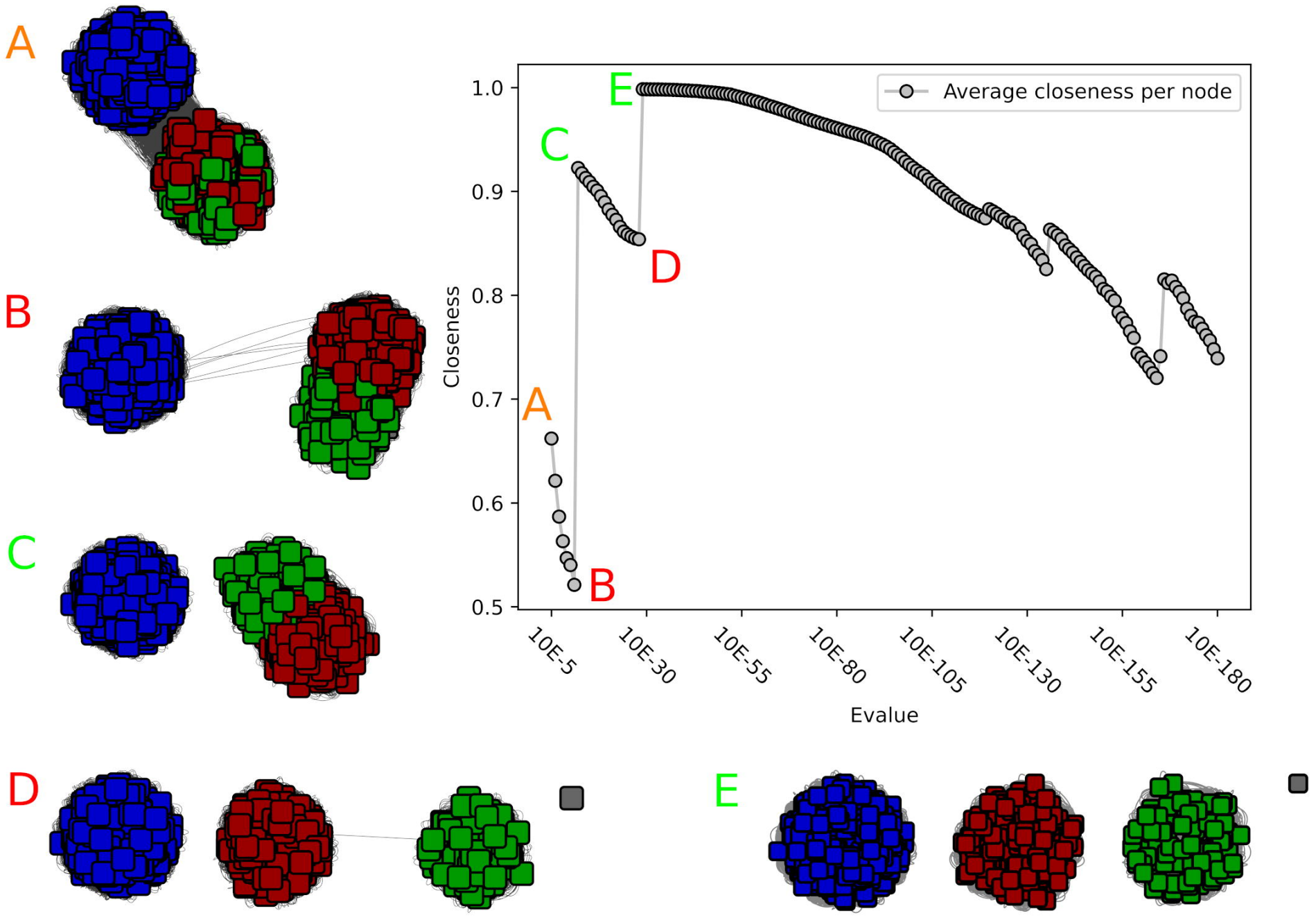
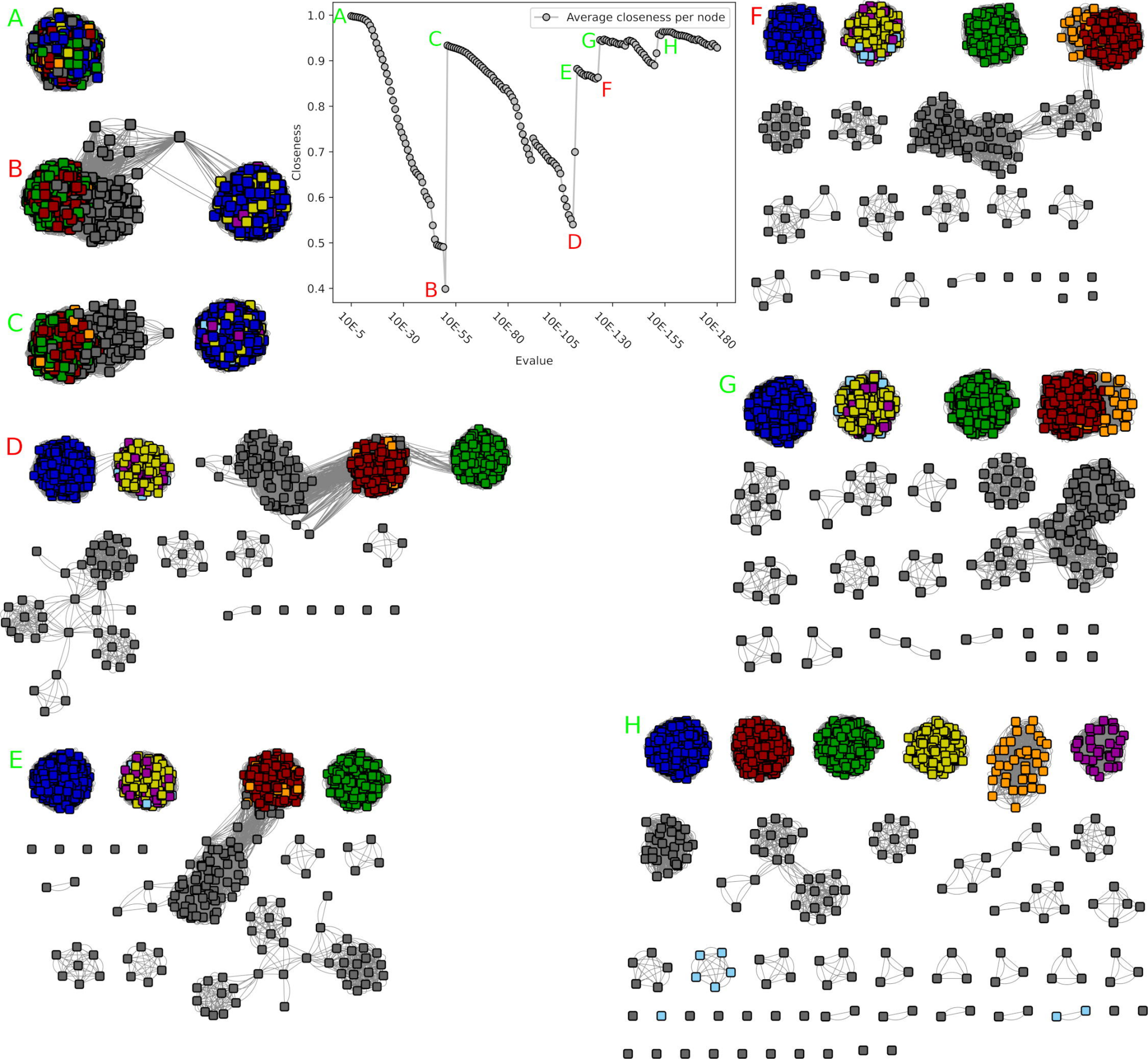
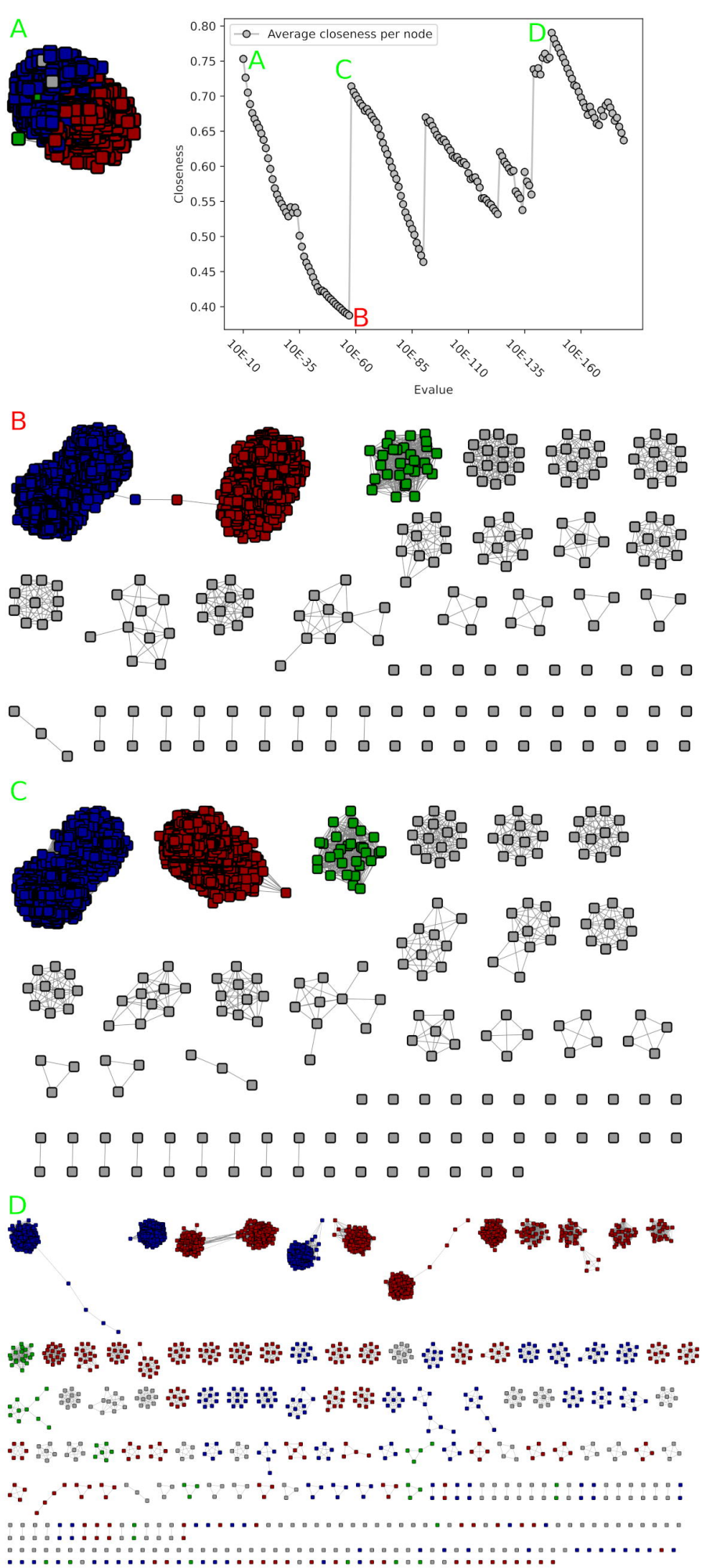
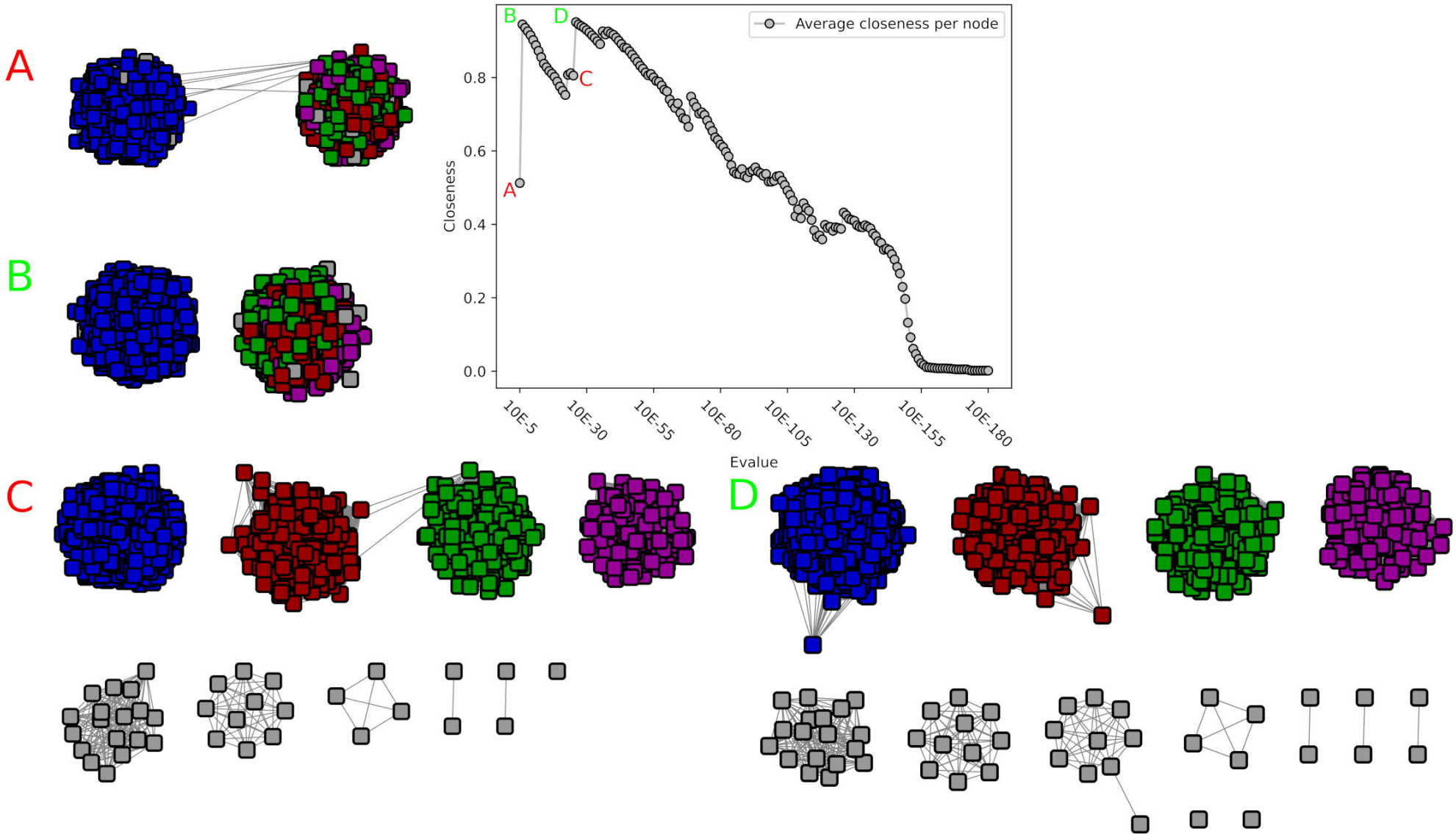
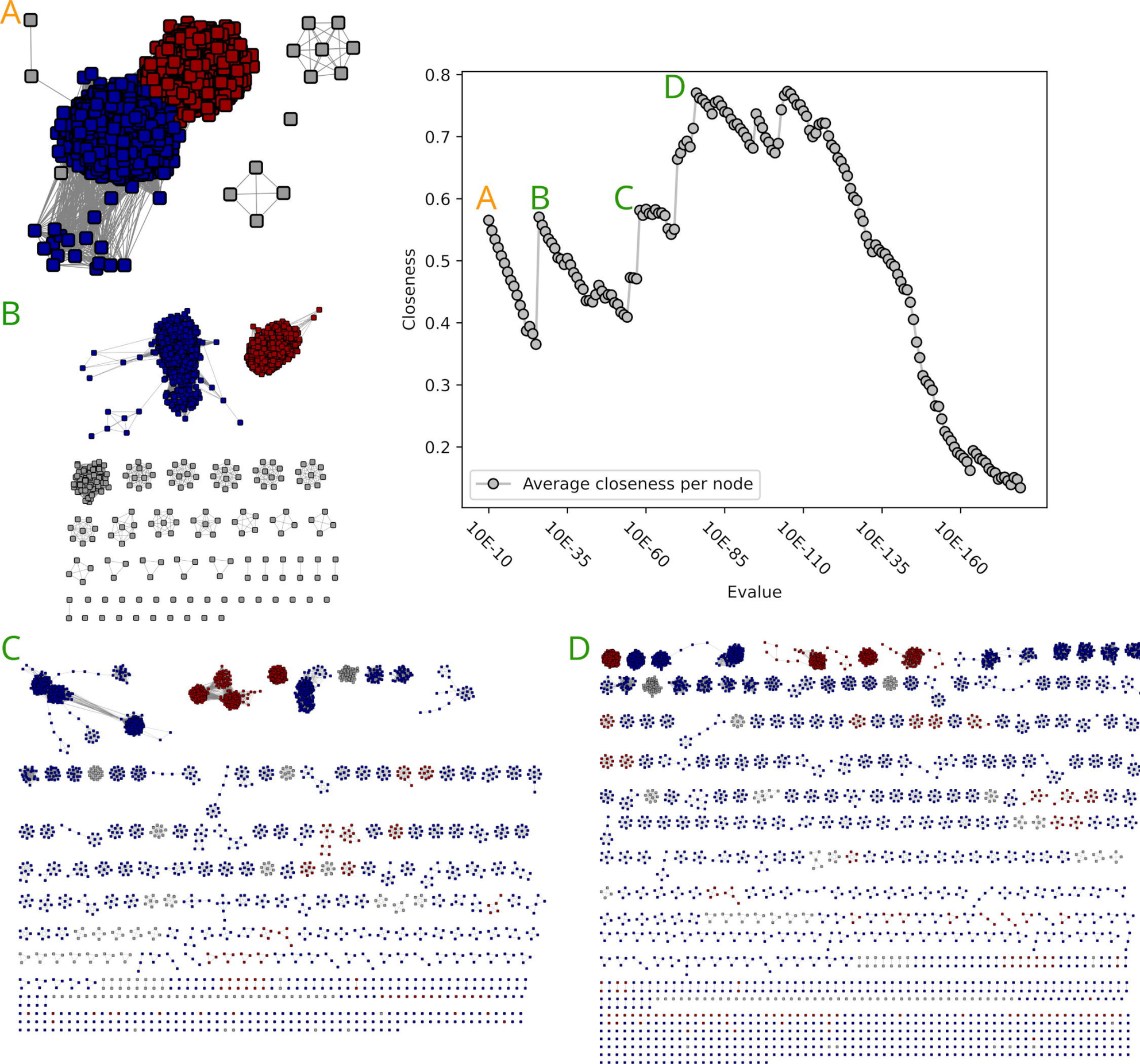

A
B
C
G
F
D
E
Closeness
Amount of edges
Average closeness per node (wf_improved=False)
1.0
0.9
0.8
0.7
0.6
0.5
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400

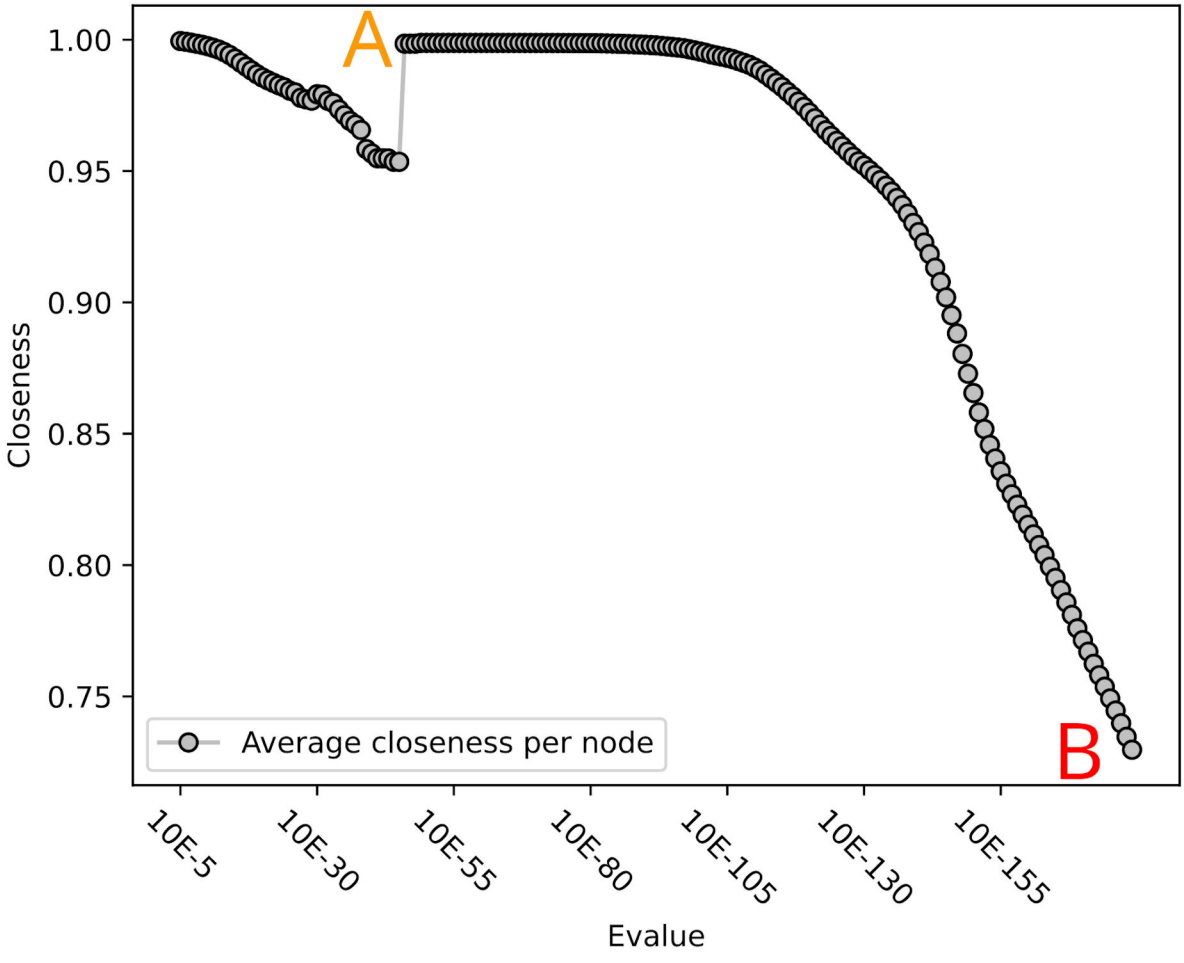

A
B
C
D
E
Average closeness per node
Closeness
1.0
0.9
0.8
0.7
0.6
0.5
10E-5
10E-30
10E-55
10E-80
10E-105
10E-130
10E-155
10E-180
Evalue

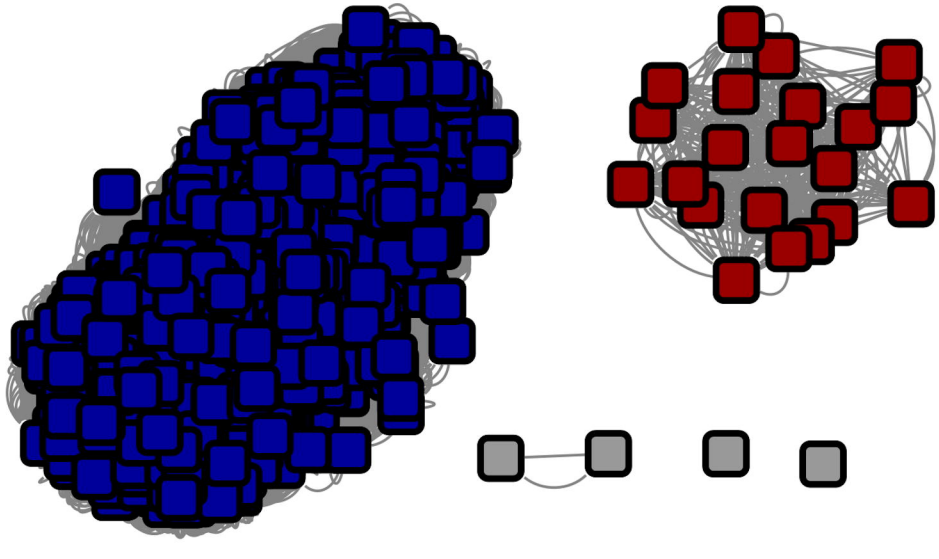

A
B
C
D
E
F
G
H
Average closeness per node
Closeness
Evalue
1.0
0.9
0.8
0.7
0.6
0.5
0.4
10E-5
10E-30
10E-55
10E-80
10E-105
10E-130
10E-155
10E-180

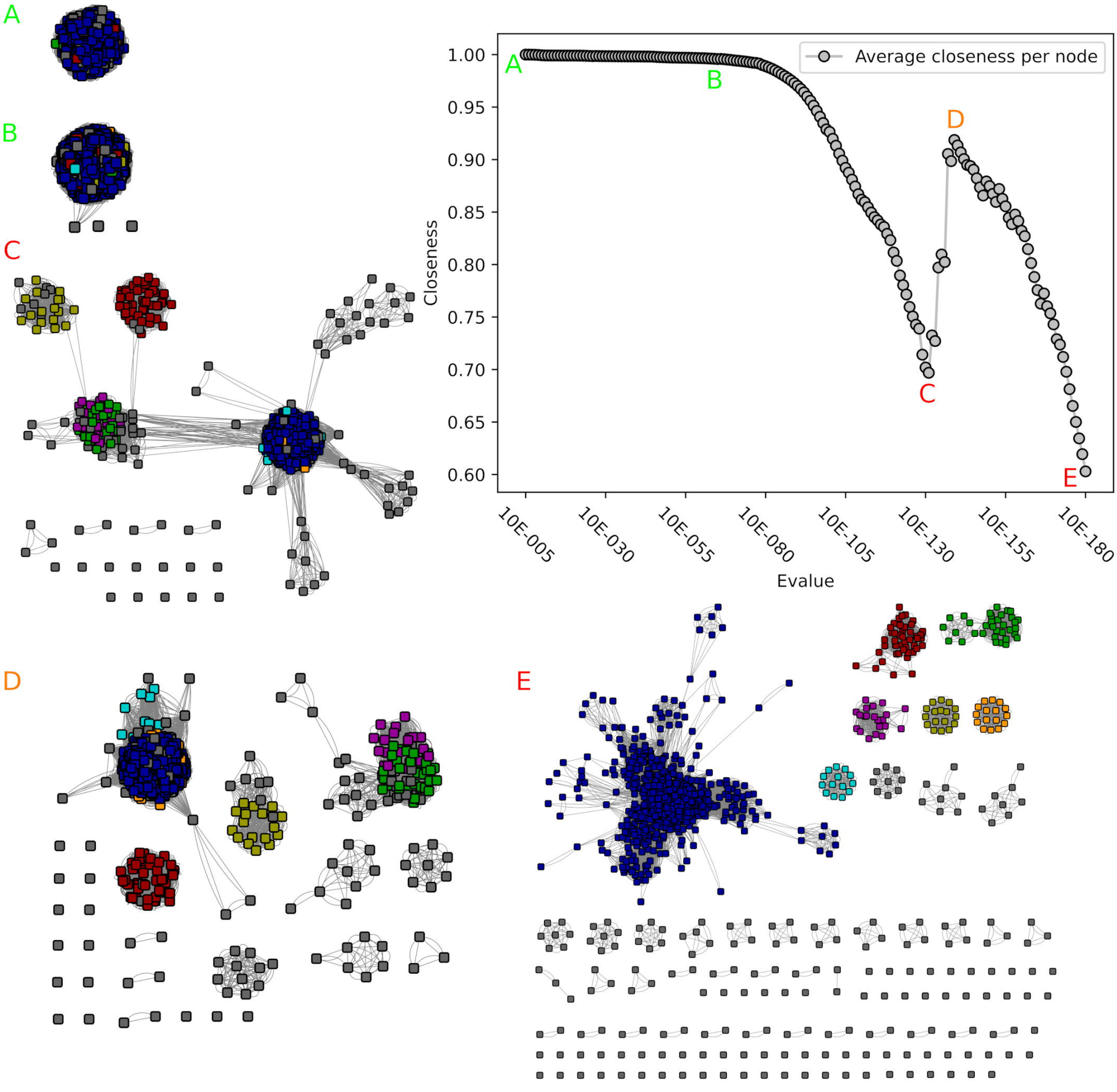

A
B
C
D
Average closeness per node
Closeness
Evalue
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
10E-10
10E-35
10E-60
10E-85
10E-110
10E-135
10E-160


A
B
C
D
Average closeness per node
Closeness
Evalue
0.0
0.2
0.4
0.6
0.8
10E-5
10E-30
10E-55
10E-80
10E-105
10E-130
10E-155
10E-180


A
B
C
D
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Closeness
Average closeness per node
10E-10
10E-35
10E-60
10E-85
10E-110
10E-135
10E-160
Evalue


A
B
1.00
0.95
0.90
0.85
0.80
0.75
Closeness
Average closeness per node
10E-5
10E-30
10E-55
10E-80
10E-105
10E-130
10E-155
Evalue




A
B
C
D
E
Average closeness per node
Closeness
Evalue
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
10E-005
10E-030
10E-055
10E-080
10E-105
10E-130
10E-155
10E-180