ViCloD, an interactive web tool for visualizing B cell repertoires and analyzing intra-clonal diversities: application to human B-cell tumors

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High throughput sequencing of adaptive immune receptor repertoire (AIRR-seq) has provided numerous human immunoglobulin (IG) sequences allowing specific B cell receptor (BCR) studies such as the antigen-driven evolution of antibodies (soluble forms of the membrane-bound Ig part of the BCR). AIRR-seq data allows researchers to examine intra-clonal differences caused primarily by somatic hypermutations and affinity maturation. Exploring the diversity of antibodies and understating how they evolve could help elucidate the generation of antibodies with high affinity or broadly neutralizing activities. Consequently, retracing their evolutionary history could also help to clarify how vaccines or pathogen exposition drive the humoral immune response. Computational methods are necessary for large-scale evaluation of AIRR-seq properties. Currently, there is no efficient and interactive tool for analyzing intra-clonal diversity, permitting users to explore AIRR-seq data and potentially discover discriminating clonal features. We developed ViCloD, a web server for large-scale visual analysis of repertoire clonality and intra-clonal diversity. ViCloD uses data preprocessed by IMGT and performs evolutionary and statistical analyses, producing a set of plots. The web server presents diverse functionalities, including repertoire navigation, clonal abundance analysis, and intra-clonal evolutionary tree reconstruction. Users can download the analyzed data in different table formats and save the generated plots as image files. ViCloD is a versatile analytical tool that can help researchers and clinicians in their studies involving B cell clonal diversity. It is user-friendly, simple, accessible, and open to all users. Moreover, its pipeline is optimized to process hundreds of thousands of sequences in a few minutes, allowing intra-clonal diversity analysis in large and complex repertoires.

Availability and implementation: The web server is available at http://www.lcqb.upmc.fr/viclod/. The pipeline is available at github and allows users to run analyses locally.

key words: B-cell immune repertoire, intra-clonal diversity, B cell lineage trees, immunoinformatics

1 Introduction

B cells are critical components of the adaptive immune system, recognizing and eliminating various foreign molecules (antigens). B cells recognize antigens through a specific protein complex, the B cell Receptor (BCR), attached to the cell surface. The BCR is made up of a recognition unit formed by an immunoglobulin (IG) protein and an associated signaling unit, the Igα an Igβ molecules. Besides this membrane-bound form, IG can also be secreted in the blood plasma as circulating antibodies.

The immune system can respond to almost any antigen to which it is exposed, partially through the vast diversity of BCRs expressed by an individual's B cells. Such diversity lies in the variable region of IG and results from complex genetic mechanisms that occur during B cell ontogeny. This process, called VDJ recombination (*1*), joins different types of genes contributing to the variable region: V (variable), D (diversity) and J (joining). The resulting combinatorial diversity is further enhanced by a junctional diversity due to the imprecise joining of these genes with deletion and insertion of random nucleotides at the recombination sites, thereby creating an extremely diverse zone within the variable region, the complementary determining region 3 (CDR3) (*2*). Further diversity occurs after the antigen encounter, with a process called affinity maturation which occurs in specialized structures of secondary lymphoid organs (*3*). There, point mutations are introduced in the variable region of IG genes at very high rates, about 10^6 times higher than observed for other genes, by a mechanism termed somatic hypermutation (SHM) (*4*), a robust protective response against the antigens in case of infection. This phenomenon is known as clonal expansion and plays a fundamental role in an efficient immune response (*5*). Therefore, the generation and selection of somatic antibody mutants are decisive for increasing antigen affinity, contributing to the B cell immune repertoire diversity.

The adaptive immune receptor repertoire sequencing (AIRR-seq) has greatly contributed to characterizing immune repertoires, potentially revealing the high-dimensional complexity of the immune receptor sequence landscape. AIRR-seq data is often used to address many scientific and clinical questions. For instance, an extremely diversified BCR repertoire reflects the expected diversity in healthy individuals. However, such diversity can be significantly affected by different factors such as autoimmune diseases (*6–12*), allergy (*13–15*), cancer (*16, 17*), and aging (*18, 19*). The exploration of

AIRR-seq data is thus an active field of research, mainly to analyze BCR composition and diversification. Analyzing mutational sequence patterns of a given clonal lineage can provide insights into the diversification and selection processes that lead to B cell clonal evolution. The examination of (intra-)clonal compositions can also help in understanding the response to infections (*20*), vaccines (*21, 22*), the mechanisms of autoimmune diseases (*23*) and allergy (*24*), among others. Rapid advances in high-throughput sequencing have opened up new possibilities for studying B cell clonal expansion and (intra-)clonal compositions. With decreasing costs of DNA sequencing technology, numerous IG sequences with inherent complexity, variability, and mutational load are accessible, including in clinical contexts. Thus, the availability of AIRR-seq data has motivated researchers with different backgrounds (biological, computational, and statistical) to investigate and examine the adaptive immune complexity.

Several methods and computational tools have been developed to treat different AIRR-seq analysis steps, producing multiple integrated context-specific software (*25–28*). Most of the tools were designed for V(D)J assignments (*29*), and identification of groups of clonally-related sequences (*30–32*). Some solutions exist to tackle and determine the heterogeneity of BCR repertoires (*28, 33–36*). Such tools quantify the diversity of a BCR repertoire by estimating clonal group sizes, characterizing CDR3 regions, identifying the most used V, D, and J genes (alleles), and the most frequent VJ combinations. However, few existing tools allow analyzing intra-clonal diversity/evolution of a B-cell repertoire (*37–41*). Such analyses can potentially reconstruct antibodies' evolutionary (clonal) development and identify mutable positions that correlate with antigen-binding sites. Most existing methods are stand-alone programs requiring computational skills for installation and pre-processing tasks such as clonal grouping and lineage tree inferences. Moreover, they not provide interactive plots that could help users to decipher their data rather than static outputs. Thus, we believe that a large-scale, time-efficient and versatile AIRR-seq tool for deeply analyzing (intra-)clonal diversity and evolution is currently lacking.

We propose ViCloD, a web-based interactive tool for visualizing B cell receptor repertoire clonality and analyzing (intra-)clonal diversity in B cell repertoires. ViCloD enables entire repertoire

analysis and proposes a flexible repertoire topology panorama. Users can navigate among B-cell clonal lineages and explore their intra-clonal diversity. For this purpose, we have developed a set of algorithms able to analyze millions of IG sequences within a few minutes (*30, 42*). Such algorithms are time efficient and compatible with both research and clinical settings. Users can download all analyzed data, save the generated plots, perform their analyses locally, and visualize them in the ViCloD webserver. ViCloD is a user-friendly data visualization to help the research/medical community select helpful information in BCR repertoire analyses. It allows non-computational experts to analyze (intra-)clonal diversities that could boost their research within the immune repertoire investigation. ViCloD provides unique functionalities absent in other webservers dedicated to AIRR data processing, analysis, and visualization. It is a versatile AIRR-seq tool with the potential to help experimental/clinical investigations of immune repertoires. To demonstrate its functionalities and potential, we applied it to repertoires of human B-cell tumors. However, ViCloD can be used to investigate B-cell clonal expansion and intra-clonal compositions of any repertoire.

2 Material and Methods

2.1 ViCloD workflow

The ViCloD workflow is presented in Figure 1; it consists of five main steps: user's input, Data processing (pipeline), repertoire composition, clonal composition, and lineage architecture, described below.

2.1.1 User inputs

ViCloD can analyze BCR Immunoglobulin Heavy Chain (IGH) repertoires. In practice, IGH is frequently used as a signature for BCR sequences since it is more diversified than IGL chains. IGH sequences contain the CDR3 region that plays a key role in antigen recognition (*2*). After sequencing, IGH sequences should be annotated to determine their IGHV, IGHD, IGHJ genes and localize their CDR3 region. ViCloD accepts data from IMGT/HighV-QUEST (*43*), the international standard web software for parsing BCR sequences, see Figure 1-A. In principle, the output of any V(D)J annota-

tion tool could be used as long as it provides the minimum information necessary for the analysis, see http://www.lcqb.upmc.fr/viclod/Format. ViCloD requires AIRR formatted input files (*44*). Users can upload the AIRR file on the main page of the ViCloD webserver and enter their email address to receive a link to their outputs. The analysis is relatively fast; for instance, it can take around three minutes to analyze a monoclonal repertoire containing around 300K sequences. Indeed, the computational time can vary depending on the structure of the sample (the size/amount of clonal lineages and sequence mutations). Alternatively, users can upload ViCloD formatted files, processed and downloaded from a previous analysis. These files were generated either by the web server or by the stand-alone version. Thus, users can store and re-analyze their data without any time constraints.

2.1.2 Data processing

Once IGH sequences are uploaded, the first step is to group them into clonally-related sequences. We use MobiLLe (*30*), a multi-objective-based clustering for inferring B-cell clonal lineages from high-throughput AIRR-seq data. Then, within each B-cell clonal lineage, detected by MobiLLe, we group sequences with the same IGHV, IGHJ genes, and identical CDR3 amino acid sequences, forming *sub-clones*. Note that we adopted the IMGT (amino acid) clonotype definition (*45*). For each clonal lineage, we try to reconstruct its evolutionary tree by using the N most abundant sub-clones. We used ClonalTree (*42*), a time-efficient and accurate approach to reconstructing B-cell lineage trees that incorporates genotype abundances into minimum spanning tree algorithms. ClonalTree requires a file in FASTA format containing sequences and their abundances as input. Each sequence in the input file represents a sub-clone. Since sequences in a given sub-clone can differ due to SHM, we select the most abundant sequence to represent it. The abundance required by ClonalTree is the total number of sequences within a sub-clone. ClonalTree also requires the hypothetical ancestral unmutated sequence (HAUS) representing the tree root. To infer it, we consider the IGHV and IGHJ germline sequences provided by IMGT/HighV-QUEST. For the junction part, we selected the sequence with the lowest number of mutations on the IGHV gene compared to the germline, then we extract its junction region. Finally, we concatenated the three parts (IGHV, IGHJ, and junction region) to obtain the HAUS. The

Data processing pipeline is illustrated in Figure 1-B.

2.1.3 Repertoire composition

We used nested circles to represent B-cell clonal lineages within a repertoire, Figure 1-C. The outer circle represents the entire BCR repertoire, while internal circles correspond to B-cell clonal lineages. The size of each circle correlates to the clonal lineages' abundance. A bar plot on the same web page shows the abundance of the ten most predominant clonal lineage. Users can choose between a standard or logarithmic scale; they can also select a threshold of clonal lineage abundance when analyzing the clonality of a repertoire, for instance, above 5%. When such a threshold is set, only B-cell clonal lineages with an abundance greater than this value are retained in the BCR repertoire visualization; all others are hidden. A table at the bottom of the page summarizes some essential properties of each inferred B-cell clonal lineage, such as the identifier, the abundance, the IGHV/IGHJ gene annotations, the CDR3 amino acid sequence and the functionality status of the most abundant clonal lineage sequence.

2.1.4 Clonal composition

Users can explore clonal composition by clicking on one of the circles representing a B-cell clonal lineage. The circle expands and other circles, representing sub-clones, appear (see dotted arrow in Figure 1-CD). Note that we label only the sub-clones with abundance greater than 1%. The circle area correlates with the sub-clone abundance within the B-cell clonal lineage; see panel "Clonal lineage population" in Figure 1-D. For the five most abundant B cell lineages, a circular B-cell lineage tree generated by ClonalTree is displayed, representing the evolutionary relationships among such sub-clones and the HAUS. Several options for tree representation are available. For instance, nodes can be colored by sub-clones, a different color for each one, or by functionality, where green and red nodes represent productive and unproductive rearrangements, respectively. Note that unproductive sequences contain stop codon(s). It is also possible to display the length of tree branches representing distances between sequences. The HAUS is highlighted by a triangle and the most abundant sub-clone by a square to facilitate tree interpretation. At the bottom of the page, additional information about

sub-clones, such as their identifiers, abundances, and CDR3 amino acid sequences are displayed.

The lineage tree inferred by the ClonalTree algorithm can represent (at most) 200 of the most abundant sub-clones. Interpreting the complete lineage tree can be challenging for large groups containing many sub-clones. Thus, we propose two simplified tree topologies while conserving nodes with high abundance and their path until the root. We developed two strategies for pruning the tree (*46*) for a more comprehensible visualization, Figure S1. These simplified versions may be more valuable than an entire tree that involves many details. The first pruning strategy transforms the tree shown in Figure S1-A in the tree of Figure S1-B; it eliminates nodes with no descendant or an abundance lower than a predefined threshold (by default 0.1%). This approach is more helpful for trees containing few nodes. Trees with many sub-clones can require the second pruning strategy that eliminates nodes with low abundance if they present high similarity with more abundant sub-clones (Figure S1-BC). For both strategies, we eliminate just leaf nodes and keep the N most abundant sub-clones in the simplified tree (by default, N=10). We try to preserve the tree's evolutionary paths by eliminating only leaves' nodes, which could be destroyed if an internal node is removed. We keep the N largest sub-clones since they contain relevant information to analyze the lineage tree. We apply the first strategy once, and on the resulting tree, we repeat the second one several times until achieving at most 30 nodes. Users can navigate between the complete lineage tree and pruned trees on the website.

2.1.5 Lineage architecture

The last ViCloD module provides more details about the B-cell lineage inferred tree, Figure 1-E. Users can explore the simplest tree to examine intra-clonal diversities. ViCloD provides four types of representations: an interactive tree, a circular bar chart (mutational profile), a CDR3 logo, and a multiple sequence alignment.

Interactive lineage tree

We kept the most pruned tree with at least N=10 most abundant sub-clones to better examine the lineage tree. Colored circles represent observed sub-clones, while white circles represent unobserved

nodes, which represent unobserved sequences that were not sampled or disappeared during affinity maturation (cf Clonaltree algorithm). A triangle represents the HAUS. The branch length represents the number of SHM between the representative sequences of connected sub-clones or the HAUS. We identify sub-clones by a number, sorted by their abundance, which means that sub-clone 1 is the most abundant in the B-cell lineage. Nodes representing the five largest sub-clones have a bold border in the tree and are enumerated from 1 to 5. Sub-clone details (identifier, abundance, and functionality) appear when passing the mouse over nodes. The option ”display abundance” shows each sub-clone abundance by increasing the node's diameter. In this visualization mode, nodes are labeled with their abundance (%) rather than with a sequential number. Each sub-clone may contain multiple unique sequences (clonotypes). Choosing ”Display proportion of unique sequence” will turn each node into a pie chart, illustrating the numerical proportion of unique sequences within the sub-clone. A pie chart for each node can be viewed on a separate page by right-clicking on the node and selecting “See the distribution of clonotypes” (Figure S2).

Circular bar chart - Mutational profile

The circular bar chart, also termed as ”Mutational profile”, shows the cumulative path distances for a set of selected sub-clones; By default, ViCloD displays the five most abundant sub-clones, but any sub-clone can be included and/or removed from the plot; a maximum of eight items are displayed. Each colored section is related to a sub-clone and represents the number of mutations observed between a given sub-clone and its parent in the tree. To highlight the tree path from a sub-clone until the root, users should hover the mouse over their desired sub-clone identifiers; to highlight a branch in the tree, one can hover the mouse over each section of the circular bar; it also displays the number of mutations between the pair of connected sub-clones.

Multiple sequence alignment

To illustrate the conservations/mutations between the representative sequence of each sub-clone and the HAUS, we build a multiple sequence alignment (MSA) with the MUSCLE program (*47*). For each

sub-clone, we display in a table: the identifier, the percentage and number of reads, the divergence rate (number of mutations) between the sub-clone representative sequence and the HAUS, and the percent identify of IGHV sequence when compared to the HAUS. Only mutated nucleotides of the sub-clone representative sequence are shown in the alignment, whereas a dot represents conserved positions. CDR and framework sections are highlighted with different colors, and the IGHD gene is underlined in each sequence; see an example in Figure 5. Users can sort out sequences in the table based on each column. The table can be downloaded into a text file by clicking on the download button. It is also possible to select one, multiple, or all sequences and send them directly to the IMGT/V-quest software. A button on the top-right allows users to visualise a simplified MSA that can make easier its interpretability.

CDR3 logo

Sequence logos provide a more detailed and precise description of sequence similarities and are frequently used to unravel significant features that are harder to visualize in the multiple sequence alignment. In the ”Lineage architecture” page, users can generate a sequence logo of the CDR3 region by clicking on the ”CDR3 Logo” button. The logo is split into two sections; the upper one shows the most frequent residues that differ from the HAUS, while the lower section shows the residues of the HAUS. The height of the stack letter represents the frequency of each residue in the alignment, showing how often the HAUS was mutated in the clonal lineage; see an example in Figure 4.

2.2 Implementation

The server-side of ViCloD web-site was implemented in python (version 3) and Bash script. We also use the MySql database (mysql.com/) to control the user's pending jobs. The client-side web interface was implemented using HTML5, CSS, and JavaScript (javascript.com/) along with several libraries, including D3.js (d3js.org/), FileSaver.js (github.com/eligrey/FileSaver.js/), saveSVGAsPNG (github.com/exupero/saveSvgAsPng), PDFKit (pdfkit.org/), and JSZip (stuk.github.io/jszip/). PHP (version 7.4.3, http://php.net/) was used

for interactive data processing. Users submit their input files and can provide an email address to receive their results. We first check if the input file contains all the required information. Then, data are processed by the ViCloD Data processing tools, and all analyses are directly available or by clicking on the link sent by email. Users' files (inputs and outputs) are available for download (during 10 days) and can be retrieved during this time. Users can also download JSON files (`json.org`) that can be input into the ViCloD web server later. It enables data to be stored locally and re-analyzed without time constraints.

2.3 Tutorials

The "Tutorial" section of the website includes a variety of text-based tutorials to help users get started with ViCloD. Multiple analyzed data sets are provided on the "Example" page to assist users in following the tutorials step by step. Several sections under the "Overview tab" provide additional information, such as references, algorithms used, and the server's versions.

2.4 Experimental data

IGH-VDJ rearrangement sequences were obtained from patients with B cell malignancies during standard routine diagnostic or prognostic procedures at the Pité-Salpêtrière Hospital. Informed consent was obtained from patients according to local regulations. Genomic DNA was extracted from peripheral blood or tissue biopsy, and IGH-VDJ next-generation sequencing was performed as previously published (*48*). Four patients with either follicular lymphoma or chronic lymphocytic leukemia were selected based on the findings of intraclonal diversity in their tumor clonal IGH-VDJ gene rearrangements.

3 Results

BCR immune repertoire studies are having a significant impact in a variety of research and clinical areas. Several works have reported a wide range of intra-clonal diversity when analyzing BCR repertoires of patients with different lymphoproliferative disorders (refs). In the following section,

we show how ViCloD can help examine patients' BCR repertoires, providing a set of visualizations for exploratory analysis.

3.1 Clonal heterogeneity in a case of follicular lymphomas

Follicular lymphoma (FL) is a tumor of mature B lymphocytes which typically develop in lymph nodes. Lymphomatous cells initially localize within germinal centers and display a follicular pattern of centrocytes and centroblasts, but later invade the lymph nodes appearing as a diffuse infiltration. FL cells express surface IG which carry high level of SHM in their IG variable regions, comparable with that of normal germinal center B cells (*49*). The process of SHM may continue in lymphomatous cells leading to clonal evolution and emergence of subclones over time (*50, 51*).

We used the IGH repertoire of a patient (labeled patient 1) with FL to demonstrate all types of analyses provided by ViCloD, see http://www.lcqb.upmc.fr/viclod/Analysis/559667. Around 186K sequences from the patient were inputted into ViCloD web-server, but around 46K ($\approx$ 25%) were analyzed after quality control and filtering, see Section 2.1.1. Figure 2-A shows the repertoire composition of the patient 1, with the presence of dominant clonal lineage accounting for more than 94% of the repertoire, annotated with IGHV3-23*01 and IGHJ4*02 genes. Such clonal proliferation is expected in lymphoproliferative disorders. Minor unrelated clonal lineages each at a frequency below 0.2% were also detected, see Table S1. ViCloD allows users to examine the five most abundant clonal proliferation, labeled in Figure 2-A.

Users can click on a given clonal lineage to inspect its composition, see Section 2.1.4. Figure 2-B shows sub-clones for the most predominant clonal lineage C1. For this example, we labeled only six sub-clones, with abundance greater than 1%, but a total of 87 were detected, indicating a high intra-clonal diversity for C1 lineage. Sequences of all sub-clones are productive. Sub-clones C1-1 and C1-2 are the most abundant of the C1 lineage, grouping around 60% and 22% of IGH sequences, respectively. We also observed intra-clonal diversity for other clonal lineages; more details are available on the "Example tab" at ViCloD website, see the URL above.

ViCloD also provides lineage architecture details for a given clonal lineage. Figure 3 shows the

B-cell lineage tree and mutational profile for the clonal lineage C1. In the tree, Figure 3-A, the HAUS is indicated by a triangle, while the most relevant sub-clones are by circles. The circle area is proportional to the sub-clonal abundance, and pie charts indicate the sub-clonal composition: the number of unique sequences, also called clonotypes. For instance, the sub-clone C1-1 is composed of 536 different clonotypes, while C1-2 of 255; see clonotype distributions for the five most abundant sub-clones in Figure S2. The tree topology confirms the high intra-clonal diversity of lineage C1. The reconstructed tree has several levels, revealing the intricate evolutionary relationships of the most abundant/relevant sub-clones. According to the tree, the sub-clone C1-1 gave origin to seven sub-clones, and C1-2 has evolved from a less abundant sub-clone C1-6. The sub-clone C1-38, which accounts for less than 0.05% of the clonal lineage C1, is the most conserved, with a lower mutation rate (19 nucleotide differences) compared to the HAUS. The longest path in the tree is until the sub-clone C1-8, C1-30, and C1-39, which group 0.49%, 0.07%, and 0.05% of the lineage, respectively, and harbor the highest number of mutations (n = 47). Interestingly, the sub-clone C1-4 presents several descendants, but none of them is among the most abundant.

Figure 3-B shows a circular plot with cumulative distances for the seven most abundant/relevant sub-clones of the C1 lineage. Each colored arc represents a sub-clone, and the arc length corresponds to the number of mutations for its direct parent. The number of cumulative mutations (CM) of a given sub-clone is the sum of all arc lengths and corresponds to the path length from the tree root to a specific sub-clone. For instance, we observe 47 cumulative mutations from C1-8 and only 19 for C1-38, as can be seen when comparing arc lengths of C1-8 and C1-38 in Figure 3-B. The circular plot (mutational profile) is interactive, allowing users to add/remove sub-clones by clicking on nodes in the tree; a maximum of eight sub-clones can be represented simultaneously. Moving the mouse over a sub-clone on the circular plot shows its evolutionary path until the root (highlighted in red in the tree).

Mutated positions can be better examined in Figures 4 and 5: the former shows multiple sequence alignment (MSA) along the entire IGH-VDJ rearrangement, while the later displays a sequence logo centered on the CDR3 region. Parts of sequences in MSA are colored according to their structural

framework regions (FR, yellow) and complementary determining regions (CDR, violet). Conserved positions are indicated by dots, while the presence of a nucleotide indicates a mutation in that position. The IGHD gene is underlined within the CDR3, which is the most diverse part of IGH sequences. The sequence logo helps examine mutations in that region, Figure 4.

3.2 ViCloD identifies different types of clonal lineage architectures

High-throughput IGH repertoire sequencing has revealed recently a much higher intraclonal diversity than anticipated, although the exact mechanisms leading to this ongoing SHM activity remains elusive (*52*). We selected three patients with chronic lymphocytic leukemia (CLL) to demonstrate how ViCloD can analyze repertoire with different levels of intra-clonal diversity. To illustrate, we selected the major clonal lineage from the repertoire of patients 2, 3 and 4 that correspond to 96%, 99% and 99% of the repertoire, respectively.

Figure 6 shows B-cell lineage tree topologies and mutational profiles for the most abundant clonal lineages of selected individuals. When comparing the clonal trees of the three patients (Figure 6-A,C,E), we observe different topologies. The clonal tree of patient 2 is very branched; the most abundant sub-clone C1-1 descends from C1-2, the second most abundant. C1-2 presented eight cumulative mutations, while C1-1 14; we observe six point mutations between them. We can also see the high intra-clonal diversity of patient 2 in the mutational profile shown in Figure 6-B. The sub-clone C1-33 is the closest to the HAUS with seven nucleotide differences, while C1-51 has the most mutated sequence with 27 cumulative mutations. The third most abundant sub-clone, C1-3, is also highly mutated with 22 cumulative mutations. C1-3 descends from a less abundant sub-clone C1-5 that originates from C1-6.

In patient 3, we also observed some intra-clonal diversity, but with a different tree topology and mutational profile, when compared to patient 2; compare Figure 6-AB and Figure 6-CD. The most abundant sub-clone of patient 3 grouped 61.93% of clonal lineage sequences and has 13 cumulative mutations. The sub-clone C1-2, with an abundance of 35.69%, presented five cumulative mutations; between C1-1 and C1-2 we observed ten nucleotide differences. Both sub-clones share a few point

mutations, which is why the ClonalTree algorithm puts them in different tree branches. The intra-clonal diversity is also evident in the mutational profile of patient 3, Figure 6-D shows a minimum of four (C1-50) and a maximum of 19 (C1-53) cumulative mutations.

On the other hand, patient 4 shows lower intra-clonal diversity, which is clearly observed in its tree topology and mutational profile, Figure 6-EF. This patient's repertoire has a highly abundant sub-clone, representing more than 95% of both clonal lineage and entire repertoire. C1-1 has just one cumulative mutation, while the sub-clone C1-50 has zero. We also observed a tree with few levels (only three), see Figure 6-E. A total of 86 sub-clones were detected. All sub-clones (except C1-50) descend from C1-1, and all C1-1 descendants present just two cumulative mutations. Note that all mutations were observed in the CDR3 regions.

3.3 Comparison with other clonal lineage visualization tools

To compare the functionalities of ViCloD with other existing methods, we chose four AIRR-seq tools that provide both evolutionary analysis (clonal lineage tree/graph) and intra-clonal visualizations. Table 1 shows the main differences between ViCloD and the considered tools.

AncesTree (*37*) is a stand-alone graphic interface to interactively explore clonal lineage trees. It does not carry out any data processing; instead, it parses the outputs of Dnaml (*53*) and IgPhyML (*54*). AncesTree displays pre-computed trees, the number of nucleotide and amino acid mutations, and multiple sequence alignments. Trees are interactive, and users can visualize the detailed location of each mutation. To explore intra-clonal diversity, users need to pre-process several tasks, such as clonal grouping, clone selection and lineage reconstruction. Another stand-alone tool is GLaMST (*38*) that uses the minimum spanning tree algorithm to infer clonal lineage trees from high-throughput sequencing data. GLaMST runs in Matlab and requires input sequences in FASTA format, where the first sequence represents the root tree. Like Ancestree, GlaMST does not process the raw data, and the analysis of intra-clonal diversity requires computational experience. Also, it does not provide neither interactive plots or mutational profiles.

Alakazam (*39*) is part of the Immcantation framework. To use it, users need to run Change-O (*32*)

to detect clonal groups, and then select a clonal lineage of interest, for example, the most abundant one. The clonal lineage tree inference is performed by dnapars (*?*), which constructs a maximum parsimonious tree of a given clonal lineage. Alakazam provides a set of trees with different layouts, but they are not interactive, and they do not display clonal abundance. An overview of the repertoire composition is not direct, but can be obtained by running other tools from the Immcantation framework (*35*). However, Alakazam computes several diversity measures, such as the species richness, and the Shannon index, and provides gene usage, and chemical properties of the amino acid sequences, which are absents in ViCloD.

IgIDivA (*40*) differs from the above tools and ViCloD since it assumes that SHMs are acquired sequentially. It first identifies all nucleotide variants (here termed as clonotypes) inside a sub-clone. Second, it constructs a network centred on the most abundant clonotype rather than a phylogenetic tree. Two clonotypes are connected only if their connection is consistent with the progressive acquisition of SHMs. Additionally, IgIDivA computes a set of graph network metrics, such as average degree and maximal path length in the network, among others, to give an overview of intra-clonal diversity. Since IgIDivA analyzes clonotypes instead of sub-clones, its outputs are not directly comparable to ViCloD ones. Moreover, it does not adopt community standards input files, making its usage more difficult with other V(D)J assignment tools rather than IMGT/High-V-Quest.

All the above tools require some computational skills to manage installations and resolve dependencies. Therefore, their usage can be limited to research environments since, most of the time, the clinical environment has software installation constraints. On the other hand, ViCloD is an anywhere-access tool being more flexible and versatile. Moreover, ViClod offers an original view of the entire repertoire composition and allows users to easily navigate among the five most abundant clonal lineages rather than only one as other tools. All data and figures are easily downloaded and can be exported to other applications for further inspection.

4 Discussion

High-throughput sequencing analysis has made possible quantitative studies in immunology, where adaptive immune receptor repertoires (AIRR) can contain millions of different receptor gene variants. Individual repertoire analysis is now routinely possible and affordable, resulting in many practical applications in biology and medical research. Several web servers devoted to analyzing AIRR data obtained by high-throughput sequencing (AIRR-Seq) have been recently reported, most of them for pre-processing, V(D)J gene annotation, clonal grouping, repertoire diversity, and mutation analysis (refs). However, only some tools permit the analysis of intra-clonal diversities and the investigation of the dynamic/evolution of an individual repertoire. To fill the gap, we propose ViCloD, an interactive web server for visualizing B cell repertoire composition and analyzing intraclonal diversities.

To demonstrate ViCloD usefulness, we used it to analyze several BCR repertoires from patients with different B cell malignancies known for bearing intraclonal diversification in their IG variable regions. This clinical condition was chosen since the B cell population is dominated by a single clonal IGH-VDJ rearrangement (or two in case of biallelic rearrangements) which helps visualize intra-clonal diversity due to the large number of sequences which can be obtained. ViCloD has constructed different B-cell lineage tree topologies and mutation profiles for the four analyzed repertoires. We have observed trees with higher depths and more cumulative mutations in patients with higher intra-clonal diversity. These study cases demonstrated how ViCloD could analyze this kind of data in detail.

Our web-server can process and analyze data coming directly from IMGT/V-Quest, the international standard web software for parsing BCR repertoire sequence data. However, it is not restricted to IMGT/V-Quest outputs since it adopts the AIRR file format (*44*). In principle, the output of any V(D)J annotation tool can be used as long as it provides the minimum information necessary for the analysis. Although we have applied ViCloD to two lymphoproliferative diseases, the server is not limited to analyzing a specific repertoire and can be applied to other clinical conditions (*55*).

ViCloD offers several interactive plots, including B-cell lineage trees and mutation profiles. Such tools allow users to interact with their data, which is more complicated with other tools that pro-

vide pre-defined outputs (*37–40*). ViCloD calculations are performed in real-time, and the results are shown immediately on the screen (or, eventually, a link is sent by email). Therefore, the user can analyze complex data very efficiently and quickly. The web-sever is user-friend and versatile, particularly for analyzing high-throughput sequencing repertoire data, providing several views of BCR repertoire, which can help users to extract important features from their analyses. ViCloD is available to all users without requiring registration or any computational requirement. Users' data are stored temporarily, but they can be downloaded in a pre-defined format that allows re-analyzing posteriorly.

Additional features will be implemented in the future, such as (i) intra-clonal analysis at the amino acid level, including Physico-chemical properties, (ii) diversity measures, such as the species richness, and the Shannon index, and gene usage plots, (iii) metrics to measure the intra-clonal diversity level (*40*), (iv) the possibility of visualizing evolutionary events within a give sub-clone, (v) comparisons of several repertoires, which will allow users to analyze repertoires from the same individual at various time points or to compare repertoires of different individuals. ViCloD's flexibility offers the possibility of further extensions, including the estimation of B cell migration and differentiation (*41*).

5 Conclusion

We have produced a new AIRR bioinformatics tool, ViCloD, which can help process, analyze and visualize B cell receptor repertoire clonality and (intra-)clonal diversity in B cell populations. ViCloD provides interactive modules for analyzing and visualizing the repertoire structures, clonal compositions, and clonal lineage architectures. ViCloD distinguishes itself from other AIRR bioinformatics tools by several features. It is flexible, accepting AIRR formatted files generated by the most used tools. It is user-friendly, providing a graphical web interface that clinicians, immunologists, and researchers without strong computational backgrounds can easily use. It does not require any software installation, dependencies, or requirements. It provides interactive plots and allows users to examine thoroughly their data, including sequences, plots, and figures, which are easily exportable to other applications for further analysis. Finally, ViCloD run-time and memory requirements have been minimized in order to be compatible with clinical applications. With the growing importance of immune

repertoire research, we believe ViCloD will play an important role in identifying specific features within immune repertoires. ViCloD can help users without computational background to decipher the high-dimensional complexity of the adaptive immune system.

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Tables

Table 1: **Comparing ViCloD with other clonal lineage visualization tools**

	ViCloD	IgIDivA	Alakazam	AncesTree	GlaMST
Data-processing					
V(D)J assigment	no	no	yes	no	no
Clonal grouping	yes	yes	yes	no	no
Evolutionary inference	yes	yes	yes	no	yes
Visualization					
Entire repertoire	yes	no	no	no	no
Clonal lineages	yes	yes	yes*	no	no
Intraclonal diversity (tree/graphs)	yes	yes	yes	yes	yes
Mutation profile	yes	yes	yes	yes	no
Interactive plots	yes	no	no	yes	no
Diversity measures	no	no	yes	no	no
Clonal/sub-clonal abundance	yes	yes	yes	no	no
Other features					
Adoption of community standards	yes	no	yes	no	no
Web server	yes	no	no	no	no
Computational experience	no	yes	yes	yes	yes
Programming language	Python, Php, JS	R	R	Java	Matlab

Figures

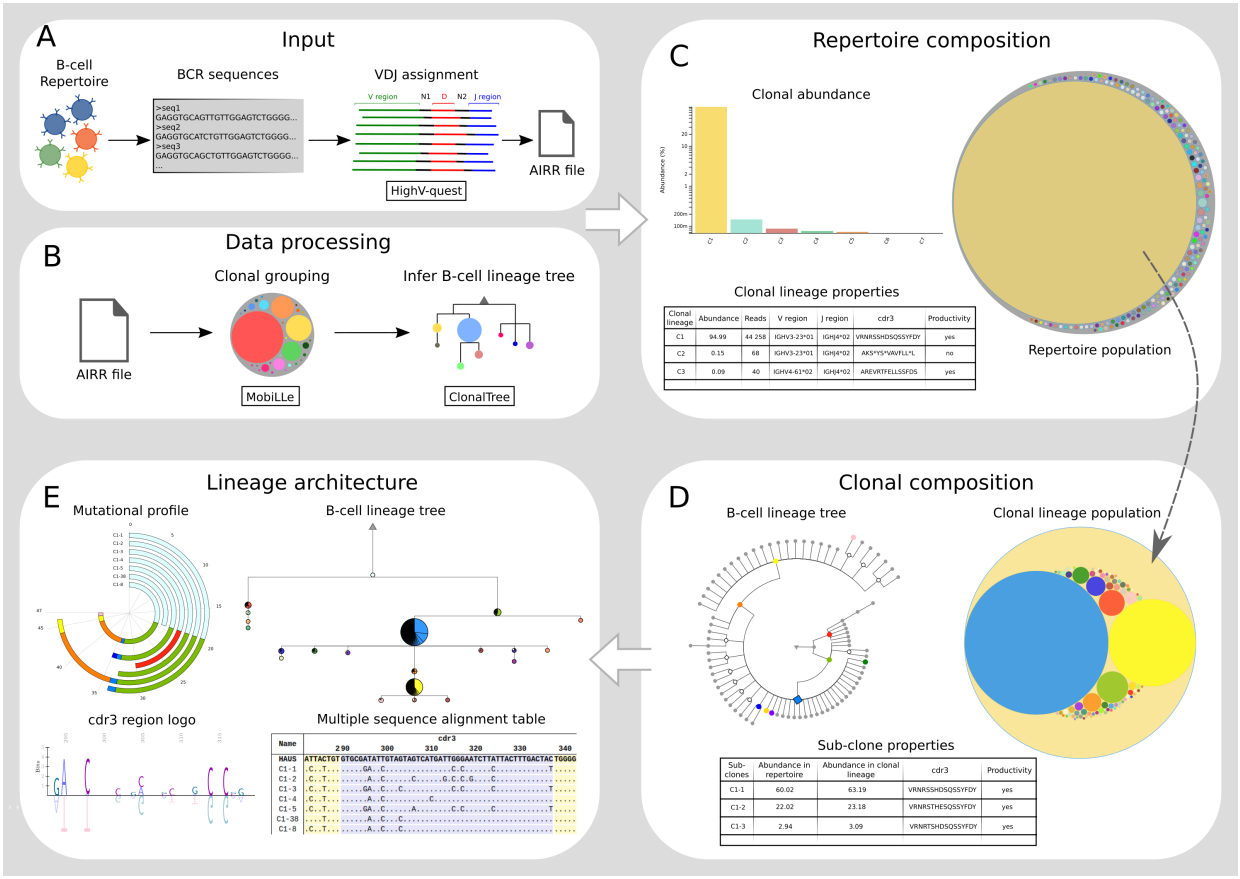

Figure 1: **Flowchart of ViCloD.**

Supporting information

S1 Table: Properties for the five most abundant clonal lineage.

S1 Figure: Simplifying tree topology.

S2 Figure Clonotype distribution.

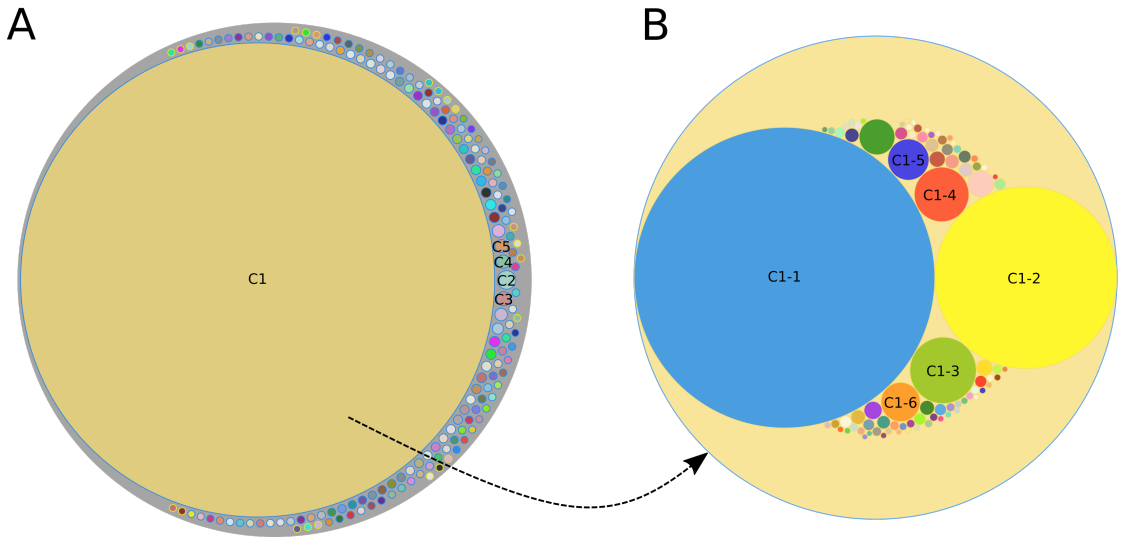

Figure 2: **Repertoire composition**

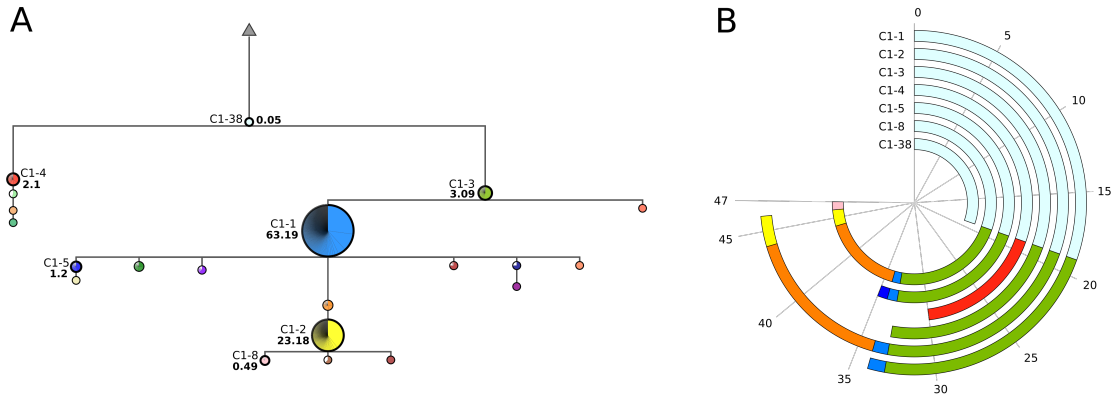

Figure 3: **Lineage architecture.**

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Name                 fwr1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
                     cdr1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
                     fwr2
                     10        20        30        40        50        60        70        80        90        100       110       120       130       140
HAUS                 GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTG
C1-1                 .........T................AT.............................................G...........................................................
C1-2                 .........T................AT.......................................T...G...A.....G.............................................
C1-3                 .........T................AT.............................................G...........................................................
C1-4                 ............C.............AT.................................................T...........A.............................................
C1-5                 .........T................AT.............................................G...........................................................
C1-38                ...........................T.............................................................................................
C1-8                 .........T................AT.......................................T...G...A.....G.............................................

Name                 cdr2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
                     fwr3
                     150       160       170       180       190       200       210       220       230       240       250       260       270       280
HAUS                 GGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTAT
C1-1                 .......AG.....A....G.....CA..TT...........................G...............................T...T...A.........G.............C.........A.G.
C1-2                 .......AG...............CA..T.....A.....................G...............................T...T...A.........G.............C.........A.G.
C1-3                 .......AG.....A....G.....CA..TT...........................G...............................T...T...A.........G.............C.........A.G.
C1-4                 .......AG........C......CA.TTTG...........................A...............................T.......A.......C.G.............C.........A.G.
C1-5                 .......AG.....A....G.....CA..TT...........................G...............................T...T...A.........G.............C.........A.G.
C1-38                .......AG...............CA..TTC..........................A...............................T.......A.........G.............C.........A.G.
C1-8                 .......AG...............CA..T.....A.....................G...............................T...T...A.........G.............C.........A.G.

Name                 cdr3                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
                     fwr4
                     290       300       310       320       330       340       350       360       370
HAUS                 ATTACTGTGTGCGATATTGTAGTAGTCATGATTGGGAATCTTATTACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG
C1-1                 .C..T.....GA..C...............C.C......C............T.................................
C1-2                 .C..T.....A..C.....C.....G.C.C.G....C.............................................
C1-3                 .C..T.....GA..C...C...........C.C......C............T.................................
C1-4                 .C..T.....A..C..........C.....................................................
C1-5                 .C..T.....GA..C......A........C.C......C............T.................................
C1-38                ....T.....A..C...C.............................................
C1-8                 .C..T.....A..C...C.............................................
```

Figure 4: **Multiple sequence alignment.**

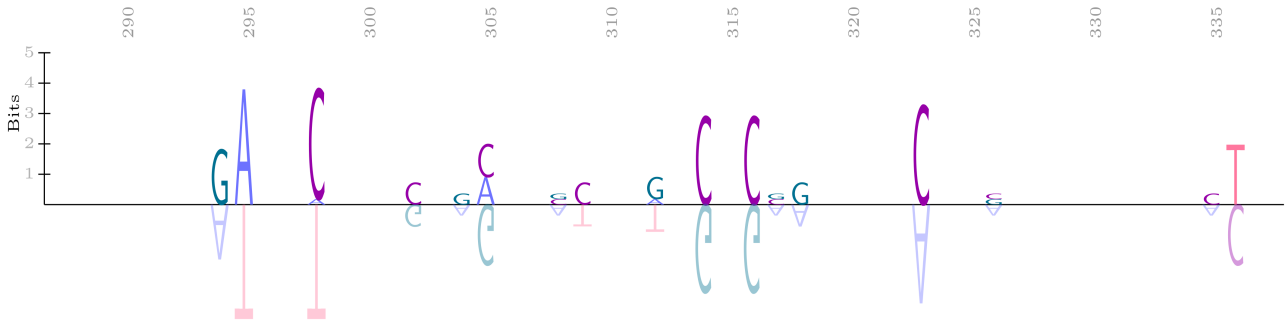

Figure 5: **CDR3 logo.**

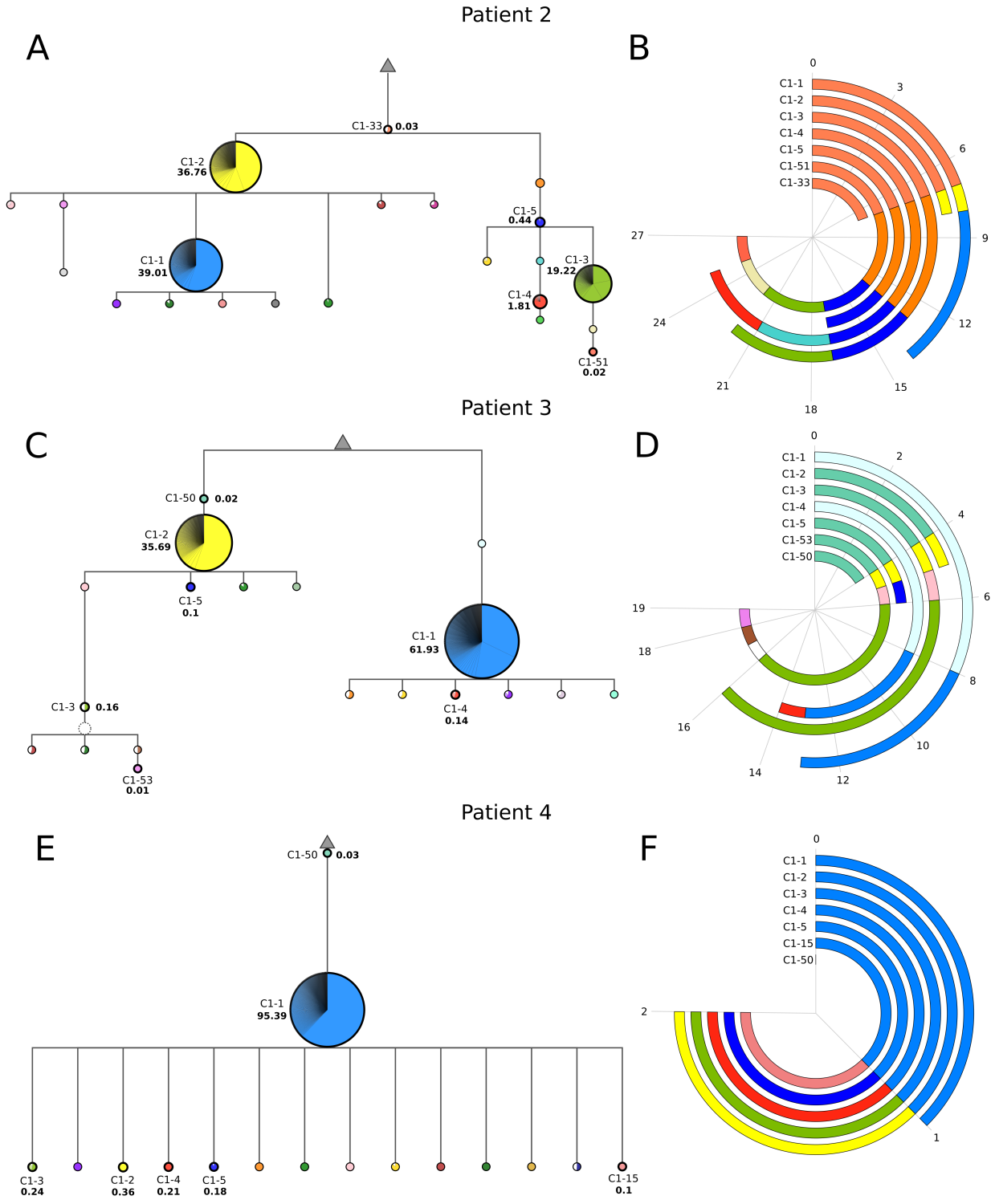

Figure 6: **Clonal lineage architectures for three patients with CLL.**