Vignette rKOMICS
application example

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1 The rKOMICS package

The core features of the rKOMICS package include data aggregation, analyses and visualization that allows to examine, summarize and extract meaningful information from minicircle sequence alignments as obtained by KOMICS or a custom bioinformatic pipeline, and from USEARCH cluster format (UC) files as generated by USEARCH or VSEARCH. In addition to storing data files, rKOMICS stores the analyses and visualization results into single list objects that can be called by the user at a later stage.

rKOMICS incorporates multiple methods of visualizations using the ggplot2 R package to plot the foundation of graphs. By adding ggplot2 functions to the rKOMICS visualization functions, the user has direct control over the finishing touches of the graph's appearances. Our package also utilizes sample-specific metadata that allows multi-group data visualizations to facilitate exploratory analysis. The overall data set can be examined using barplots, heatmaps, PCA plots and box plots that are generated for each specified minimum percent identity. This makes it possible to visualize population structure and diversity based on minicircle sequence composition.

To show the functionality of rKOMICS, we performed an example analysis using whole-genome sequencing data from a recently published study on the history of diversification of the *Leishmania braziliensis* species complex in Peru. This species complex comprises two closely related species: the lowland and zoonotic *L. braziliensis* parasite circulating in a diverse range of wild mammals in Neotropical rainforests, and the highland anthroponotic *L. peruviana* parasite that is largely endemic to the Pacific slopes of the Peruvian Andes. A total of 67 *Leishmania* parasites from 47 localities in Peru were cultured and subjected to whole genome sequencing.

```
data(exData, package = "rKOMICS")
table(exData$species)
```

```
         hybrid L. braziliensis    L. peruviana
             13              23              31
```

2 Required R-packages

```
library(ggplot2)
library(rKOMICS)
library(ggpubr)
library(viridis)
```

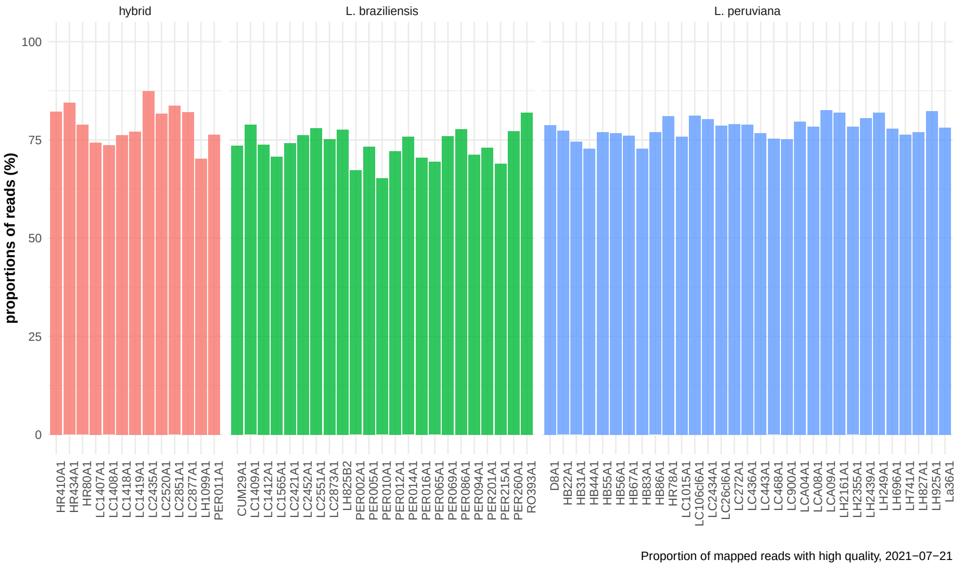
3 Quality of the assembly

The `msc.quality` function allows you to examine the quality of the assembly by alignment of reads to the assembled minicircles (see `https://github.com/FreBio/komics` for tutorial). We found that on average 77% of all mapped reads aligned with a mapping quality larger than 20 (Figure 1) and on average 84% aligned in proper pairs. On average 93% of all CSB3-containing reads aligned against the assembled minicircle contigs and 88.5% aligned perfectly, suggesting that KOMICS was able to retrieve a large proportion of the minicircle classes.

```
map <- msc.quality(mapstats = system.file(”extdata”,
                                          exData$mapstats,
                                          package = ”rKOMICS”),
                   groups = exData$species)
lapply(map$proportions, mean)$MR_HQ
```

```
[1] 77.04245
```

```
map$plots$MR_HQ + labs(caption = paste0('Proportion␣of␣mapped␣reads␣with␣
    high␣quality,␣', Sys.Date()))
```



Proportion of mapped reads with high quality, 2021−07−21

Figure 1: Proportion of reads with a mapping quality higher than 20 per Leihmania isolate.

4 Minicircle copy numbers

The depth statistics (see `https://github.com/FreBio/komics` for tutorial) include average, median, minimum and maximum per site read depth of every minicircle contig that has been assembled. The `msc.depth` function allows you to summarize those statistics (Figure 2) and to estimate minicircle copy numbers by standardizing median read depths per minicircle contig to the median genome-wide read depths.

```
depth <- msc.depth(depthstats = system.file(”extdata”,
                                            exData$depthstats,
                                            package = ”rKOMICS”),
                   groups = exData$species,
                   HCN = exData$medGWD/2) # haploid copy number
depth$CN
```

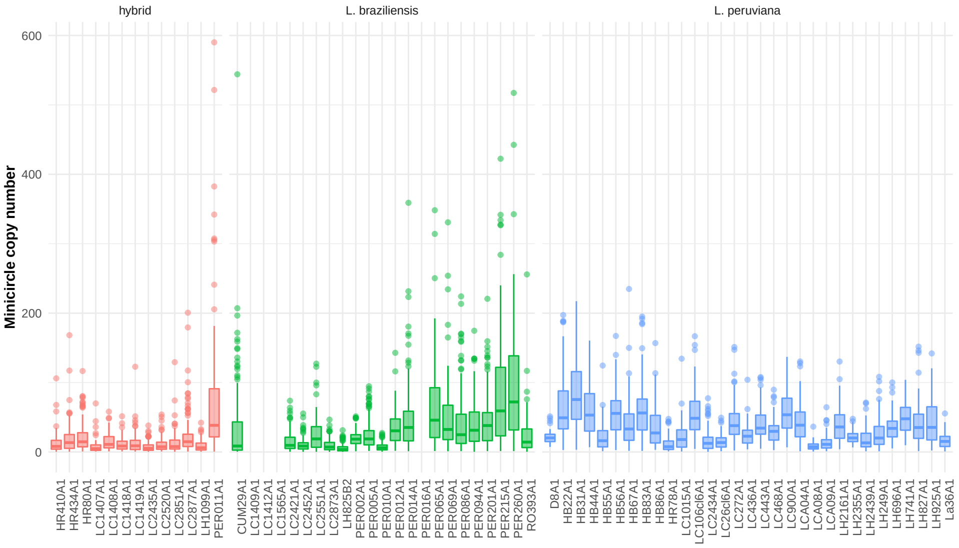


Figure 2: Minicirle copy number distribution per Leihmania isolate.

5 Inspect minicircle lengths

A combined total of 7,760 minicircles were assembled for 67 *Leishmania* isolates. When examining the length distribution of the circularized minicircle sequences using the function `msc.length`, we found that the majority of minicircles (95.2%) were 720-760 bp long, which is within the expected length range of minicircles in *Leishmania* parasites (Figure 3). 294 minicircle contigs (4.8%) showed twice this length (1400-1700 bp) (Figure 3), which may suggest that these are artificial minicircle dimers introduced by the assembly process, and were removed.

```
bf <- msc.length(file = system.file("extdata",
                                     "all.minicircles.fasta",
                                     package = "rKOMICS"),
                  samples = exData$samples,
                  groups = exData$subspecies)
bf$plot
```

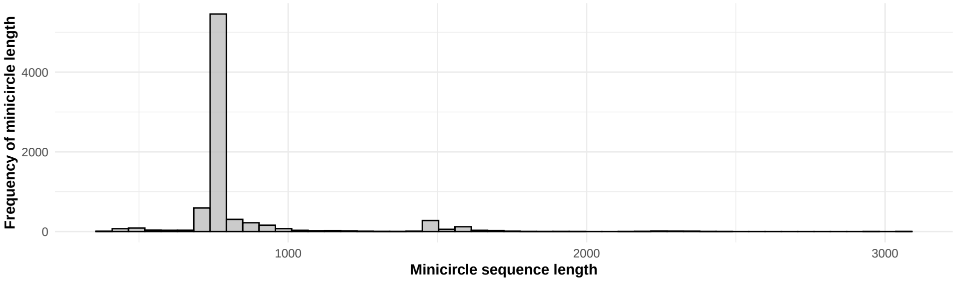


Figure 3: Length distribution of the 7,760 assembled minicircles in 67 Leishmania isolates.

```
c(length(bf$length),
  length(which(bf$length < 800)),
  length(which(bf$length > 1400)))
```

```
[1] 7760 6329  576
```

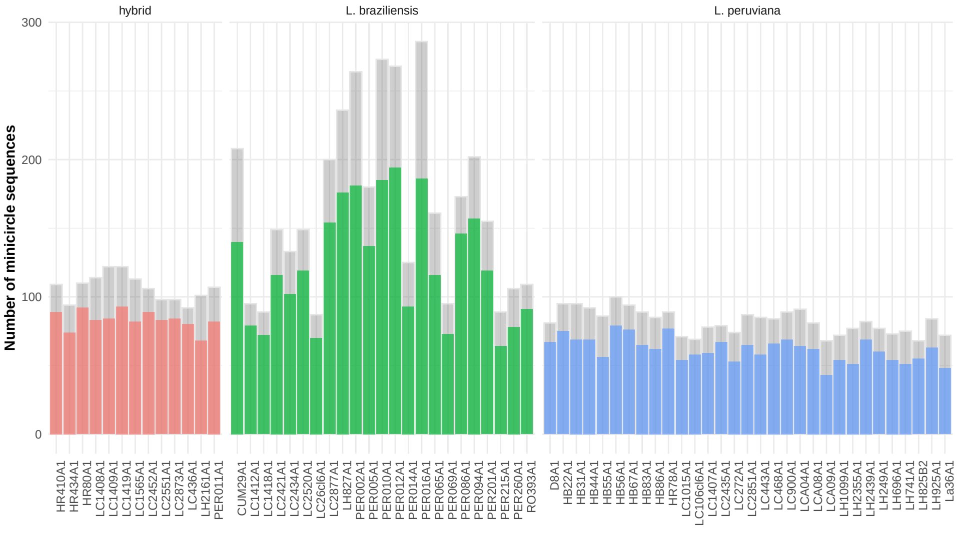
6 Filter minicircle sequences

For downstream analyses, we only retained the circularized minicircles of the expected length (720-760bp) using the `preprocess` function (Figure 4; coloured barplots), resulting in a final set of 5,849 minicircles.

```
pre <- preprocess(files = system.file(”extdata”,
                                      exData$fastafiles,
                                      package = ”rKOMICS”),
                  groups = exData$species, circ = TRUE,
                  min = 500, max = 1200,
                  writeDNA = FALSE)
pre$summary
```

```
beforefiltering  afterfiltering
           7760            5849
```

```
pre$plot +
  labs(caption = paste0(’N␣of␣MC␣sequences␣before␣and␣after␣filtering,␣’,
     Sys.Date()))
```



N of MC sequences before and after filtering, 2021−07−21

Figure 4: Gray barplots show the total number of minicircles found per Leishmania isolate, and coloured barplots indicate the number obtained after retaining only circularized minicircles of the expected length.

7 Clustering results

We used the function `msc.uc` to then examine the combined number of minicircle sequence classes (MSCs) (based on overall identity) across all 67 isolates, and we identified a total of 3,811 MSCs at 100% identity. This number decreased sharply to 918 MSCs at 97% identity and 603 MSCs at 95% identity (Figure 5). The proportion of perfectly aligned minicircle sequences (i.e. alignments without any insertion/deletion) during the clustering process decreased from 100% (only perfect alignments) at 100% identity to 79% (79% of the alignments were perfect) at 97% identity and 68% at 95% identity (Figure 5).

```
ucs <- msc.uc(files = system.file(”extdata”, exData$ucs, package = ”rKOMICS
    ”))
c(ucs$MSCs[”100”], ucs$MSCs[”97”], ucs$MSCs[”95”])
```

```
 100   97   95
3811  918  603
```

```
ucs$plots$`MSCs and perfect aligments`
```

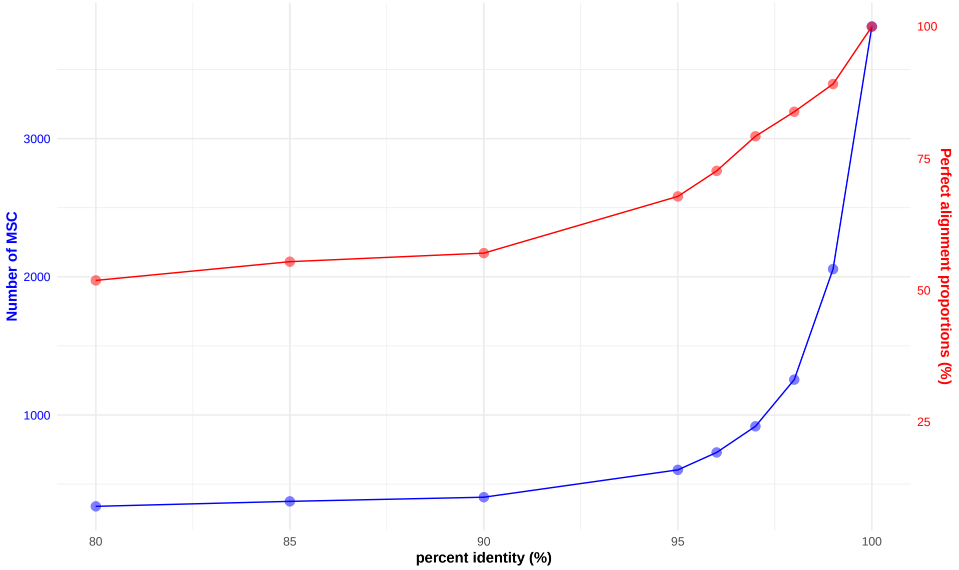


Figure 5: Number of MSCs (blue) and proportion of perfect alignments (red) as obtained following clustering analyses for a range of percent identities.

While insertions were mostly 1 bp long (Figure 6; left), the number of insertions per alignment increased with decreasing percent identity (Figure 6; right). Most notably, below 97% identity, we found a steady increase in alignments with 3 or 4 insertions (Figure 6; right). Similar results were obtained for deletions (results not shown). Hence, we decided to focus most of our downstream analyses at the 97% identity threshold, as this would capture sufficient minicircle sequence classes (Figure 5) while minimizing the number of alignment gaps (Figure 6).

```
ucs$plots$insertions
```

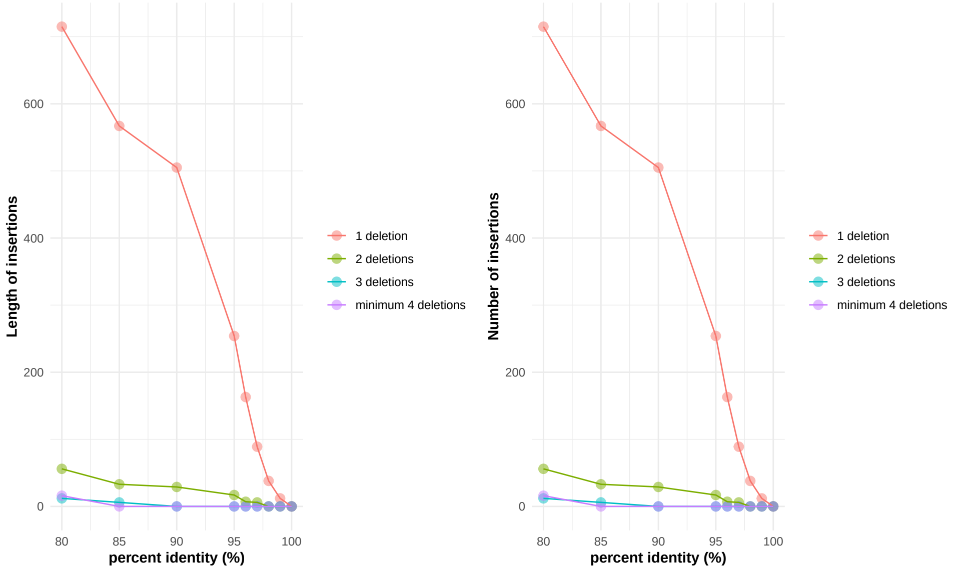


Figure 6: Length and number of insertions in MSC alignments following clustering analysis for a range of percent identities.

8 Build MSC matrix

Clustering based on a percent identity, performed with the VSEARCH tool, will generate files in uc format. The `msc.matrix` function will transform every input file into a cluster matrix. The columns of the matrix correspond to the samples and the rows of the matrix correspond to the minicircle sequence cluster (MSC). The absence of a MSC in a sample is indicated with the value of zero while the presence of a MSC in a sample will be indicated with a value >= 1.

```
matrices <- msc.matrix(files = system.file("extdata",
                                           exData$ucs,
                                           package = "rKOMICS"),
                       samples = sort(exData$samples),
                       groups = exData$species[order(exData$samples)])
### or: data(matrices, package = "rKOMICS")
# rowSums(matrices[["id97"]]) # --> frequency of MSC across all samples
colSums(matrices[["id97"]]) # --> number of MSC per sample
```

```
   CUM29A1       D8A1      HB22A1      HB31A1      HB44A1      HB55A1      HB56A1
       140         67          75          69          69          56          79
    HB67A1     HB83A1      HB86A1     HR410A1     HR434A1      HR78A1      HR80A1
        76         65          62          89          74          77          92
    La36A1   LC1015A1 LC106cl6A1    LC1407A1    LC1408A1    LC1409A1    LC1412A1
        54         58          59          83          84          79          72
  LC1418A1   LC1419A1    LC1565A1    LC2421A1    LC2434A1    LC2435A1    LC2452A1
        93         82         116         102          67          89         119
  LC2520A1   LC2551A1   LC26cl6A1     LC272A1    LC2851A1    LC2873A1    LC2877A1
        83         70          53          65          84         154          80
   LC436A1    LC443A1     LC468A1     LC900A1     LCA04A1     LCA08A1     LCA09A1
        58         66          69          64          62          43          54
  LH1099A1   LH2161A1    LH2355A1    LH2439A1     LH249A1     LH696A1     LH741A1
        68         51          69          60          54          51          55
   LH825B2    LH827A1     LH925A1    PER002A1    PER005A1    PER010A1    PER011A1
       176         63          48         181         137         185          82
  PER012A1   PER014A1    PER016A1    PER065A1    PER069A1    PER086A1    PER094A1
       194         93         186         116          73         146         157
  PER201A1   PER215A1    PER260A1     R0393A1
       119         64          78          91
```

The `msc.heatmap` function generates a heatmap of the input cluster matrix that summarizes the presence or absence of Minicircle Cluster Sequences (MCSs) between groups of samples.

```
msc.heatmap(clustmatrix = matrices[["id97"]],
            groups = exData$species,
            samples = exData$samples)
```

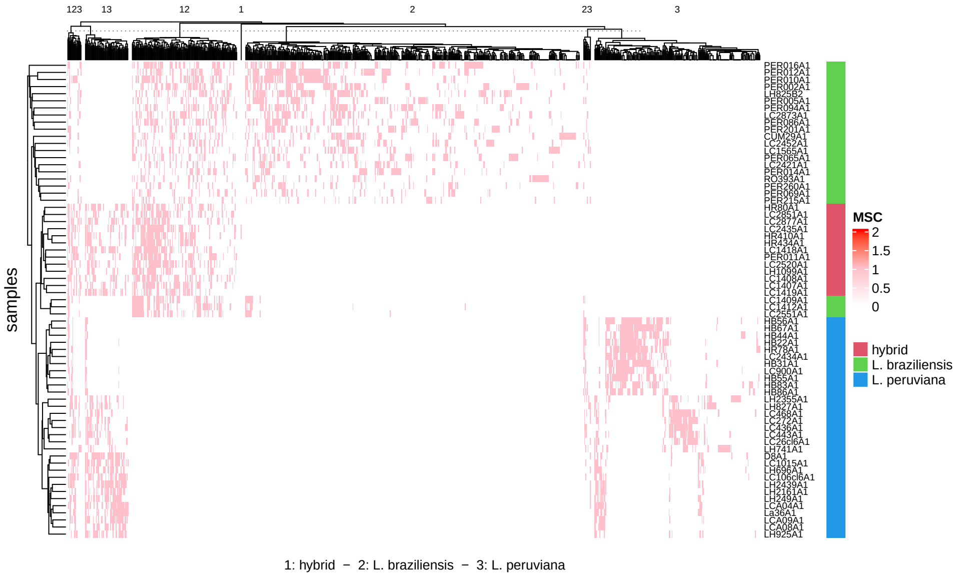


Figure 7: Heatmap of the MSC matrix at a percent identity of 97.

9 MSC Richness

Focusing on the results at 97% identity, we observed that the Andean and near-clonal *L. peruviana* parasites harbored substantially less MSCs (mean = 62 MSCs per isolate) compared to the Amazonian and recombining *L. braziliensis* parasites (mean = 124 MSCs per isolate).

```
richness <- msc.richness(matrices,
                         samples = exData$samples,
                         groups = exData$species)
apply(richness$table[which(richness$table$group=="L.␣peruviana"),-(1:2)],
   2, mean)
```

```
    id80     id85     id88     id89     id90     id91     id92     id93 
61.80645 61.80645 61.80645 61.80645 61.80645 61.80645 61.80645 61.80645 
    id94     id95     id96     id97     id98     id99    id100 
61.80645 61.83871 61.83871 61.87097 61.87097 61.87097 61.87097 
```

```
apply(richness$table[which(richness$table$group=="L.␣braziliensis"),-(1:2)
   ], 2, mean)
```

```
    id80     id85     id88     id89     id90     id91     id92     id93 
117.2174 119.8261 121.0000 121.1739 121.3478 121.4783 121.7826 122.3913 
    id94     id95     id96     id97     id98     id99    id100 
122.7826 123.2609 123.5217 123.8261 123.8261 123.8261 123.8261 
```

```
apply(richness$table[which(richness$table$group=="hybrid"),-(1:2)], 2, mean
   )
```

```
    id80     id85     id88     id89     id90     id91     id92     id93 
79.69231 80.00000 80.07692 80.46154 80.76923 81.46154 81.38462 81.53846 
    id94     id95     id96     id97     id98     id99    id100 
81.84615 82.38462 83.07692 83.30769 83.30769 83.30769 83.30769 
```

```
richness$plot
```

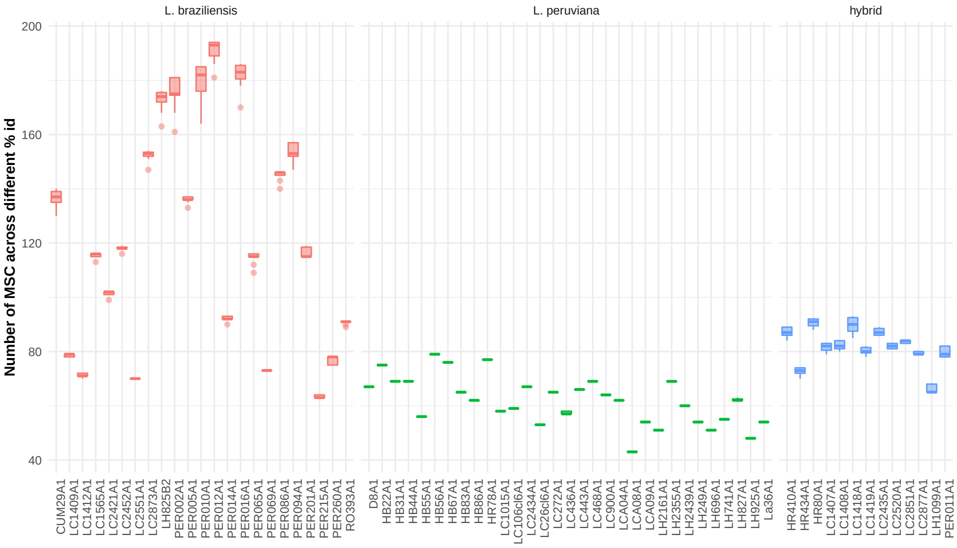


Figure 8: Number of MSC per isolate across different percent identities.

10 Similarity

Using the function `msc.similarity`, we found that 48.9% and 25.9% of the MSCs were unique to *L. braziliensis* and *L. peruviana*, respectively, while hybrid *L. braziliensis* x *L. peruviana* parasites shared MSCs with both parents (Figure 9). This confirms that hybrid parasites inherited minicircles from both Leishmania parental species. We also confirmed that the Andean and near-clonal *L. peruviana* parasites harbored substantially less MSCs (mean = 62 MSCs per isolate) compared to the Amazonian and recombining *L. braziliensis* parasites (mean = 124 MSCs per isolate).

```
sim <- msc.similarity(matrices,
                      samples = exData$samples,
                      groups = exData$species)
sim$relfreq.plot + scale_fill_viridis(discrete = TRUE)
```

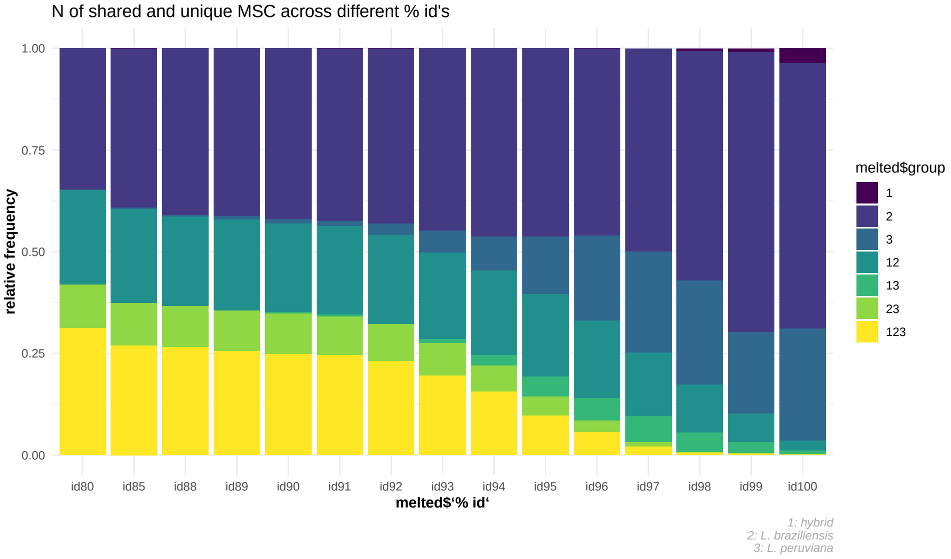


Figure 9: Barplots show the proportion of minicircle sequence classes that are unique or shared between L. braziliensis, L. peruviana and their hybrids, for each % identity threshold used during the clustering analyses.

```
c(sim$relfreq$id97["2"]*100,
  sim$relfreq$id97["3"]*100)
```

```
       2        3
49.89107 24.72767
```

11 PCA

Principal Component Analysis based on minicircle sequence similarity (i.e. MSC presence/absence per isolate) separated *L. braziliensis* from *L. peruviana* on the first axis and three *L. peruviana* populations on the second axis (Figure 10). The three *L. peruviana* populations correspond to the Porculla lineage that circulates in the tropical deciduous forests of Peru, and the two Surco lineages that circulate in desert shrubland on the Pacific Coast (Surco North/Central and Surco Central/South). Hybrids did not cluster with either parental species, in contrast to what was observed for the uniparentally inherited kinetoplast, but instead occupied an intermediate position between *L. braziliensis* and the *L. peruviana* Surco Central/South lineage (Figure 10), again consistent with mixing of the parental minicircle populations.

```
res.pca <- lapply(matrices, function(x) msc.pca(x, samples = exData$samples
    ,
                                    groups = exData$species, n=30, labels=FALSE, title=NULL))
res.pca$id97$plot
```

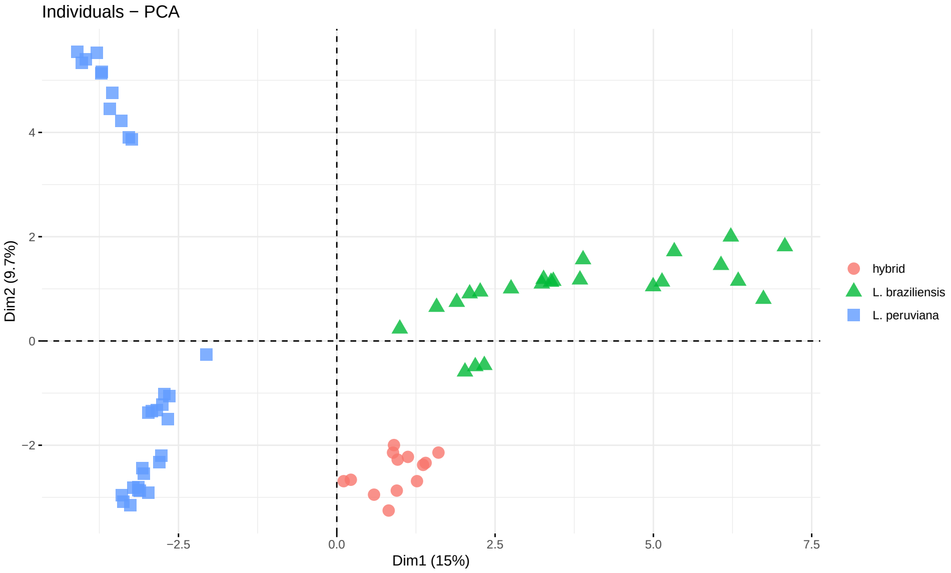


Figure 10: Principal Component Analysis based on sequence similarity between MSCs at 97% identity.