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Conformational characterization of disulfide bonds: a tool for protein classification

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Abstract

Background

Throughout evolution, mutations in particular regions of some protein structures have resulted in extra covalent bonds that increase the overall robustness of the fold: disulfide bonds. The two strategically placed cysteines can also have a more direct role in protein function, either by assisting thiol or disulfide exchange, or through allosteric effects. In this work, we verified how the structural similarities between disulfides can reflect functional and evolutionary relationships between different proteins. We analyzed the conformational patterns of the disulfide bonds in a set of disulfide-rich proteins that included twelve *SCOP* superfamilies: thioredoxin-like and eleven superfamilies containing small disulfide-rich proteins (*SDP*).

Results

The twenty conformations considered in the present study were characterized by both structural and energetic parameters. The corresponding frequencies present diverse patterns for the different superfamilies. The least-strained conformations are more abundant for the *SDP* superfamilies, while the “catalytic” +/-RHook is dominant for the thioredoxin-like superfamily. The “allosteric” -RHSaple is moderately abundant for BBI, Crisp and thioredoxin-like superfamilies and less frequent for the remaining superfamilies. Using a hierarchical clustering analysis we found that the twelve superfamilies were grouped in biologically significant clusters.

Conclusions

In this work, we carried out an extensive statistical analysis of the conformational motifs for the disulfide bonds present in a set of disulfide-rich proteins. We show that

the conformational patterns observed in disulfide bonds are sufficient to group proteins that share both functional and structural patterns and can therefore be used as a criterion for protein classification.

Keywords: Disulfide bond, conformer, cluster analysis, protein classification.

Introduction

Disulfide bonds are a common motif in Nature. These structural elements have a significant role in the thermal stability and function of proteins (Bhattacharyya *et al.*, 2004; Creighton, 1988; Hogg, 2003; Klink *et al.*, 2000; Sardiù *et al.*, 2007). From an evolutionary perspective, these bonds are a relatively recent addition to protein structure (Brooks and Fresco, 2002; Brooks *et al.*, 2002; Jordan *et al.*, 2005; Schmidt and Hogg, 2007). According to the respective functions, the disulfide bonds can then be classified as structural, catalytic or allosteric (Schmidt *et al.*, 2006; Schmidt and Hogg, 2007). Schmidt *et al.* (2006) have performed a thorough analysis of disulfides present in the X-ray structures of the PDB database, and found that both catalytic and allosteric disulfides fell into particular structural categories. The two groups had a higher average potential energy, which reflected their functional role that implied easy bond breaking (Schmidt *et al.*, 2006).

The disulfide three-dimensional structure is highly conserved in Nature and has been used for protein clustering (Cheek *et al.*, 2006; Chuang *et al.*, 2003; Harrison and Sternberg, 1996; Thangudu *et al.*, 2007). Different schemes have been introduced to classify the disulfide conformers (Harrison and Sternberg, 1996; Hutchinson and Thornton, 1996; Ozhogina and Bominaar, 2009; Schmidt *et al.*, 2006; Srinivasan *et al.*, 1990) and in this work we adopted the scheme proposed by Schmidt *et al.* (2006). We analyzed a sample of disulfide bonds associated with a protein set extracted from SCOP data base (Andreeva *et al.*, 2004; Andreeva *et al.*, 2008; Murzin *et al.*, 1995). The protein set included eleven superfamilies of small disulfide-rich proteins (SDP) and the thioredoxin-like super-family. Each superfamily selected for the protein set had to fit

the following criteria: (i) contain a minimum of thirty disulfide bonds, (ii) have a minimum of five *PDB* structures available, (iii) have X-ray structures with a resolution higher than 2.5 Å and (iv) have only uncomplexed structures. In order to understand whether or not the structure of the disulfides reflected functional or evolutionary relationships between the different proteins, we grouped the disulfide from the 12 superfamilies in different clusters using a Hierarchical Clustering Analysis (*HCA*) and a structural-based distance protocol. The results demonstrate that the clusters' aggregate superfamilies share both functional and structural patterns, therefore we conclude that the use of disulfide bonds conformational patterns is a valid protein classification criterion.

Methodology

The scheme used in this work to classify the disulfide conformers was based on five relevant torsion angles (Figure 1). The disulfide species were treated as symmetrical. In this context, only twenty conformational categories had to be considered (Table 1). For example the –RHHook conformational category can be obtained by either combinations of torsion angles (-,+,+,-) or (-,-,+,+,-). This classification was based on structural patterns (Schmidt *et al*, 2006) that included main, orientational and peripheral motifs (Table 2).

[Insert Figure 1]

[Insert Table 1]

[Insert Table 2]

Representative structures for the different conformational categories are presented in Tables 3 to 5.

[Insert Table 3]

[Insert Table 4]

[Insert Table 5]

The protein set under study is characterized in Table 6. We determined the five relevant torsion angles (χ_1 , χ_2 , χ_3 , χ_2' and χ_1') for each disulfide bond. Additionally, the (C_α - C_α' and C_β - C_β') distances and the dihedral strain energy (*DSE*) were also evaluated.

[Insert Table 6]

The *DSE* quantity was expressed, as a function of the five above-mentioned torsion angles, by the empirical equation (Katz and Kossiakoff, 1986; Weiner *et al.*, 1984):

$$\begin{aligned} DSE(kJ\ mol^{-1}) = & 8.37(1 + \cos(3\chi_1)) + 8.37(1 + \cos(3\chi_1')) + \\ & 4.18(1 + \cos(3\chi_2)) + 4.18(1 + \cos(3\chi_2')) + \\ & 14.64(1 + \cos(2\chi_3)) + 2.51(1 + \cos(3\chi_3)) \end{aligned} \quad (1)$$

The *DSE* quantity provided a useful ranking of the most favored disulfide conformations. The minimum ($2.5\ kJ\ mol^{-1}$) and the maximum ($84.5\ kJ\ mol^{-1}$) values of *DSE* correspond to the torsion angles combinations (60° , 60° , $\pm 83^\circ$, 60° , 60°) and (0° , 0° , 0° , 0° , 0°), respectively (Schmidt *et al.*, 2006). Despite its simplicity, this equation has been successfully applied for a semi-quantitative evaluation of the strain energy in disulfide bonds (Schmidt *et al.*, 2006; Schmidt and Hogg, 2007).

Representative conformations of the different types of disulfide bonds (structural, catalytic or allosteric) are identified in Table 7. We will be referring to bonds with the conformations +/-RHHook as “catalytic”, and -RHStaple as “allosteric”, because these

two types of bonds were found to be intimately associated with those conformational categories (Schmidt *et al.*, 2006).

A computer program, designated by *Disulph*, was developed to perform the calculations. The disulfide bonds propensity Pr_A , for a superfamily **A** with np_A PDB structures, was calculated as,

$$Pr_A = (1/np_A) \sum_{k=1}^{np_A} 100 \times nss_k / nres_k, \quad (2)$$

where nss_k and $nres_k$ were respectively the number of disulfide bonds and the number of coded residues in the PDB structure **k**. This quantity evaluates the frequency of the disulfide bonds within a superfamily. It is calculated as the average frequency associated with a correspondent sample of *PDB* structures.

The frequencies associated with all the conformational categories, defined in Table 1, were then evaluated for each super-family and for the sample. These quantities were used to build a square Euclidean distances matrix, whose elements ($d_{Euclidean}^2(A, B)$) were defined as:

$$d_{Euclidean}^2(A, B) = \sum_{i=1}^{20} (freq(i, A) - freq(i, B))^2; A=1, \dots, 12 \text{ and } B=1, \dots, 12 \quad (3)$$

In equation (3), $freq(i, A)$ and $freq(i, B)$ are respectively the frequency of conformational category *i* in the superfamilies **A** and **B**. The square Euclidean distances matrix defines a metric for evaluating the similarities between objects in n-dimensional spaces and therefore can be used in cluster analysis.

In order to represent this matrix, we adopted the intuitive formalism introduced by (Xie *et al.*, 2000). The coordinates of the original objects (the twelve superfamilies) were projected in the 3D Cartesian space by minimizing the square deviation cost function **SD**:

$$SD = \sum_{A=1}^{12} \sum_{B=1}^{A-1} \left(d(A, B) - d_{Euclidian}^2(A, B) \right)^2, \quad (4)$$

where $d(A, B)$ was the distance between the projections the superfamilies **A** and **B** in the 3D Cartesian space. We used the Newton method to carry out the iterative minimization process. The procedure associated with equation (4) was introduced for visualizing large chemical databases (Xie *et al.*, 2000). The minimization of this equation provided an appropriate representation of the original high-space of the chemical descriptors in a low dimensional space (2D or 3D).

The square Euclidean distances matrix was then used for a *HCA* procedure (Johnson and Wichern, 2007), which provided a classification of the superfamilies in different clusters. We evaluated the consistency of the *HCA* partitioning, by the evaluation of the square Euclidean distances matrix in the cluster space. The elements of this matrix were all the mean-square distances between a cluster **C_i** with n_{C_i} superfamilies and a cluster **C_j** with n_{C_j} superfamilies ($MSd_{Euclidean}^2(C_i, C_j)$) and within a cluster **C_i**

($MSd_{Euclidean}^2(C_i)$):

$$MSd_{Euclidean}^2(C_i, C_j) = (1/(n_{C_i} \times n_{C_j})) \times \sum_{A=1}^{n_{C_i}} \sum_{B=1}^{n_{C_j}} d_{Euclidian}^2(A, B) \quad (5)$$

$$MSd_{Euclidian}^2(C_i) = (2/(n_{C_i} \times n_{C_i})) \times \sum_{A=1}^{n_{C_i}} \sum_{B=1}^{A-1} d_{Euclidian}^2(A, B) \quad (6)$$

This matrix was defined according to the mean linkage criterion within the *HCA* procedure (Johnson and Wichern, 2007). The dissimilarity between two clusters C_i and C_j increased with the increasing of the correspondent non-diagonal element

($MSd_{Euclidean}^2(C_i, C_j)$). On the other hand, the similarity within a cluster C_i increases with the decreasing of the correspondent diagonal element ($MSd_{Euclidean}^2(C_i)$).

In this work we used the *HCA* divisive method which partitioned successively an initial set with n objects into finer clusters. The correspondent algorithm was the following:

- (i) Assign the n objects to a single cluster.
- (ii) Compute a distance matrix in the cluster space using an appropriate metric.
As was mentioned above, we adopted a square Euclidean metric in this work.
- (iii) Find the least similar objects and separate them in different clusters.
- (iv) Repeat steps (ii) and (iii) until the diagonal elements of this matrix being significantly smaller than the non-diagonal ones.

Results

The characterization of the disulfide conformational categories found in our sample is presented in Table 7. The –LHSpiral is the most frequently observed category (28.9 %) and has the lowest *DSE* (11.5 kJ mol⁻¹). Additionally, six least strained categories (-

LHSpiral, +/-RHSpiral, +/-LHSpiral, -RHSpiral, +RHSpiral and -/+RHHook) are clearly prevalent (63.1 %) relative to the remainder of the most strained categories (36.9 %). The representative conformations for catalytic (+/-RHHook) and allosteric (-RHStaple) disulfide bonds have moderate *DSE* values. We found the $d(C_{\alpha}-C_{\alpha}')$ distances to be more relevant for disulfide conformational specificities than the $d(C_{\beta}-C_{\beta}')$ distances (Table 7). The $d(C_{\alpha}-C_{\alpha}')$ distances were quite insensitive to the nature of conformational categories (varies from 3.3 Å to 4.0 Å), while the $d(C_{\beta}-C_{\beta}')$ distances had a significant variation over the series (from 4.4 Å to 6.0 Å). For instance, in agreement with Schmidt *et al* (2006), the -RHStaple conformation was characterized by significant lower $d(C_{\alpha}-C_{\alpha}')$ distances than the other conformational categories.

[Insert Table 7]

[Insert Table 8]

[Insert Figure 2]

The frequencies for the different conformational categories, calculated for each superfamily, are presented in Table 8 and Figure 2. From this figure, it is evident that thioredoxin-like and *SDP* superfamilies exhibit very distinct conformational patterns. The least strained conformations are significantly abundant in *SDP* superfamilies present significant abundances (from 43.4 % to 86.5 %), but occur at a very low frequency in thioredoxin-like superfamily (13.8 %). This is obvious for the most stable conformation (-LHSpiral) for which the *SDP* superfamilies present frequencies at least four times larger than the thioredoxin-like frequency (from 12.1 % to 43.8 % against 3.1 %; Table 8 and Figure 2). Most of the disulfide bonds of thioredoxin-like superfamily (50.8 %) are associated with the “catalytic” +/-RHHook conformation,

whereas this is relatively rare (from 0.0 % to 7.7 %) for the *SDP* superfamilies (Table 8 and Figure 2). On the other hand, the “allosteric” –RHSaple is moderately abundant for BBI (24.2 %), Crisp (24.1 %) and thioredoxin-like (16.9 %) superfamilies and scarce (from 0.0 % to 5.7 %) for the remainder superfamilies.

[Insert Figure 3]

Further insight into how the structural similarities between disulfides can reflect relationships between different proteins was obtained with a *HCA* procedure, whose dendrogram (Murtagh, 1984) is presented in Figure 3. The 3D-cartesian projection of the respective square Euclidean distances matrix is represented in Figure 4 together with the six clusters identified by this analysis. Four clusters reflect the main structural and functional motifs identified in the sample:

- Cluster 1 includes the catalytic proteins of thioredoxin-like superfamily, with the lowest disulfide propensities and a dominant α/β secondary structure;
- Cluster 4 includes most of the metabolic superfamilies (Cystine-Knot, EGF-Laminin and Plant lectins), with a dominant β secondary structure;
- Cluster 5 includes most of the toxin/defense superfamilies (Defensin-like, omega toxins, small snake toxins and scorpions-like toxins), with moderate to high disulfide propensities and a dominant β secondary structure;
- Cluster 2 includes the plant protease inhibitors of BBI superfamily, with high disulfide propensities and a dominant β secondary structure.

[Insert Figure 4]

The remainder two clusters reflect divergences from the mentioned motifs:

- Cluster 3 includes Crisp superfamily and is a divergence from cluster 5.
This cluster includes toxin/defense proteins with low disulfide propensities and a dominant α secondary structure.
- Cluster 6 includes BPTI-like and Kringle-like superfamilies. This cluster is the least well-characterized and includes proteins with small disulfide propensities and different biological functions. The elements of this cluster share more diffuse properties as (i) they are constrained by three disulphide bonds with the same disulfide topology (1-6, 2-4 and 3-5) and (ii) they are associated with the regulation of similar biological processes (binding mediation, proteolytic activity, blood clotting, etc.).

We represent the Euclidean distances matrix for the cluster space in Table 9. From the analysis of this Table, we can verify that the mean-square distances between the clusters are significant larger than within the clusters. These results strongly indicate that the *HCA* partitioning is consistent.

[Insert Table 9]

Conclusions

In this work, we carried out an extensive statistical analysis of the conformational motifs for the disulfide bonds found in set of disulfide-rich proteins from twelve *SCOP* superfamilies.

The frequencies of the twenty conformational categories provided a near-spectral representation of the 12-dimension hyperspace under study. The general trends observed in this sample were quite consistent with the results obtained by other authors (Schmidt *et al.*, 2006; Schmidt and Hogg, 2007) for three different protein sets. We calculated the root mean-square deviations between our and the previously obtained frequencies. The three values obtained were all lower than 2.6 %.

The *HCA* partitioning of the data using a square Euclidean distances matrix resulted in a number of clusters, the majority of which aggregates superfamilies sharing both functional and structural patterns. The only exception is cluster 6, whose elements presented more diffuse connections. We therefore suggest the use of disulfide bonds conformational patterns as a criterion in *SDP* classification, as well as to recognize main divergences between *SDP* and other disulfide-rich superfamilies. However, the generalized application of this methodology for protein classification has to be subjected to further investigation.

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Figures captions

Figure 1: Graphical representation of the five torsion angles used to classify the disulphide conformers.

Figure 2: Frequencies for the disulfide conformational categories.

Figure 3: Dendrogram for the hierarchical clustering analysis. The following notation was adopted: (1) Crisp, (2) Cystine-Knot, (3) Defensin-like, (4) EGF-Laminin, (5) Omega toxins, (6) Plant lectins, (7) Small snake toxins, (8) Scorpion-like toxins, (9) BBI, (10) BPTI-like, (11) Kringle-like and (12) Thioredoxin-like.

Figure 4: .Projected 3-D Cartesian representation of the square Euclidean distances matrix and clusters obtained by the hierarchical clustering analysis. The following notation was adopted: (1) Crisp, (2) Cystine-Knot, (3) Defensin-like, (4) EGF-Laminin, (5) Omega toxins, (6) Plant lectins, (7) Small snake toxins, (8) Scorpion-like toxins, (9) BBI, (10) BPTI-like, (11) Kringle-like and (12) Thioredoxin-like.

Table 1. Classification of disulphide bonds in conformational categories (Schmidt *et al*, 2006).

Disulphide category [#]	χ_1	χ_2	χ_3	χ_2'	χ_1'
-LHSpiral	-	-	-	-	-
-RHHook	-	+	+	-	-
+/-RHSpiral	+	+	+	+	-
+/-LHSpiral	+	-	-	-	-
-RHSpiral	-	+	+	+	-
+/-RHHook	+	-	+	+	-
+RHSpiral	+	+	+	+	+
-LHHook	-	-	-	+	-
-/+RHHook	-	-	+	+	+
-RHStaple	-	-	+	-	-
+/-LHHook	+	-	-	+	-
-/+LHHook	-	-	-	+	+
+/-LHStaple	+	+	-	+	-
-LHStaple	-	+	-	+	-
+LHSpiral	+	-	-	-	+
+LHHook	+	-	-	+	+
+RHHook	+	+	+	-	+
+/-RHStaple	+	-	+	-	-
+LHStaple	+	+	-	+	+
+RHStaple	+	-	+	-	+

[#]LH: Left-handed oriented; RH: Right-handed oriented; -: Negative value for the respective torsion angle; +: Positive value for the respective torsion angle.

Table 2. Characteristic conformational motifs used for disulphide classification.

Main motifs	χ_2 χ_3 χ_2'			Orientational motifs	χ_3		Peripheral motifs	χ_1 χ_1'			
	Spiral	+	+		+	LH		-	+	+	+
		-	-		-			-	-	-	
	Staple	+	-		+	RH		+	-	-	-
		-	+		-				+/ -	+	-
	Hook	+	+		-				- / +	-	+
		-	+		+						
		+	-		-						
		-	-		+						

Table 3. Representative structures for the spiral conformational categories.

Table 4. Representative structures for the staple conformational categories.

Table 5. Representative structures for the hook conformational categories.

<p>-LHHook</p>	<p>-RHHook</p>
<p>+LHHook</p>	<p>+RHHook</p>
<p>+/-LHHook</p>	<p>+/-RHHook</p>
<p>-/+LHHook</p>	<p>-/+RHHook</p>

Table 6. Characterization of the protein set under study. The sample used in the statistical analyses is considered to include all the disulphide bonds identified in this protein set.

Superfamily	Dominant secondary structure	Propensity [#]	No. of PDB structures	No. of disulphide bonds	Function
Crisp	α	5.3 %	6	54	Toxins/defense
Cystine-Knot	β	3.7 %	13	112	Metabolic
Defensin-like	β	7.4 %	15	47	Toxins/defense
EGF-Laminin	β	6.4 %	27	121	Metabolic
Omega toxins	β	8.9 %	28	88	Toxins/defense
Plant lectins	β	9.9 %	8	100	Metabolic
Small snake toxins	β	6.5 %	40	209	Toxins/defense
Scorpion-like toxins	β	7.9 %	70	247	Toxins/defense
BB1 (Bowman <u>B</u> irk <u>I</u> nhibitors)	β	9.6 %	5	33	Protease inhibition
BPTL-like	$\alpha+\beta$	5.1 %	12	42	Protease inhibition
Kringle-like	β	3.7 %	12	53	Metabolic
Thioredoxin-like	α/β	0.8 %	43	66	Isomerase catalysis

[#] Calculated by equation 2.

Table 7. Average parameters for the disulphide bonds conformational categories in the sample under study. Representative conformations for structural (-LHSpiral), catalytic (+/-RHHook) and allosteric (-RHStaple) disulphide bonds are represented in bold.

Conformational category	Frequency	DSE/kJ mol ⁻¹	d(C _α -C _{α'})/Å	d(C _β -C _{β'})/Å
-LHSpiral	28.9%	11.5	5.7	3.7
-RHHook	9.9%	25.0	5.7	4.0
+/-RHSpiral	8.6%	14.5	5.9	3.8
+/-LHSpiral	7.9%	17.9	6.0	3.7
-RHSpiral	7.0%	18.9	6.0	3.8
+/-RHHook	6.1%	19.4	5.3	3.8
+RHSpiral	6.0%	12.8	5.8	3.7
-LHHook	5.2%	37.0	5.7	4.1
-/+RHHook	4.7%	17.9	5.5	3.9
-RHStaple	4.0%	21.1	4.4	4.0
+/-LHHook	2.2%	26.8	5.9	4.0
-/+LHHook	1.9%	32.7	6.1	4.0
+/-LHStaple	1.6%	30.3	5.0	3.7
-LHStaple	1.5%	31.4	5.5	3.9
+LHSpiral	1.4%	20.8	6.2	3.9
+LHHook	1.2%	29.3	5.9	3.8
+RHHook	0.7%	30.7	6.1	4.1
+/-RHStaple	0.6%	32.3	5.9	4.1
+LHStaple	0.4%	39.3	5.4	3.3
+RHStaple	0.1%	24.9	5.9	3.3
Least strained [#]	63.1%	15.6	5.8	3.8
Most strained	36.9%	28.6	5.6	3.9

[#]The six conformational categories with the smallest DSE have a grey background.

Table 8. frequencies for the different conformational categories.

Superfamily Category	1	2	3	4	5	6	7	8	9	10	11	12	Sample
-LHSpiral	35.2%	43.8%	26.4%	46.1%	13.8%	29.0%	27.3%	28.3%	12.1%	28.6%	32.7%	3.1%	28.9%
-RHHook	0.0%	5.4%	18.9%	8.7%	20.7%	24.0%	6.7%	9.3%	0.0%	4.8%	3.8%	10.8%	9.9%
+/-RHSpiral	7.4%	14.3%	7.5%	5.2%	8.0%	20.0%	3.8%	12.1%	3.0%	4.8%	3.8%	1.5%	8.6%
+/-LHSpiral	11.1%	4.5%	3.8%	9.6%	2.3%	22.0%	4.3%	2.8%	0.0%	19.0%	30.8%	6.2%	7.9%
-RHSpiral	14.8%	9.8%	3.8%	2.6%	3.4%	0.0%	17.2%	3.2%	21.2%	4.8%	1.9%	1.5%	7.0%
+/-RHHook	0.0%	3.6%	3.8%	3.5%	2.3%	0.0%	2.9%	7.7%	0.0%	2.4%	0.0%	50.8%	6.1%
+RHSpiral	0.0%	3.6%	1.9%	3.5%	4.6%	4.0%	12.9%	9.7%	0.0%	0.0%	1.9%	1.5%	6.0%
-LHHook	0.0%	1.8%	7.5%	6.1%	5.7%	1.0%	5.3%	10.1%	6.1%	2.4%	3.8%	1.5%	5.2%
-/+RHHook	5.6%	0.0%	0.0%	1.7%	11.5%	0.0%	2.9%	4.9%	12.1%	23.8%	15.4%	0.0%	4.7%
-RHStaple	24.1%	0.0%	5.7%	1.7%	4.6%	0.0%	2.4%	0.4%	24.2%	0.0%	0.0%	16.9%	4.0%
+/-LHHook	0.0%	0.0%	9.4%	1.7%	5.7%	0.0%	2.9%	2.0%	0.0%	0.0%	1.9%	3.1%	2.2%
-/+LHHook	0.0%	1.8%	5.7%	0.0%	3.4%	0.0%	3.8%	2.4%	0.0%	0.0%	0.0%	0.0%	1.9%
+/-LHStaple	1.9%	0.9%	0.0%	0.0%	10.3%	0.0%	1.4%	0.0%	6.1%	0.0%	1.9%	3.1%	1.6%
-LHStaple	0.0%	0.9%	3.8%	0.9%	2.3%	0.0%	1.0%	1.6%	6.1%	9.5%	0.0%	0.0%	1.5%
+LHSpiral	0.0%	8.9%	0.0%	0.0%	1.1%	0.0%	1.0%	1.2%	0.0%	0.0%	0.0%	0.0%	1.4%
+LHHook	0.0%	0.0%	1.9%	2.6%	0.0%	0.0%	2.4%	1.2%	6.1%	0.0%	0.0%	0.0%	1.2%
+RHHook	0.0%	0.9%	0.0%	1.7%	0.0%	0.0%	0.5%	1.2%	0.0%	0.0%	1.9%	0.0%	0.7%
+/-RHStaple	0.0%	0.0%	0.0%	0.9%	0.0%	0.0%	1.4%	0.8%	3.0%	0.0%	0.0%	0.0%	0.6%
+LHStaple	0.0%	0.0%	0.0%	2.6%	0.0%	0.0%	0.0%	0.8%	0.0%	0.0%	0.0%	0.0%	0.4%
+RHStaple	0.0%	0.0%	0.0%	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%

Table 9. Square Euclidian distances matrix for the cluster space.

Cluster	1	2	3	4	5	6
1	0.00%	34.89%	40.29%	45.79%	32.03%	44.03%
2	34.89%	0.00%	8.77%	25.38%	13.42%	21.20%
3	40.29%	8.77%	0.00%	12.00%	11.24%	12.72%
4	45.79%	25.38%	12.00%	6.18%	19.99%	24.61%
5	32.03%	13.42%	11.24%	19.99%	3.98%	19.42%
6	44.03%	21.20%	12.72%	24.61%	19.42%	1.83%

Figure 1

Figure 2

4. Figure