**Original article**

**Inferring hypothesis-based transitions in clade-specific models of chromosome number evolution along the sedges’ phylogeny (Cyperaceae, Poales).**

José Ignacio Márquez-Corro1, Marcial Escudero2, Santiago Martín-Bravo1, Daniel Spalink3 & Modesto Luceño1

1. Department of Molecular Biology and Biochemical Engineering, Pablo de Olavide University, Carretera de Utrera km 1, ES-41013 Seville, Spain.

2. Department of Plant Biology and Ecology, University of Seville, Reina Mercedes sn, ES-41012 Seville, Spain.

3. Department of Botany, University of Wisconsin-Madison, WI-53706 Madison, USA.

Author for correspondence: José Ignacio Márquez-Corro, e-mail: [jimarcorr@gmail.com](mailto:jimarcorr@gmail.com).

**Abstract**

Lineage diversification has been intensely studied in plenty of organisms. Some events, like chromosomal rearrangements, have been showed to trigger diversification. For instance, angiosperms evolution has been linked with several polyploidization events. As counterpart, phenomena of fusions and fissions have been overlooked in most works regarding evolutionary effects of changes in chromosome numbers. In this article, we aim to elucidate the role of chromosomal rearrangements on lineage diversification by analyzing the two most recent family trees of the sedges. Mode of chromosome evolution will be inferred to the complete phylogeny as null hypothesis. In order to discern patterns of diversification shifts and chromosome number changes within family tree, we pruned the complete phylogeny in several clades/subtrees according to previously reported increments of diversification rates. Results show a link between diversification and changes in chromosome evolution, with two clear patterns: one in genus *Carex*, that evolved mainly by gains and losses of chromosomes (fissions and fusions), and in a lineage of *Cyperus*, characterized by duplications of the genome content.

**Keywords**

ChromEvol, chromosome evolution, dysploidy, diversification rates, holocentric chromosomes, polyploidy

**1. Introduction**

Chromosomal rearrangements are usual in eukaryotes and have been proved to lead species differentiation (Coghlan et al., 2005). These rearrangements could be produced by a sole mechanism or a combination of translocations, inversions, duplications, deletions, dysploidies (fusions and fissions of parts or complete chromosome sets) and polyploidies (whole genome duplication –WGD–) (Coghlan et al., 2005). Whereas some of these events could produce changes in the linkage disequilibrium of genes (Butlin, 2005), others could affect directly in the amount of gene content either erasing (i.e. deletions and fissions –except for some lineages–) or increasing it (i.e. duplications and polyploidies) (Coghlan et al., 2005). These events aid speciation by provoking changes in species fitness, adaptability to new habitats, reproductive isolation or shifts in recombination rates (Butlin, 2005; Coghlan et al., 2005; Coyne and Orr, 2004; Navarro and Barton, 2003a, 2003b; S P Otto and Whitton, 2000; Rieseberg, 2001; Soltis et al., 2009).

In angiosperms, it has been especially discussed the role of polyploidy and its consequences on speciation, with a remarkably interest in ancient polyploid events in some of the richest lineages (Debodt et al., 2005; Smith et al., 2017; Soltis et al., 2009; Soltis and Soltis, 2016), which has led to understand polyploidization as a possible driver for lineage radiation (Comai, 2005; Hegarty and Hiscock, 2007, 2008; Levin, 1983; Otto, 2007; Otto and Whitton, 2000; Soltis and Soltis, 2016, 2000; Van de Peer, 2011). On the other hand, although dysploidy is more frequent than polyploidy and aneuploidy (duplication or deletion of a chromosome) in angiosperm (Grant, 1981), its consequences in diversification have been disregarded, despite some authors pointed out dysploidy as important events in species diversification (Gitaí et al., 2014; Lee and Namai, 1993, 1992; Orellana et al., 2007; Vallès et al., 2012; Vickery, 1995; Weiss‐Schneeweiss et al., 2009). Nonetheless, its importance has been recently suggested to be neutral in terms of lineage diversification, probably because these events do not involve changes in genetic material content (Escudero et al., 2014).

The cosmopolitan family of sedges (Cyperaceae, ca. 5500 species; Govaerts et al. 2017) is the tenth richest angiosperm family and it is mainly diversified on the tropics, with exception of genus *Carex* L. that is distributed mostly along the temperate regions (Reznicek, 1990). Moreover, sedges are the angiosperm family with highest chromosomal variation (2n=4–224; Roalson 2008). Because of its high species richness and wide range of chromosome numbers, Cyperaceae constitutes a model taxa for implementing studies on biodiversity, evolution and systematics, especially the genus *Carex* (e.g. Hipp 2007). This genus also present a wide variation of chromosome numbers (2n=12–124; Roalson 2008) which have encourage important works on this matter (e.g. Hipp 2007 and Roalson 2008b). Variation in the number of chromosomes and changes in the mode of evolution have been suggested as a possible driver of species richness increment in *Carex* (Escudero et al., 2014, 2012b), the most diversified genus of the family (ca. 40%; Govaerts et al. 2017).

Shifts in diversification have been detected in four main nodes of Cyperaceae. Previously, Escudero et al. (2012b) had already found an increment in diversification rates in the node that comprises Core *Carex*, Caricoid *Carex* and *Carex* subgenus *Vignea*, which has been confirmed in a recent study by Spalink et al. (2016). Escudero and Hipp (2013) found the node including the tribes Scirpeae, Dulichieae, and Cariceae plus *Khaosokia* *caricoides* (SDC clade) and the tribes Fuireneae, Abildgaardieae, Eleocharideae, and Cypereae (FAEC clade) to present changes in the diversification rate based on Hinchliff and Roalson (2013) phylogeny. Spalink et al. (2016), showed shifts in three different lineages inside the clade reported by Escudero and Hipp (2013), obtaining the same node as Escudero et al. (2012b). Moreover, Spalink et al. (2016) also found a shift in the FAEC clade and in the represented taxa of the C4 photosynthetic pathway *Cyperus* within Cypereae 2 clade.

The huge continuous variation in chromosome number of this family is explained by the presence of holocentric chromosomes, which means that the kinetochoric activity is present along the chromosomes, contrary to those monocentric, that present a clear primary constriction in which kinetochoric activity is concentrated (Hipp et al., 2013; Melters et al., 2012; Mola and Papeschi, 2006). In lineages that present holocentric chromosomes (see revision in Márquez-Corro et al. 2017), fusions and fissions (named symploidy and agmatoploidy, respectively) are more common (Grant, 1981). This occur even within species level, due to the characteristics of the kinetochoric plate (Hipp et al., 2013; Melters et al., 2012; Mola and Papeschi, 2006) and the neutral balance in DNA content (Escudero et al., 2014).

As stated above, dysploidy (Gitaí et al., 2014; Lee and Namai, 1993, 1992; Orellana et al., 2007; Vallès et al., 2012; Vickery, 1995; Weiss‐Schneeweiss et al., 2009) and polyploidy (Comai, 2005; Hegarty and Hiscock, 2007, 2008; Levin, 1983; Otto, 2007; Sarah P Otto and Whitton, 2000; Soltis and Soltis, 2016, 2000; Van de Peer, 2011) have been considered as possible diversification drivers. Cyperaceae family present lineages with prevalence of different modes of chromosomal evolution (e.g. *Carex* karyotype evolves mainly via agmatoploidy and symploidy; Heilborn 1924; Davies 1956). Thus, this hyperdiverse family and its wide range of karyotypic variation constitute a perfect taxon to study how changes in diversification rates and chromosome evolution are related. In light of these studies, we hypothesize that some shifts in lineage diversification could be caused, at least in part, by changes in the mode of chromosome evolution, probably leading to different mechanism of adaptation or reproductive isolation (Butlin, 2005; Coghlan et al., 2005; Coyne and Orr, 2004; Navarro and Barton, 2003a, 2003b; S P Otto and Whitton, 2000; Rieseberg, 2001; Soltis et al., 2009).

Recently (Glick and Mayrose, 2014; Mayrose et al., 2010), some hypothesis-testing probabilistic models of chromosome number evolution have been formulated. These models include different parameters, with the simplest ones calculating the rate of gains, losses and ploidy augments in chromosome number along a phylogeny. Complexes models allow identifying linear dependency between the starting number of chromosomes and the rate of ascending and decreasing dysploidy. Because our hypothesis is focused on the shifts of gains/losses of specific lineages chromosome number, and not in the relation between the latter and the parameters, we overlook the linear models. We applied these models to the latest sedges phylogenies (Hinchliff and Roalson, 2013; Spalink et al., 2016) and the different pruned lineages with a treatment similar to that proposed by O’Meara et al. (2006) and already carried out by Hipp (2007) on north American *Carex* sect. *Ovales* species.

The aim of this study is (i) to elucidate the role of chromosome evolution in diversification of sedges family by the implementation of probabilistic models in an unprecedented manner, and (ii) to evaluate the potential usability of the nested models treatment in studies of chromosome evolution for high species richness lineages in order to discern different evolution patterns within a phylogeny. The null hypothesis is that chromosome number changes along the family at constant rate, independent of the diversification rates. On the other hand, the alternative hypothesis is that there are transitions in the mode of chromosome evolution, somewhat followed by a shift in diversification rates.

**2. Materials** **and** **methods**

**2.1. Family tree and chromosome counts**

Latest phylogenies of Cyperaceae family (Hinchliff and Roalson, 2013; Spalink et al., 2016) were used as backbone for analyses performance. Species haploid number were collected from online databases IPCN (Index to Plant Chromosome Numbers, Goldblatt and Johnson 2017) and CCDB (Chromosome Counts Database, Rice et al. 2015), and some chromosome number reports (see Appendix 1).

Regarding Hinchliff and Roalson (2013) phylogeny, *Cyperus* *cyperoides*, *C*. *cuspidatus* and *Kyllingiella* *microcephala* were not taken as part of the clade that comprises others C4 photosynthetic pathway *Cyperus* because those taxa were not sampled in Spalink et al. (2016) and, thus, it is unclear whether the increment of diversification rate event would also include aforementioned species. In this way, the analysis would be more conservative.

Due to the holocentric characteristic of sedges chromosomes, counts could range widely even within single species (Roalson, 2008). Because we aimed to detect shifts in chromosome number evolution along the Cyperaceae family tree, we assigned to the tips the most frequent number in the species dominated by simploidy/agmatoploidy events, and the record with the lower chromosome number for the taxa presenting polyploidy (see Appendix 1).

**2.2. Implementation of the analyses**

Latest Cyperaceae phylogenies (Hinchliff and Roalson, 2013; Spalink et al., 2016) were analyzed with chromosome information using ChromEvol v.2.0 (Glick and Mayrose, 2014; Mayrose et al., 2010) in order to elucidate the mode of chromosome evolution. This software allows determining the probability of a certain model to explain the given data along the phylogeny, based on the two first or more of the following parameters: (i) gain or (ii) loss of a single chromosome, (iii) polyploidization, (iv) increment on half of the chromosome number (demi-polyploidization) and (v) increment with regard of the same base-number. Furthermore, two more parameters permit detecting linear dependency between the starting haploid number and the rate of (vi) gain and (vii) loss of chromosomes. Because we aim to elucidate the link between diversification rates shifts and the mode of chromosome evolution, and not with the initial haploid number, we discard those models regarding linear parameters.

As stated above, shifts in diversification have been detected in four main nodes of Cyperaceae (Escudero et al., 2012b; Escudero and Hipp, 2013; Spalink et al., 2016), so the analysis was carried out not only for the complete phylogenies but for several pruned clades as well. The analysis was implemented on the entire phylogeny, single clades that have been proved to exhibit diversification rates shifts, background of every clade, and further combinations of clades and backgrounds. This methodology have previously allowed (see O’Meara et al. 2006; Hipp 2007) the identification of different processes throughout the evolutionary history of the study group. We started covering from simplest to most complex scenarios, analyzing and comparing the models that best fit the data by calculating the sum of the respective Akaike information criterion score (AIC, Mayrose et al. 2010). Therefore, if the mode of chromosome evolution of the entire phylogeny presents less explanatory statistical power than the sum of the respective subtree, a shift in diversification rate might be related to a change in the mode of chromosome evolution.

**3. RESULTS**

**3.1. Chromosome data**

Chromosomes counts were downloaded for a total of 255 taxa (Appendix 1), of which 72 taxa were represented in both phylogenies. A total of 207 and 120 chromosome data was obtained for Hinchliff and Roalson (2013; ca. 45% of the species represented in the phylogeny –435 species–) and Spalink et al. (2016; ca. 35% –345 species–), respectively (Appendix 2). Nonetheless, chromosome base number of the family was impossible to infer phylogenies due to the frequent agmatoploidy/symploidy events, the incomplete phylogenies (6–8% of Cyperaceae species in both studies) and lack of karyological reports for the represented species.

**3.2. Models of chromosome evolution**

Best-fitting models for the complete trees were Const\_Rate\_Demi\_Est for Hinchliff and Roalson (2013) and Const\_Rate\_Demi for Spalink et al. (2016) phylogenies, with AIC values of 1489.68 and 893.625, respectively (see Table 1). The Const\_Rate\_Demi model implies a constant rate of single chromosome increment, decrement and a constant and equal rate of polyploidy and demi-polyploidy (Mayrose et al., 2010). In contrast, Const\_Rate\_Demi\_Est calculates independent rates of polyploidy and demi-polyploidy (Mayrose et al., 2010). The analysis of separate pruned trees and clades showed a significant decrement of the AIC score in both family phylogenies: ΔAIC=−24.88 and ΔAIC=−32.34 for Hinchliff and Roalson (2013) and Spalink et al. (2016), respectively (see Table 1).

The best scenario for Hinchliff and Roalson (2013) was a background model of evolution based on gains (97.7 events for all branches of that “phylogeny slice”) and losses (93.7 events) of single chromosomes and a rate of chromosome change equal to 3.7e-2 with a chromosome base number x = 17 (Base\_Num model, Figure 1). At the node 1 (Eleocharideae-Abildgaardieae-Fuireneae-Cypereae clade), the mode of evolution changed to the Constant\_Rate\_Demi model, with 7.3 total events of gain, 3.8 events of loss and 1.3 events of duplication (either demi-polyploidization or WGD). Finally, the node 3 (C4 *Cyperus* lineage) continued the Constant\_Rate\_Demi model, but experienced a change in the parameters. Fissions events decreased (1.4e-10), as well as duplication (demi-ploidy = polyploidy, 0.5 events), whereas fusions augmented remarkably (28.5 events).

On the other hand, Spalink et al. (2016) showed a Constant\_Rate background with a total of 20.1 gain events, 13.5 loss events and 1.3 duplication (i.e. polyploid) events along the phylogeny. Two shifts in the mode of chromosome evolution were detected. The first at the node 2 (most *Carex* genus), in which gains and losses increased (56.6 and 40.9 events, respectively), and no duplication was identified (Constant\_Rate\_No\_Dupli model). The second shift (to Constant\_Rate\_Demi model) is produced at the node 3, with 1.4e-10 gain events, 36.8 loss events and 0.4 duplication (demi-ploidy = polyploidy) events, similarly to the corresponding clade of Hinchliff and Roalson (2013).

The results of the remaining model combinations are included in Table 1, with the three best-fitting models depicted in Figure 1. Analysis output files with all the chromosome rate transitions of every model studied are electronically available on [github.com/jimarcor/ChromEvolCyp](https://github.com/jimarcor/ChromEvolCyp).

**4. Discussion**

**4.1. Chromosome evolution on the family**

Although the models vary slightly among the best scenarios of the two family trees used, this dissimilarity is explained mainly by the different sampling between the phylogenies, which only share 72 species. Thus, congruency in the results was found when implementing the analysis in both phylogenies (Figure 1, Table 1). Because of this reason, parameters of fissions, fusions and duplication of chromosomes are similar when comparing between models of each clade or subtree (see Figure 1). There are two clear patterns of karyological evolution showed in both trees’ best fitting-models.

The first one shows the evolution of the C4 *Cyperus* species represented (Node 3), related with genome duplication. Accordingly, polyploidy has been suggested previously for *Cyperus* *esculentus* (Arias et al., 2011; De Castro et al., 2015), sister species of *C. rotundus*, this latter represented in the studied phylogenies. Despite neo-polyploids have been argued to not trigger higher diversification rates (Escudero et al., 2014), this *Cyperus* lineage would constitute a counterexample of the trend. This case is very remarkable and should be studied in detail. In this work, lineage diversification agrees with the change in the mode of chromosome evolution from a constant rate to an evolutionary scenario dominated by genome duplication. Nevertheless, this increment of the diversification rate could be related with other innovative mechanisms of the lineage, such as the evolution to the C4 photosynthetic pathway (Larridon et al., 2013).

Other pattern supported by the analysis is the change in the genus *Carex* –that now comprises former genera *Kobresia*, *Schoenoxiphium*, *Uncinia* and *Cymophyllus* (Global *Carex* Group, 2015)–, in which no duplication has been inferred, contrary to the background tree. Models regarding this clade imply the evolution of chromosomes by events of agmatoploidy (fission) and symploidy (fusion). This phenomenon have been historically suggested (Davies, 1956; Hipp et al., 2009; Hoshino, 1981; Wahl, 1940), but it has never been statistically tested at this level. *Carex* constitutes ca. 40% of the Cyperaceae species (Govaerts et al., 2017), so understanding how diversification rates shifts is related to karyotypic change is key to comprehend chromosome evolution as output, trigger or part of speciation process, as well as to elucidate whether this change is mediated by intrinsic (e.g. linkage disequilibrium), extrinsic (e.g. ecology) or the interplay of both factors. Elucidating what kind of effect climate change or new niche availability could have in lineage diversification in these species and other organisms with holocentric chromosomes will shed light in a straightforward direction to understand chromosome evolution on these organisms. In this regard, ecological conditions have been stated to play an important role in *Carex* karyotype, as extreme and unstable habitats correlates with low chromosome number and, thus, with low recombination events (Escudero et al., 2012a).

Finally, although less represented among the best-fitting models (Figure 1), maybe due to the lower number of taxa included in the phylogenies, shifts on nodes 1 and 4 have been detected. This could be explained by partially hidden pattern of change in chromosome numbers that have been disregarded due to misrepresentation of specimens with chromosome counts in the phylogenies and differences between the phylogenies implemented, that only shared 72 taxa. A more complete family tree is required in order to find further possible lineage diversification events and, then, a more detailed work on the possible relation with more chromosome data yet to study.

**4.2. Notes on the methodology**

Implementing the methodology of studying full trees and subtrees separately has led to a significant increment in the adjusting (>25 AIC score decrement) of the chromosome data to the distribution of different models of chromosome evolution along the phylogeny. It worth mentioning that the results remain analogous independently of the phylogeny sampling. Thus, the methodology is prove again as favorable (see also O’Meara et al. 2006; Escudero et al. 2012) in order to study evolutionary process such as chromosome evolution at higher taxa levels. Moreover, this is also relevant in the study of organisms with holocentric chromosome, which labile karyotype could evolve differently throughout the phylogeny of the lineages, as it has been perceived in this study.

**4.3. Future research on chromosome evolution**

Due to the fact that each day data and information are more available and complete (i.e. resolved family trees and chromosome counts), studies at this level of complexity are more viable than ever. Further comparable research on other organisms’ lineages will lead to uncover linking patterns between diversification rates, karyotype evolution, genetic mutations and ecological factors. One example would be the study of the relation between chromosome number and ecological conditions, as aforementioned (see section 4.1.; Escudero et al., 2012a), not only in a major scale including organisms with holocentric chromosomes, but in a broader sense including different lineages of monocentric organisms.

Summing up, this study propose (i) the use of separate pruned trees and clades vs. complete phylogenies as a feasible approach to the study of chromosome evolution in relation to other evolutionary processes; (ii) that, in our case, has resulted in a correspondence between an increment of diversification rates with a change in the way how chromosomes evolves; (iii) evidencing agmatoploidy/symploidy as main mean of chromosome evolution in genus *Carex* (except *Siderostictae* clade), (iv) and duplication for, at least, part of the lineage of C4 *Cyperus* species.

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**Figure captions:**

Fig. 1. Three best-fitting scenarios of chromosome evolution for (a) Spalink et al. (2016) and (b) Hinchliff and Roalson (2013) Cyperaceae phylogenies. Numbered nodes correspond to those in which a shift in diversification rate have been detected (1, FAEC clade; 2, *Carex* lineage; 3, *Cyperus* lineage; 4, SDC+FAEC clade).

**Table 1. AIC score values obtained for every model carried on ChromEvol (Glick and Mayrose, 2014; Mayrose et al., 2010).**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Spalink et al. (2016)** | | | | | | | **Hinchliff & Roalson (2013)** | | | | | |
| **Clade / subtree** | **Base Num** | **Base Num Dupl** | **Const Rate** | **Const Rate Demi** | **Const Rate Demi Est** | **Const Rate No Dupli** | **Base Num** | **Base Num Dupl** | **Const Rate** | **Const Rate Demi** | **Const Rate Demi Est** | **Const Rate No Dupli** |
| **Complete tree** | **907.725** | **903.823** | **899.767** | **893.625** | **895.477** | **964.142** | **1510.83** | **1503.91** | **1499.91** | **1490.54** | **1489.68** | **1648.87** |
| **1BG** | 541.905 | 539.900 | 535.920 | 532.147 | 533.556 | 543.686 | 874.05 | 878.96 | 874.98 | 875.56 | 878.39 | 908.07 |
| **1** | 360.629 | 361.143 | 360.030 | 356.154 | 357.493 | 380.763 | 623.86 | 613.35 | 610.96 | 602.78 | 601.65 | 638.70 |
| **SUM** | **1BG + 1** | | | **888.301** | | |  | | | **1475.69** | | |
| **2BG** | 651.422 | 642.7 | 640.563 | 639.879 | 640.42 | 732.8 | 1043.39 | 1033.45 | 1030.38 | 1020.61 | 1020.16 | 1117.54 |
| **2** | 232.824 | 234.593 | 230.379 | 230.379 | 232.407 | 228.683 | 461.92 | 467.80 | 464.54 | 465.26 | 465.77 | 488.52 |
| **SUM** | **2BG + 2** | | | **870.258** | | |  | | | **1482.08** | | |
| **3BG** | 839.218 | 832.924 | 828.933 | 822.238 | 824.62 | 874.76 | 1431.95 | 1422.12 | 1418.21 | 1411.49 | 1411.94 | 1532.09 |
| **3** | 68.454 | 72.041 | 68.508 | 68.265 | 70.291 | 69.699 | 65.69 | 67.69 | 66.21 | 64.56 | 65.87 | 65.93 |
| **SUM** | **3BG + 3** | | | **890.503** | | |  | | | **1476.05** | | |
| **4BG** | 173.163 | 172.596 | 171.784 | 171.889 | 173.584 | 203.711 | 247.85 | 246.18 | 242.15 | 242.97 | 245.07 | 256.75 |
| **4** | 727.852 | 728.705 | 724.709 | 719.564 | 720.171 | 742.179 | 1259.07 | 1255.56 | 1251.56 | 1248.8 | 1247.99 | 1325.64 |
| **SUM** | **4BG + 4** | | | **891.348** | | |  | | | **1501.22** | | |
| ***1+2BG*** | 289.538 | 289.138 | 289.270 | 283.817 | 282.938 | 317.807 | 415.63 | 418.41 | 415.56 | 413.06 | 414.33 | 415.13 |
| **SUM** | **1 + 2 + *1+2BG*** | | | **867.775** | | |  | | | **1476.63** | | |
| ***2+3BG*** | 578.124 | 568.485 | 564.333 | 564.426 | 565.219 | 645.091 | 964.923 | 953.422 | 949.733 | 943.486 | 944.406 | 1012.49 |
| **SUM** | **2 + 3 + *2+3BG*** | | | **861.281** | | |  | | | **1469.97** | | |
| ***4-2*** | 477.589 | 470.274 | 471.548 | 470.251 | 486.541 | 513.650 | 794.19 | 788.14 | 786.84 | 779.14 | 779.56 | 814.32 |
| **SUM** | **4BG + *4-2* + 2** | | | **870.718** | | |  | | | **1483.63** | | |
| ***1-3*** | 288.382 | 284.223 | 285.295 | 284.488 | 285.971 | 306.846 | 543.40 | 535.51 | 529.71 | 526.192 | 527.34 | 553.34 |
| **SUM** | **1BG + *1-3* + 3** | | | **884.635** | | |  | | | **1464.80** | | |
| ***4-2-3*** | 400.855 | 395.161 | 396.550 | 396.520 | 405.842 | 436.318 | 710.799 | 709.18 | 705.40 | 702.64 | 702.23 | 723.49 |
| **SUM** | **4BG + 2 + 3 + *4-2-3*** | | | **863.893** | | |  | | | **1470.86** | | |
| ***4-1*** | 359.638 | 361.417 | 357.533 | 355.696 | 355.520 | 355.653 | 628.267 | 630.282 | 630.081 | 630.761 | 632.092 | 653.479 |
| **SUM** | **4BG + *4-1* + 1** | | | **883.458** | | |  | | | **1473.20** | | |

**BG**: Background of the node. **SUM**: Sum of the models. **1+2BG**: Background of Node 1 and 2. **2+3BG**: Background of Node 2 and 3. **4-2**: Node 4 without species of Node 2. **1-3**: Node 1 without species of Node 3. **4-2-3**: Node 4 without species of Nodes 2 and 3. **4-1**: Node 4 without species of Node 1.