

A comparative karyological study of *Helianthemum* (Cistaceae): karyotype size, karyotype symmetry and evolution of chromosome number

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Abstract. In this study we assessed karyotype size and symmetry for a comprehensive taxonomic and geographic representation of *Helianthemum* and reconstructed chromosome number evolution in the genus. Using root tips, we photographed mitotic metaphase spreads to obtain chromosome number, total haploid (monoploid) length of the chromosome set (THL), karyotype formula, Stebbins' classification of karyotype asymmetry, interchromosomal coefficient of variation of chromosome length (CV_{CL}) and intrachromosomal mean centromeric asymmetry (M_{CA}) using MATO (Measurement and Analysis Tools). We found that shifts in chromosome number are not a major driver in the evolution of *Helianthemum*, whose chromosome number evolved at a constant rate of single chromosome gain or loss. Karyotype asymmetry is very low and little variable in all taxonomic categories studied, with a predominance of metacentric and submetacentric small to medium-sized chromosomes about 3 μ m at the genus level. However, total karyotype length varies from 16.91 μ m to 47.84 μ m at the species level, with a cytogenetic signature that is not conserved within subgenera and most sections. Overall, *H. subg. Plectolobum* shows both the longest and the most symmetrical karyotypes. We hypothesize that the variation in karyotype size in *Helianthemum* is likely a consequence of chromosome rearrangements that have occurred under selective pressures.

Resumen. En este estudio analizamos el tamaño y la simetría del cariotipo de un elevado número de especies de *Helianthemum* y reconstituimos la evolución del número cromosómico. Para ello, analizamos metafases mitóticas de ápices radicales para obtener el número cromosómico, valores de la longitud (monoploide) total del cariotipo (THL), la fórmula cariotípica, el grado de asimetría según la clasificación de Stebbins, el coeficiente de variación intercromosómico de la longitud cromosómica (CV_{CL}) y la asimetría intracromosómica media (M_{CA}), parámetros obtenidos mediante MATO (Measurement and Analysis Tools). Encontramos que la diversificación en *Helianthemum* no está ligada a cambios en el número de cromosomas, el cual ha evolucionado de forma lenta y constante mediante bajas tasas de pérdida o ganancia de cromosomas. La asimetría del cariotipo es escasa y poco variable en todas las categorías taxonómicas, y existe una predominancia de cromosomas meta o submetacéntricos cuya longitud media a nivel de género es de c. 3 μ m. En cambio, la longitud total del cariotipo oscila entre 16,91 y 47,84 μ m y *H. subg. Plectolobum* posee los cariotipos de mayor longitud y simetría. Proponemos la hipótesis de que las diferencias en el tamaño del cariotipo en *Helianthemum* son consecuencia de reajustes cromosómicos sucedidos bajo presiones selectivas.

Keywords. ChromEvol, comparative cytogenetics, karyotype, plant cytotoxicology.

Palabras clave. Cariotipo, ChromEvol, citogenética comparativa, citotaxonomía vegetal.

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INTRODUCTION

The comparative study of karyotype diversity among species of a lineage, including variation in chromosome number, size, and symmetry, is an essential cytotaxonomic information for understanding evolutionary patterns in plants (Weiss-Schneeweiss & Schneeweiss 2013). For example, trends potentially related to evolutionary processes have been described, such as lower chromosome numbers selected under unstable environmental conditions (Carta & al. 2018) or small chromosome size characterizing species with large geographical distributions (Elliot & al. 2022). Indeed, environmental factors may favour large versus small genomes and affect the performance of organisms (Aparicio & al. 2019; Cacho & al. 2021).

The full length of the chromosome set is correlated with genome size (i.e., the amount of DNA contained in a cell nucleus), but not with chromosome number (Soltis & al. 2005; Greilhuber & Leitch 2013; Weiss-Schneeweiss & Schneeweiss 2013), and the mechanisms of karyotype evolution entail both increases and decreases in the length of chromosome arms and the position of centromeres in monocentric chromosomes (Stebbins 1971; Lysák & al. 2006; Schubert & Lysák 2011; Weiss-Schneeweiss & Schneeweiss 2013). Thus, it is essential for comparative cytotaxonomy to assess not only if chromosome number is a stable feature across the studied lineage but also to estimate karyotype diversity and size.

Helianthemum Mill. is a monophyletic lineage within the family Cistaceae Juss. composed of three subgenera, 10 sections and about 140 species and subspecies (Martín-Hernanz & al. 2021a). It is distributed in the Palearctic region along a wide variety of environmental conditions (Martín-Hernanz & al. 2021b) and includes significant variability in life form (therophytes to chamaephytes) and breeding systems (autogamous, facultatively xenogamous and xenogamous; Martín-Hernanz & al. 2023). From a cytotaxonomic perspective, the somatic numbers known so far for most species are $2n = 20$ and $2n = 22$, with the occasional $2n = 10$ and $2n = 24$ restricted to *H. squamatum* (L.) Dum.Cours. and *H. caput-felis* Boiss., respectively. Based on the chromosome number of *H. squamatum*, it was assumed that $x = 5$ was the base chromosome number in *Helianthemum* being most species ancient tetraploids (e.g., Dalgaard 1986). However, it has recently been inferred (Aparicio & al. 2019) that the chromosome number in *H. squamatum* is the result of a recent large dysploid genome reorganization, and that $x = 10$ is the most likely ancestral base chromosome number of the genus; thus, all species in *Helianthemum* can be considered diploid (but see below).

Although abundant information on chromosome number is available for *Helianthemum*, with about 65% of the species already known (Goldblatt & Johnson 1979; Rice &

al. 2015; Aparicio & al. 2019), other constituent karyotype features such as karyotype size and asymmetry remain virtually unknown. In this study we aimed to increase the number of *Helianthemum* species for which the chromosome number is known and to analyse karyotype features such as the full size (length) of the karyotype and the interchromosomal and intrachromosomal components of karyotype asymmetry (Peruzzi & Eroğlu 2013). We analysed these characteristics at the genus, subgenus, section, and species level to assess whether karyotypes are conserved within these taxonomic categories. This information will be essential to unravel the genomic mechanisms that operated in the evolutionary history of the genus, which has expanded and diversified widely around the Mediterranean basin since the Late Miocene, entailing shifts in life history traits and, remarkably, in environmental niches (Albaladejo & al. 2021; Martín-Hernanz & al. 2021b; Martín-Hernanz & al. 2023).

MATERIAL AND METHODS

Sampling and nomenclature

We designed the sampling of this study aiming to include a broad geographical (Palearctic region) and taxonomic (three subgenera and 10 sections) representation of *Helianthemum* (see Fig. 1). Except for one species, all the seeds came from wild plants sampled in the field. We considered karyotype features to be constant at species level, so we included seeds from one or two populations per species. In every population we harvested ripe capsules from 5 to 15 different plants which were pooled in paper bags. Then, the capsules were carefully opened in the laboratory to extract the seeds. The accessions of clean seeds were kept in a dry and cool place until study. We additionally included seeds from four species stored in seed banks (Millennium Seed Bank and Israel Plant Gene Bank) (Appendix 1). In total, we karyotyped mitotic metaphase plates obtained from about 350 seeds, representing 85 populations and 78 species and subspecies.

In this study, we followed the taxonomic adscriptions and nomenclatural recommendations for the genus *Helianthemum* proposed by Martín-Hernanz & al. (2021a). Notice that we present the results for *H. sect. Helianthemum* (s.l.) separated into two groups: (1) *H. sect. 'Helianthemum Canarian clade'*, which include all the 15 species of *H. sect. Helianthemum* endemic to the Canary Islands (see Table 1), and (2) *H. sect. 'Helianthemum p.p.'* for the rest of the species in *H. sect. Helianthemum*. This is because the species from the Canary Islands conform a cohesive monophyletic lineage within *H. sect. Helianthemum* that rapidly diversified during the Pleistocene in the archipelago with idiosyncratic genomic, morphological, biogeographical, and ecological features (Aparicio & al. 2017; Martín-Her-

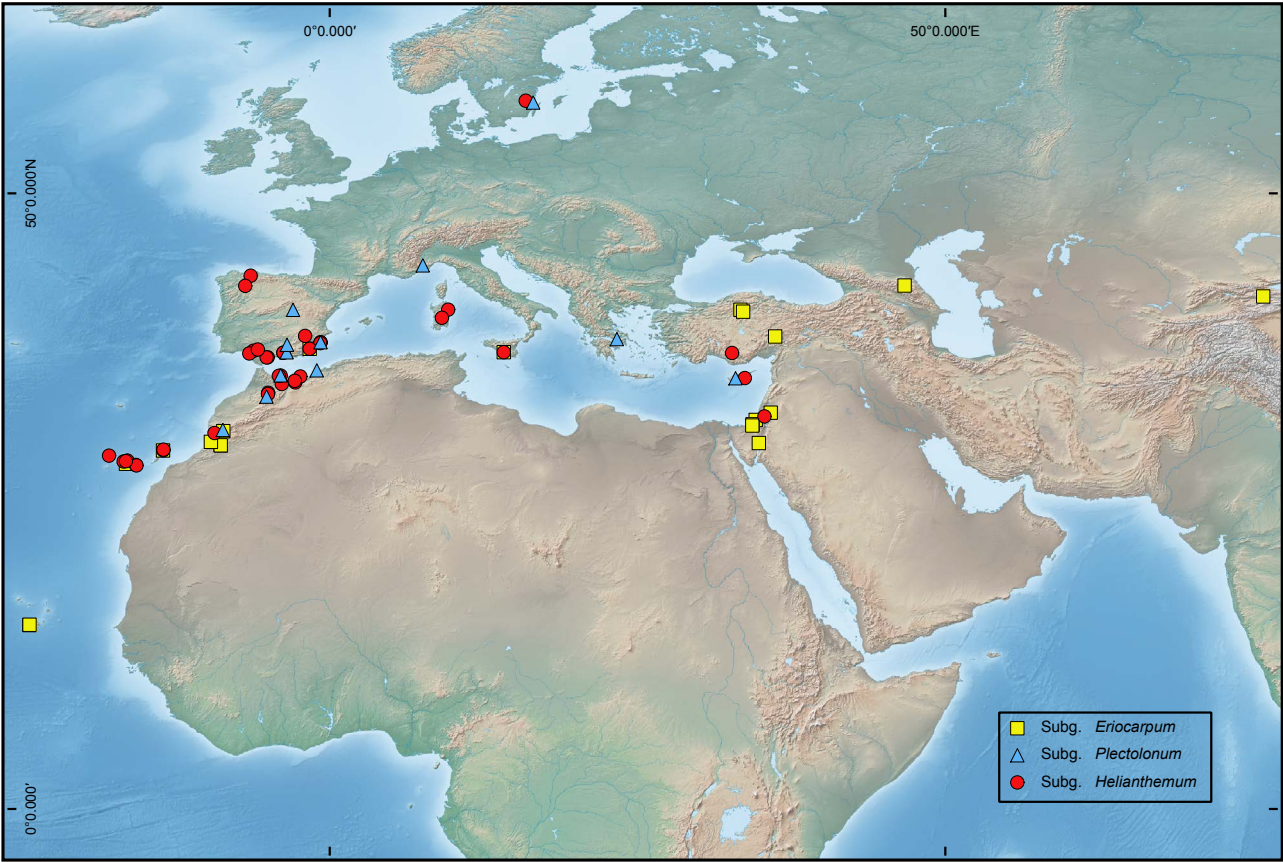


Fig. 1. Location of the 85 populations of *Helianthemum* whose seeds were analysed in this study.

Table 1. Karyological data obtained in this study for the genus *Helianthemum* at subgenus, section, and species level. 2n, somatic chromosome number; Pop, source population; N, sample size (number of mitotic metaphase spreads); THL, mean total haploid (monoploid) length of chromosome set (Altinordu & al. 2016); CV (%), coefficient of variation of mean THL; Karyotype formula (Levan & al. 1964); SKA, Stebbins' karyotype asymmetry classification (Stebbins 1971); CV_{CL} (Mean ± SD), coefficient of variation of chromosome length (Paszko 2006); MCA (Mean ± SD), mean centromeric asymmetry (Peruzzi & Eroğlu 2013). Nomenclature follows Martín-Hernanz & al. (2021a). Asterisks indicate new chromosome counts. *Helianthemum dagestanicum* Rupr. is ascribed to *H.* sect. *Pseudomacularia* following Martín-Hernanz & al. (unpublished).

	2n	Pop	N	THL (µm)	CV (%)	Karyotype formula	SKA	CV _{CL}	M _{CA}
HELIANTHEMUM	10, 20, 22, 24		709	31.40	26.48			15.20±3.38	22.80±4.47
Subg. <i>ERIOCARPUM</i>	10, 20, 22		183	22.58	27.53			17.11±3.15	20.93±4.32
Sect. <i>ARGYROLEPIS</i>	10		9	24.57	-			14.52	10.91
<i>H. squamatum</i> (L.) Dum.Cours.	10	239	9	24.57	7.13	10m	1A	14.52±5.39	10.91±1.12
Sect. <i>LAVANDULACEUM</i>	20		19	37.26	3.07			14.12±1.92	28.07±7.43
<i>H. motae</i> Sánchez-Gómez, Jiménez & Vera	20	277	8	36.45	9.61	4m+16sm	3A	15.47±2.27	33.32±1.66
<i>H. syriacum</i> (Jacq.) Dum.Cours.	20	17	11	38.07	9.65	14m+6sm	2A	12.76±3.23	22.81±1.87
Sect. <i>ERIOCARPUM</i>	20		104	18.93	6.87			18.80±2.63	19.52±0.94
<i>H. canariense</i> (Jacq.) Pers.	20	403	7	17.68	9.21	16m+4sm	1A	16.78±2.98	19.20±3.45
<i>H. confertum</i> Dunal*	20	525	10	19.33	9.33	18m+2sm	1A	20.19±4.17	20.18±3.31
<i>H. ellipticum</i> (Desf.) Pers.	20	374	10	19.19	8.06	16m+4sm	2A	21.04±4.63	20.79±7.56
<i>H. gorgoneum</i> Webb	20	348, 349	9	18.73	8.99	20m	1A	16.98±2.94	17.54±2.72
<i>H. kahiricum</i> Delile	20	374	8	19.71	7.63	19m+1sm	1A	22.35±3.67	18.32±1.95
<i>H. lippii</i> (L.) Dum.Cours.	20	283	6	17.52	6.91	16m+4sm	2A	22.47±3.95	19.09±2.60
<i>H. sancti-antonii</i> Schweinf.*	20	642	9	21.00	7.42	20m	1A	16.30±3.08	19.16±3.32

Table 1. Cont'd.

	2n	Pop	N	THL (µm)	CV (%)	Karyotype formula	SKA	CV _{CL}	M _{CA}
<i>H. sessiliflorum</i> (Desf.) Pers.	20	384, 385	13	16.91	9.35	15m+5sm	1A	18.07±3.37	19.24±5.01
<i>H. sicanorum</i> Brullo, Giusso & Sciandr.	20	297	8	21.21	5.11	20m	1A	20.15±2.62	20.38±2.49
<i>H. stipulatum</i> (Forssk.) C.Chr.	20	382	6	18.69	4.49	19m+1sm	1A	20.58±2.73	20.13±0.90
<i>H. thymiphyllum</i> Svent.	20	409	6	18.16	9.33	18m+2sm	1A	15.92±2.79	19.99±2.28
<i>H. ventosum</i> Boiss.	20	644	12	19.04	9.07	18m+2sm	1A	14.82±3.89	20.18±3.98
Sect. <i>PSEUDOMACULARIA</i>	22		51	25.67	14.64			14.17±1.29	24.11±1.28
<i>H. antitauricum</i> Davis & Coode*	22	647	10	27.86	9.04	10m+12sm	2A	15.79±3.12	25.91±3.57
<i>H. dagestanicum</i> Rupr.*	22	448	13	20.65	9.15	14m+8sm	1A	14.27±1.76	24.12±2.23
<i>H. germanicopolitanum</i> Bornm.*	22	645, 648	14	25.03	4.80	14m+8sm	1A	12.66±1.99	23.39±2.82
<i>H. songaricum</i> Zhao, Zhu & Cao*	22	643	14	29.12	6.59	15m+7sm	2A	13.97±1.77	23.02±3.17
Subg. <i>PLECTOLOBUM</i>	22, 24		137	38.27	16.78			14.30±1.94	20.23±4.05
Sect. <i>CAPUT-FELIS</i>	24		9	44.03	-			15.27	13.66
<i>H. caput-felis</i> Boiss.	24	275	9	44.03	5.93	24m	1A	15.27±1.58	13.66±1.66
<i>H. sect. ATLANTHEMUM</i>	22		6	24.09	-			18.70	26.04
<i>H. sanguineum</i> (Lag.) Lag. ex Dunal in DC.	22	295	6	24.09	6.83	13m+9sm	2A	18.70±2.98	26.04±3.49
<i>H. sect. MACULARIA</i>	22		14	30.82	39.20			12.70±1.94	25.24±7.69
<i>H. lunulatum</i> (All.) DC.	22	500	6	39.37	7.37	14m+8sm	2A	14.07±1.99	19.80±1.71
<i>H. pomeridianum</i> Dunal	22	352	8	22.28	9.89	5m+17sm	2A	11.32±2.75	30.68±7.00
Sect. <i>PSEUDOCISTUS</i>	22		108	40.39	4.76			14.11±1.53	19.39±1.87
<i>H. cinereum</i> subsp. <i>rotundifolium</i> (Dunal) Greuter & Burdet	22	429	12	37.14	8.88	17m+5sm	2A	13.53±1.67	19.46±2.55
<i>H. frigidulum</i> Cuatrecasas*	22	619	10	38.61	9.91	19m+3sm	2A	15.53±0.67	18.43±1.86
<i>H. hymettium</i> Boiss. & Heldr.	22	534	10	41.47	6.85	14m+8sm	2A	15.81±1.71	20.38±2.26
<i>H. marifolium</i> subsp. <i>andalusicum</i> (Font Quer & Rothm.) G.López*	22	49	14	42.22	8.85	14m+8sm	2A	14.72±1.49	21.20±1.62
<i>H. oelandicum</i> subsp. <i>conquense</i> (Borja & Rivas Goday ex G.López) Martín-Hernanz, Velayos, Albaladejo & Aparicio	22	242	10	38.95	9.71	17m+5sm	2A	12.36±1.78	18.28±2.02
<i>H. oelandicum</i> (L.) DC. subsp. <i>oelandicum</i>	22	576	7	39.30	9.98	15m+7sm	2A	15.59±3.32	20.89±2.39
<i>H. originifolium</i> subsp. <i>africanum</i> B.Crespo, M.A.Alonso, A.Vicente & J.L. Villar*	22	621	7	41.97	6.42	18m+4sm	2A	13.73±2.99	18.50±1.90
<i>H. pannosum</i> Boiss.	22	615	6	40.77	4.52	14m+8sm	2A	12.75±0.89	17.92±2.11
<i>H. polyanthum</i> (Desf.) Pers.	22	117	12	43.80	8.64	12m+10sm	2A	16.07±1.90	23.23±2.28
<i>H. raynaudii</i> Ortega Oliv., Romero García & C. Morales	22	70	8	39.34	4.86	16m+6sm	2A	13.50±2.56	16.63±2.09
<i>H. viscidulum</i> Boiss.	22	81	11	40.67	7.11	16m+6sm	2A	11.61±1.27	18.33±1.45
Subg. <i>HELIANTHEMUM</i>	20		389	32.88	19.50			14.69±2.81	24.49±2.09
Sect. <i>BRACHYPETALUM</i>	20		24	21.70	5.13			21.74±1.79	24.74±1.62
<i>H. angustatum</i> Pomel	20	82	5	21.94	8.06	12m+8sm	2A	23.60±2.18	26.08±3.02
<i>H. ledifolium</i> (L.) Mill.	20	423	5	22.83	3.97	14m+6sm	2A	22.04±3.65	22.38±2.23
<i>H. papillare</i> Boiss.	20	262	7	21.86	9.09	12m+8sm	2A	19.30±3.07	25.12±1.88
<i>H. salicifolium</i> (L.) Mill.	20	288, 297	7	20.16	5.74	12m+8sm	2A	22.03±2.20	25.38±4.47
Sect. <i>HELIANTHEMUM</i> s.l.	20		365	33.99	16.42			13.98±2.74	24.47±4.37
Sect. <i>HELIANTHEMUM</i> p.p.	20		250	37.51	9.22			14.47±3.26	24.62±5.31
<i>H. aegyptiacum</i> (L.) Mill.	20	123	11	27.80	6.16	6m+14sm	3A	17.31±1.92	29.71±2.50
<i>H. almeriense</i> Pau	20	47	8	39.53	8.30	9m+11sm	2A	15.49±2.23	24.23±2.98
<i>H. alypoides</i> Losa & Rivas Goday	20	45	8	37.47	9.22	9m+11sm	2A	13.90±1.66	24.19±4.04

Table 1. Cont'd.

	2n	Pop	N	THL (µm)	CV (%)	Karyotype formula	SKA	CV _{CL}	M _{CA}
<i>H. apenninum</i> (L.) Mill. subsp. <i>apenninum</i>	20	239	6	37.38	9.82	10m+10sm	2A	16.27±1.86	25.89±3.07
<i>H. apenninum</i> subsp. <i>stoechadifolium</i> (Brot.) Samp.	20	236	14	41.54	7.53	12m+8sm	2A	13.34±1.91	22.81±2.62
<i>H. croceum</i> (Desf.) Pers.	20	573	13	38.15	8.47	12m+8sm	2A	13.66±1.80	22.28±3.89
<i>H. fontqueri</i> Sennen	20	41	9	37.63	4.80	11m+9sm	2A	15.78±2.26	23.59±2.12
<i>H. grosii</i> Pau & Font Quer	20	118, 425	9	37.62	5.85	9m+11sm	2A	17.13±2.10	23.04±2.43
<i>H. helianthemoides</i> (Desf.) Grosser	20	426	8	34.64	5.89	10m+10sm	2A	13.68±2.64	27.41±3.04
<i>H. hirtum</i> (L.) Mill.	20	431	14	34.00	5.87	13m+7sm	2A	13.21±2.00	21.77±1.39
<i>H. kostchyanum</i> Boiss.*	20	646	10	47.84	8.37	6m+10sm+4st	2A	17.41±1.06	29.91±1.70
<i>H. marminorensis</i> Alcaraz, Peinado & Mart. Parras	20	276	10	35.25	9.60	10m+10sm	2A	13.94±3.10	24.16±3.21
<i>H. morisianum</i> Bertol.	20	574	9	36.82	5.02	12m+8sm	2A	13.03±1.48	20.69±2.09
<i>H. neopiliferum</i> Muñoz Garm. & Navarro	20	309	11	36.97	8.87	10m+10sm	2A	14.08±1.82	23.26±1.96
<i>H. nummularium</i> subsp. <i>cantabricum</i> (M.Lainz) Martín-Hernanz, Velayos, Albadalejo & Aparicio	20	373	7	37.68	4.94	12m+8sm	2A	13.96±1.77	23.21±2.27
<i>H. nummularium</i> (L.) Mill. subsp. <i>nummularium</i>	20	575	13	38.31	8.55	10m+10sm	1A	13.25±2.64	24.15±3.71
<i>H. nummularium</i> subsp. <i>tinetense</i> (M.Mayor & Fern.Benito) Martín-Hernanz, Velayos, Albadalejo & Aparicio	20	369	10	40.53	8.20	11m+9sm	2A	14.25±1.66	25.30±2.99
<i>H. pergamaceum</i> Pomel	20	114	10	34.83	7.79	11m+9sm	2A	12.39±2.75	22.81±1.76
<i>H. raskebdanae</i> Alonso, Crespo, Juan & Sáez	20	274	8	34.96	5.20	9m+11sm	2A	13.91±1.31	24.98±3.88
<i>H. ruficum</i> (Viv.) Spreng.	20	111	9	36.23	8.66	7m+13sm	2A	13.32±1.18	26.20±2.21
<i>H. sauvagei</i> Raynaud	20	282	13	39.81	6.23	8m+12sm	2A	18.14±0.77	25.73±1.86
<i>H. vesicarium</i> Boiss.*	20	641	10	40.12	8.11	10m+10sm	2A	14.07±1.89	23.22±2.00
<i>H. violaceum</i> (Cav.) Pers.	20	81, 364	13	37.07	8.46	9m+11sm	2A	13.43±2.05	24.41±3.58
<i>H. virgatum</i> (Desf.) Pers.	20	424	8	38.54	9.94	6m+14sm	2A	12.40±1.88	25.38±1.83
<i>H. viscarium</i> Boiss. & Reut.	20	41, 275	7	37.03	7.22	8m+12sm	2A	14.44±0.80	27.25±3.57
Sect. <i>HELIANTHEMUM</i> Canarian clade	20		115	28.14	9.68			13.17±1.49	24.21±2.01
<i>H. aganae</i> Marrero Rodr. & R.Mesa	20	435	8	26.02	8.98	14m+6sm	2A	14.69±1.62	23.37±2.75
<i>H. aguloi</i> Marrero Rodr. & R.Mesa	20	436	10	27.77	9.90	16m+4sm	1A	11.44±2.12	21.60±2.81
<i>H. brammwelliorum</i> Marrero Rodr.	20	412	7	30.98	6.10	9m+11sm	2A	14.66±1.49	24.14±2.24
<i>H. broussonetii</i> Dunal	20	432	5	24.18	2.97	8m+12sm	2A	12.06±2.95	24.59±1.05
<i>H. bystropogophyllum</i> Svent.	20	419	5	24.84	8.43	13m+7sm	2A	12.59±2.47	24.00±2.41
<i>H. cirae</i> A.Santos	20	432	11	30.49	9.71	12m+8sm	2A	13.92±3.36	22.86±2.66
<i>H. gonzalezferreri</i> Marrero Rodr.	20	411	9	31.88	7.07	12m+8sm	1A	13.42±1.59	22.66±3.16
<i>H. henriquezii</i> A.Rebolé, A.Acevedo & A.García	20	432	7	27.04	7.49	10m+10sm	2A	13.61±2.08	24.46±2.08
<i>H. inaguae</i> Marrero Rodr., González-Mart. & González-Art.	20	418	10	31.86	9.24	10m+10sm	2A	12.90±2.39	22.92±1.56
<i>H. juliae</i> Wildpret	20	434	5	24.09	7.04	13m+7sm	2A	12.54±0.79	22.64±2.44
<i>H. linii</i> A.Santos	20	399	9	29.67	8.96	10m+10sm	2A	11.88±1.88	23.08±2.51
<i>H. sp. nov. 1</i>	20	405	6	30.07	9.89	6m+14sm	2A	16.06±3.28	29.38±2.87
<i>H. teneriffae</i> Coss.	20	401	6	26.49	9.72	11m+7sm+2st	2A	14.20±1.99	25.50±3.07
<i>H. tholiforme</i> Bramwell, J.Ortega & B.Navarro	20	420	5	26.80	4.92	6m+14sm	2A	11.07±1.86	27.45±3.57
<i>H. tibiabinae</i> Marrero Rodr., Díaz Bertrana & S.Scholz	20	437	10	29.86	7.04	11m+9sm	2A	15.49±6.14	24.44±3.85

nanz & al. 2019; Albaladejo & al. 2021; Martín-Hernanz & al. 2021b). *Helianthemum dagestanicum* Rupr. has been ascribed to *H.* sect. *Pseudomacularia* Grosser on the basis of target sequencing according to Martín-Hernanz & al. (unpublished data). The voucher specimens from the populations studied have been deposited in the SEV herbarium (University of Seville) (Appendix 1).

Germination of seeds and karyotype analysis

The protocol for the germination of seeds in *Helianthemum* was described in detail in Aparicio & al. (2019). Briefly, all the material required for seed germination (sandpaper, Petri dishes, distilled water, filter paper, scissors, etc.) was introduced in an UV-cleaner box for about 40 minutes. Then, seeds were gently scarified by abrasion between two sheets of fine-grained sandpaper (Pérez-García & González-Benito 2006) and set for germination in Petri dishes at 20°C. Root tips were pre-treated by immersion in 2 mM 8-Hydroxyquinoline (Tjio & Levan 1950) for 4 h at 10°C, fixed in 1:3 glacial acetic acid and absolute ethanol for at least 2.5 h at 10°C, stained in alcoholic-hydrochloric acid-carmines for 24–48 h (Snow 1963) and then squashed in 45% acetic acid. For every species, clear metaphase spreads were photographed in an Olympus BX41 microscope equipped with a ColorView III digital camera.

Chromosome counts and karyotype analyses were carried out from mitotic metaphase photographs. We measured between 5–14 mitotic metaphase spreads for every species and used the tools provided by the software MATO (Measurement and Analysis Tools; Altinordu & al. 2016) to compute several karyological parameters. Aiming to assess karyotype length and karyotype heterogeneity, considering both the inter and intrachromosomal components of karyotype asymmetry (Peruzzi & Eroğlu 2013), we obtained five different parameters: (1) total haploid (monoploid) length of chromosome set (THL; Altinordu & al. 2016), (2) karyotype formula (Levan & al. 1964), (3) karyotype asymmetry classification of Stebbins (Stebbins 1971), (4) interchromosomal coefficient of variation of chromosome length (CV_{CL} ; Paszko 2006), and (5) intrachromosomal mean centromeric asymmetry (M_{CA} ; Peruzzi & Eroğlu 2013). To obtain mean THL at species level we discarded extreme values by merging in MATO only individual measurements which yielded a mean THL value with a coefficient of variation (CV) < 10%. Satellited chromosomes can be commonly observed in metaphase plates, but due to inconsistency among them we have not considered satellites in this study.

To test for significant relationships between chromosome number and the karyotype parameters, we converted the base chromosome number into a binary response variable ($n = 10$ to 0 and $n = 11$ to 1) and ran a phylogenetic logistic regression (Ives & Garland 2010) with phylolm R

package (Ho & Ane 2014). We excluded $n = 5$ and $n = 12$ because these numbers are only present in one species each (see Introduction). We accounted for the shared ancestry of chromosome numbers using the TreePL phylogenetic tree explained in the following section.

Updating chromosome number evolution analysis

Chromosome number evolution in *Helianthemum* was already reconstructed by Aparicio & al. (2019) based on a phylogenetic hypothesis derived from DNA Sanger sequences (Aparicio & Albaladejo 2017). To update this analysis, we used the time-calibrated phylogeny based on GBS (*genotyping-by-sequencing*) data obtained by the software TreePL (Martín-Hernanz & al. 2019). This phylogenetic tree was additionally modified by the inclusion of Sanger DNA sequences of *H. dagestanicum* (see methodology in Martín-Hernanz & al. 2021b) and the exclusion of *H. ordosicum* Y.Z.Zhao, Zong Y.Zhu & R.Cao until confirmation of its polyploid ($2n = 4x = 40$) status. The updated analysis was also enhanced by the inclusion of all the species of *H.* sect. *Pseudomacularia*, whose chromosome numbers were unknown until this study. The time-calibrated phylogeny was pruned to keep one single tip per species. The final data set consisted of 73 species.

The updated time-calibrated phylogeny and the chromosome numbers were analysed using ChromEvol v.2.0 (Glick & Mayrose 2014; Mayrose & al. 2010) to elucidate the mode of chromosome evolution. ChromEvol determines the probability of a certain model to explain the given data (haploid chromosome numbers) along a phylogeny, based on the combination of the first two or more of the following parameters: (i) gain or (ii) loss of a single chromosome, (iii) polyploidization, (iv) half increment of the chromosome number (demi-polyploidization) and (v) increment of the base number with regard of a rate of multiplication different from a regular duplication. Furthermore, two additional parameters permit to detect linear dependency between the current haploid number and the rate of (vi) gain and (vii) loss of chromosomes. Specifically, we performed the analyses using eight models of chromosome evolution implemented in ChromEvol that combine differently these parameters for chromosome number transitions: CONST_RATE, CONST_RATE_DEMI, CONST_RATE_DEMI_EST, CONST_RATE_NO_DUPL, LINEAR_RATE, LINEAR_RATE_DEMI, LINEAR_RATE_DEMI_EST and LINEAR_RATE_NO_DUPL.

Based on our own previous reconstruction of chromosome evolution (Aparicio & al. 2019) we run the analysis fixing the root of the phylogeny at a chromosome base number of $n = 10$. Models were compared using Akaike information criterion (AIC and ΔAIC), which allowed us to test the alternative hypotheses of chromosome evolution. The best model was plotted on the time-calibrated phylo-

geny using the ChromEvol functions v. 1 by N. Cusimano (https://www.en.sysbot.bio.lmu.de/people/employees/cusimano/use_r/) in R.

RESULTS

We obtained seeds and karyological data for 78 species and subspecies belonging to all the three subgenera and 10 sections of the genus *Helianthemum* across its distribution range (Fig. 1), including different life forms and species thriving in different environmental niches. Overall, 709 mitotic metaphase spreads were analysed meaning 9.09 ± 2.58 (mean $\pm$ SD) measurements for each species. The mean chromosome size in the genus *Helianthemum* is about 3 μm long, but above species level it may range from 1.69 μm in *H. sessiliflorum* (Desf.) Pers. to 4.91 μm in *H. squamatum*. Chromosome numbers resulted quite constant without instances of polyploidy, and mean THL ranged from 16.91 μm , in *H. sessiliflorum*, to 47.86 μm , in *H. kostchyanum* Boiss. Conversely, karyotype heterogeneity was generally low at the species, section, and subgenus level, with values of CV_{CL} and M_{CA} ranging from 11.07–23.60 and 10.91–33.32, respectively. Table 1 shows the karyological data obtained in this study at the genus, subgenus, section and species level.

Chromosome numbers and karyotype features

We obtained new chromosome counts for 11 species and subspecies, which are illustrated in Figure 2 (see also Table 1). Therefore, the number of species of *Helianthemum* whose chromosome number is known increases to c. 77%. We confirmed that the predominant somatic chromosome numbers are $2n = 20$ and $2n = 22$, while that $2n = 10$ and $2n = 24$ are restricted to just one species each (*H. squamatum* and *H. caput-felis*, respectively). However, we have unexpectedly found $2n = 22$ for all the species of *H. sect. Pseudomacularia* [*H. subg. Eriocarpum* (Dunal) Martín-Hernanz, Velayos, Albaladejo & Aparicio], a chromosome number never reported so far out of *H. subg. Plectolobum* Willk. The phylogenetic logistic regression showed that chromosome number is not correlated with the karyotype parameters analysed, except marginally and negatively with CV_{CL} (Table 2), i.e., the higher the number of chromosomes, the higher the interchromosomal homogeneity. As mentioned, satellited chromosomes are common (see for example Fig. 2a, b, c, h, i) but they have not been described in this study due to inconsistencies among metaphase spreads.

Our analyses showed that karyotype of *Helianthemum* can be considered quite symmetric (Table 1). At subgenus level, values of interchromosomal heterogeneity (CV_{CL}) ranged between 14.30 ± 1.94 in *H. subg. Plectolobum* to 17.11 ± 3.15 in *H. subg. Eriocarpum*, and intrachromoso-

mal heterogeneity (M_{CA}) between 20.23 ± 4.05 in *H. subg. Plectolobum* to 24.49 ± 2.09 in *H. subg. Helianthemum*. At the section level, values of CV_{CL} and M_{CA} were also quite homogeneous ranging CV_{CL} from 12.70 ± 1.94 in *H. sect. Macularia* Dunal to 21.74 ± 1.79 in *H. sect. Brachypetalum* Dunal, and M_{CA} from 10.91 in the monospecific *H. sect. Argyrolepis* Spach to 28.07 ± 7.43 in *H. sect. Lavandulaceum* G.López. Moreover, CV_{CL} and M_{CA} were not correlated, and *H. subg. Plectolobum* showed even a more symmetric karyotype compared to the other two subgenera, particularly in mean centromeric asymmetry (Fig. 3). The asymmetry classification of Stebbins and the karyotype formula further showed regular symmetry and a predominance of metacentric (m) and submetacentric (sm) chromosomes, with some subtelocentric (st) chromosomes only present in *H. teneriffae* Coss. and *H. kostchyanum* (Table 1; Fig. 2i). We can discard chromosome structural heterozygosity, so odd numbers in karyotype formulas are the consequence of chromosome folding in photographs or subtle angle deviation in individual measurements (Yu, pers. comm.).

Nevertheless, it is very interesting to note that the total karyotype length (THL) resulted quite variable among subgenera, sections and species. THL values for *H. subg. Eriocarpum*, *Plectolobum* and *Helianthemum* were 22.58 μm , 38.27 μm , and 32.88 μm , respectively. Sections ranged from 18.93 μm in *H. sect. Eriocarpum* Dunal to 44.03 μm in the monospecific *H. sect. Caput-felis* G.López. The entire genus *Helianthemum*, the three subgenera, and *H. sects. Pseudomacularia*, *Helianthemum* (s.l.), and *Macularia* showed high heterogeneity in mean THL values across species, with $\text{CV} > 10\%$ (Table 1; Fig. 4). Conversely, mean THL had $\text{CV} < 10\%$ in the non-monospecific sects. *Lavandulaceum*, *Eriocarpum*, *Brachypetalum* and *Pseudocistus* Dunal. Notice that *H. sect. Helianthemum* p.p. and *H. sect. Helianthemum* Canarian clade had also $\text{CV} < 10\%$.

Chromosome number evolution

The analysis of chromosome number evolution with the ancestral base chromosome number fixed at $n = 10$ showed that the best-fitting model for *Helianthemum* was CONST_RATE_NO_DUPLI with an AIC value of 66.02 (Table 3). This scenario revealed a CONSTANT_RATE background with 0.02053 gain events Myr^{-1} , 0.03258 loss events Myr^{-1} and no polyploid events across the phylogenetic tree (Fig. 5). In agreement with previous knowledge, we detected one shift in the mode of chromosome evolution in the lineage of *H. squamatum* ($n = 5$) in which the rate of chromosome losses increased several orders of magnitude (5.34359 events Myr^{-1}).

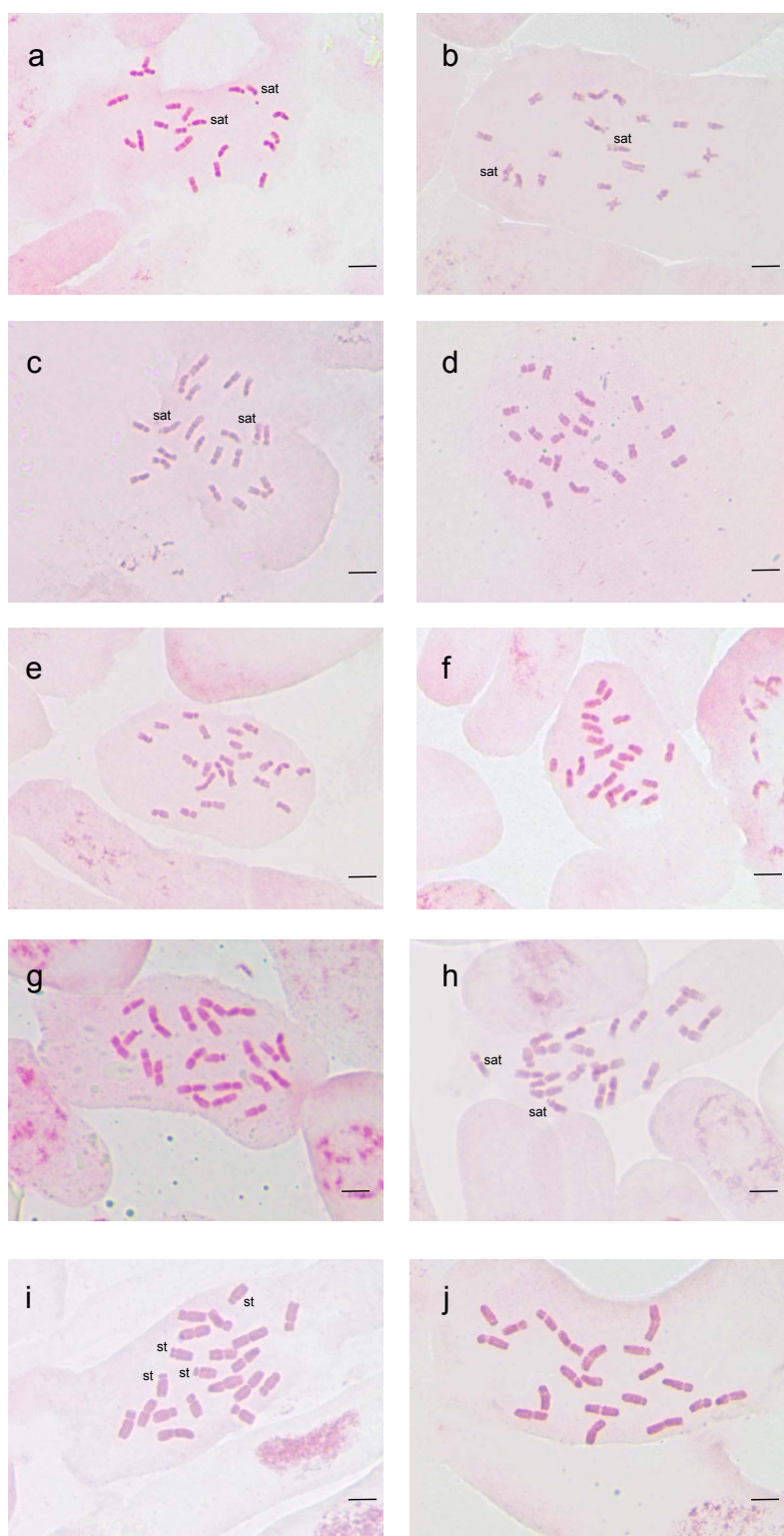


Fig. 2. Mitotic metaphase spreads and mean total haploid (monoploid) length of chromosome set (THL) newly obtained in this study for *Helianthemum* subg. *Eriocarpum* (Dunal) Martín-Hernanz, Velayos, Albaladejo & Aparicio sect. *Eriocarpum* Dunal (**a-b**), sect. *Pseudomacularia* Grosser (**c-f**); *H.* subg. *Plectolobum* Willk. sect. *Pseudocistus* Dunal (**g-h**); *H.* subg. *Helianthemum* sect. *Helianthemum* (**i-j**). **a**, *H. confertum* Dunal $2n = 20$, THL = 19.33 μm ; **b**, *H. sancti-antonii* Schweinf. $2n = 20$, THL = 20.59 μm ; **c**, *H. antitauricum* Davis & Coode $2n = 22$, THL = 27.86 μm ; **d**, *H. dagestanicum* Rupr. $2n = 22$, THL = 20.65 μm ; **e**, *H. germanicopolitanum* Bomm. $2n = 22$, THL = 24.57 μm ; **f**, *H. songaricum* Zhao, Zhu & Cao $2n = 22$, THL = 29.12 μm ; **g**, *H. frigidulum* Cuatrecasas $2n = 22$, THL = 38.61 μm ; **h**, *H. origanifolium* subsp. *africanum* B. Crespo, M.A. Alonso, A. Vicente & J.L. Villar $2n = 22$, THL = 41.98 μm ; **i**, *H. kostchyanum* Boiss. $2n = 20$, THL = 47.84 μm ; **j**, *H. vesicarium* Boiss. $2n = 20$, THL = 40.12 μm . sat = satellite chromosome. st = subtolocentric chromosome. Scale bars = 5 μm .

Table 2. Results of the logistic regression of chromosome number against mean total haploid (monoploid) length of chromosome set (THL), coefficient of variation of chromosome length (CV_{CL}) and mean centromeric asymmetry (M_{CA}).

	Estimate	SE	z-value	p-value
THL	-0.0106	0.0547	-0.1933	0.8468
CVCL	-0.3339	0.1552	-2.1518	0.0314
MCA	-0.1966	0.1214	-1.6202	0.1052

Table 3. Values of AIC for the chromosome evolution models tested for *Helianthemum*. The best model with the lowest AIC is shown in bold.

Model	Log-likelihood	AIC	ΔAIC
CONST_RATE_NO_DUPL	-31.01	66.02	0
CONST_RATE	-31.01	68.01	1.99
CONST_RATE_DEMI	-31.01	68.02	2
CONST_RATE_DEMI_EST	-30.54	69.08	3.06
LINEAR_RATE_NO_DUPL	-31.00	70.01	3.99
LINEAR_RATE	-30.53	71.07	5.05
LINEAR_RATE_DEMI	-30.53	71.07	5.05
LINEAR_RATE_DEMI_EST	-30.54	73.07	7.07

DISCUSSION

Chromosome number, chromosome size, karyotype length and karyotype symmetry are among the most used karyological attributes to describe trends in karyotype evolution (Stace 2000; Levin 2002). The explanatory mechanisms for karyotype rearrangements involve from whole genome duplications (i.e., polyploidy) to primary chromosomal rearrangements such as paracentric or pericentric inversions, multiple chromosome fusions by symmetrical reciprocal translocations or Robertsonian rearrangements, loss, or inactivation of active centromeres such as translocations, fissions, fusions, or inversions (i.e., dysploidy) (Levin 2002; Schubert & Lysák 2011; Weiss-Schneeweiss & Schneeweiss 2013). In *Helianthemum*, chromosome number and karyotype symmetry are quite conserved at the species, section, and subgenus levels, and might not be the major drivers in the evolution of the genus. However, karyotype length turn to be highly variable above species level, which leads us to consider possible frequent chromosomal rearrangements along the genus evolution in response to shifts in environmental niche or life history traits.

Chromosome size and number

Mean chromosome length in *Helianthemum* is about 3 µm long, albeit they range from small (1.69 µm) to medium-sized (4.91 µm) at section and subgenus levels.

Remarkably, *H. squamatum* is both, the species with the lowest chromosome number ($2n = 10$) and the species with the longest chromosomes (4.91 µm), likely because its genome is the consequence of a large reorganization achieved by progressive primary chromosomal rearrangements (see Aparicio & al. 2019).

Except in one case (*H. songaricum* Schrenk ex Fisch. & C.A.Mey; see below) we did not find deviating chromosome numbers with respect to previous knowledge (Goldblatt & Johnson 1979; Rice & al. 2015; Aparicio & al. 2019). Also, all the new chromosome counts here provided are coincident with those of related species, such as $2n = 20$ for *H. confertum* Dunal and *H. sancti-antonii* Schweinf. in *H. sect. Eriocarpum*, or $2n = 22$ for *H. frigidulum* Cuatrecasas, *H. marifolium* subsp. *andalusicum* (Font Quer & Rothm.) G.López and *H. origanifolium* subsp. *africanum* M.B.Crespo, M.A.Alonso, A.Vicente & J.L.Villar in *H. sect. Pseudocistus*. Nevertheless, the chromosome number found for all the species of *H. sect. Pseudomacularia* ($2n = 22$) is unexpected since this number has never been reported for any species within *H. subg. Eriocarpum* (actually, out of *H. subg. Plectolobum*). Furthermore, it is remarkable that our observation of $2n = 22$ for *H. songaricum* (see Fig. 2f) disagrees with Zhao & al. (2000) who reported $2n = 20$. Overall, it seems that the whole genus *Helianthemum* is integrated by diploid species, since only a few tetraploid populations have been reported in the literature for some therophyte species [e.g., *H. aegyptiacum* (L.) Mill., *H. le-difolium* (L.) Mill.; Goldblatt & Johnson 1979]. It is then essential to confirm the somatic number $2n = 40$ reported for *H. ordosicum* (Zhao & al. 2000), considering that this species is closely related to *H. songaricum* (in fact synonymized by Quiner & Gilbert 2007).

Karyotype symmetry

Karyotype symmetry has two components, one related to variation among chromosome size and the other to variation in centromere position (Peruzzi & Eroğlu 2013). Most species of angiosperms are characterized by uniform symmetric karyotypes with mostly meta or submetacentric chromosomes (Weiss-Schneeweiss & Schneeweiss 2013), and it has been classically assumed that asymmetric karyotypes derived from symmetric ones (Stebbins 1971). Nevertheless, cytogeneticists now believe that reversal situations may have occurred (Stace 2000) and that karyotype asymmetry is a transitory state rather than an evolutionary endpoint (Lysák & al. 2006). In this study we find that most chromosomes in *Helianthemum* are meta or submetacentric, and that indices of karyotype symmetry show consistently low values of heterogeneity and variation. Values of CV_{CL} and M_{CA} can reach 100 (or even higher), but in taxa in which variation is ostensible, the value of asymmetry indexes may span from 20–80 (see fig. 2 in Peruzzi &

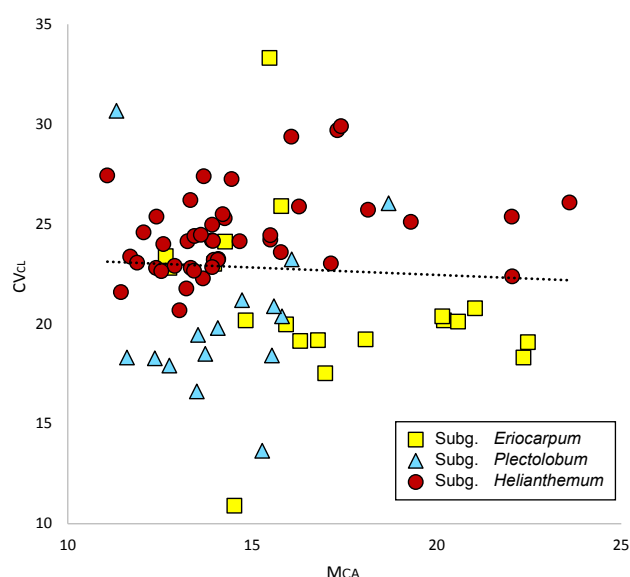


Fig. 3. Scatter plot and regression line (dotted) for the intra and interchromosomal components of karyotype asymmetry for the *Helianthemum* species analysed in this study: CV_{CL} (coefficient of variation of chromosome length) vs. M_{CA} (mean centromeric asymmetry).

Eroğlu 2013). Values of CV_{CL} and M_{CA} for *Helianthemum* were low and scarcely variable at the subgenus and section levels (overall from 10.91–33.32). At the species level, the highest values of intrachromosomal heterogeneity are

found in *H. motae* Sánchez-Gómez, Jiménez & Vera, *H. pomeridianum* Dunal and *H. kostchyanum* Boiss. (M_{CA} = 33.32, 30.68, 29.91, respectively), in which a higher proportion of submetacentric and subtelocentric chromosomes is found (see Table 1). It is interesting to note that *H.* subg. *Plectolobum* has the longest chromosomes and the lowest asymmetry (also evident in *H. squamatum*, at the species level), a result that supports that genomic processes involved in increasing chromosome size are also likely increasing chromosome symmetry (Levin 2002; Weiss-Schneeweiss & Schneeweiss 2013).

Karyotype length

The total length of the karyotype is an indirect measure of the amount of DNA contained in the cell nucleus, and is thus related to the size of the genome, whose trends of variation and evolution have received increasing attention (Levin 2002; Soltis & al. 2005; Weiss-Schneeweiss & Schneeweiss 2013; Pellicer & al. 2018). In *Helianthemum*, the values of total haploid karyotype length between species range almost 3-fold from 16.91 μ m in *H. sessiliflorum* (*H.* sect. *Eriocarpum*) to 47.84 μ m in *H. kostchyanum* (*H.* sect. *Helianthemum* p.p.), disregarding chromosome number. But when THL is analysed at the section and subgenus levels remarkably interesting results appear.

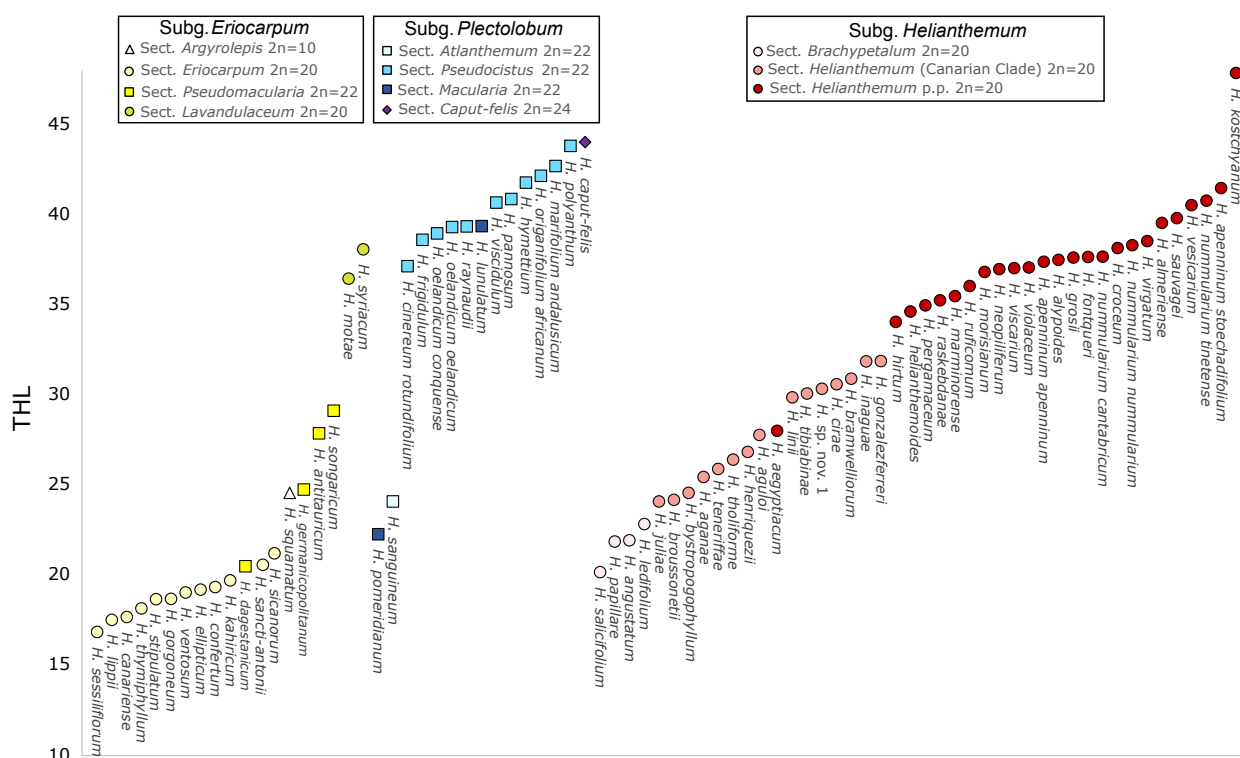


Fig. 4. Mean total haploid (monoploid) chromosome set length (THL) for the *Helianthemum* species analysed in this study, ordered from lower to higher values within each of the three subgenera. Sections are indicated by colours and chromosome numbers by shapes (see inset).

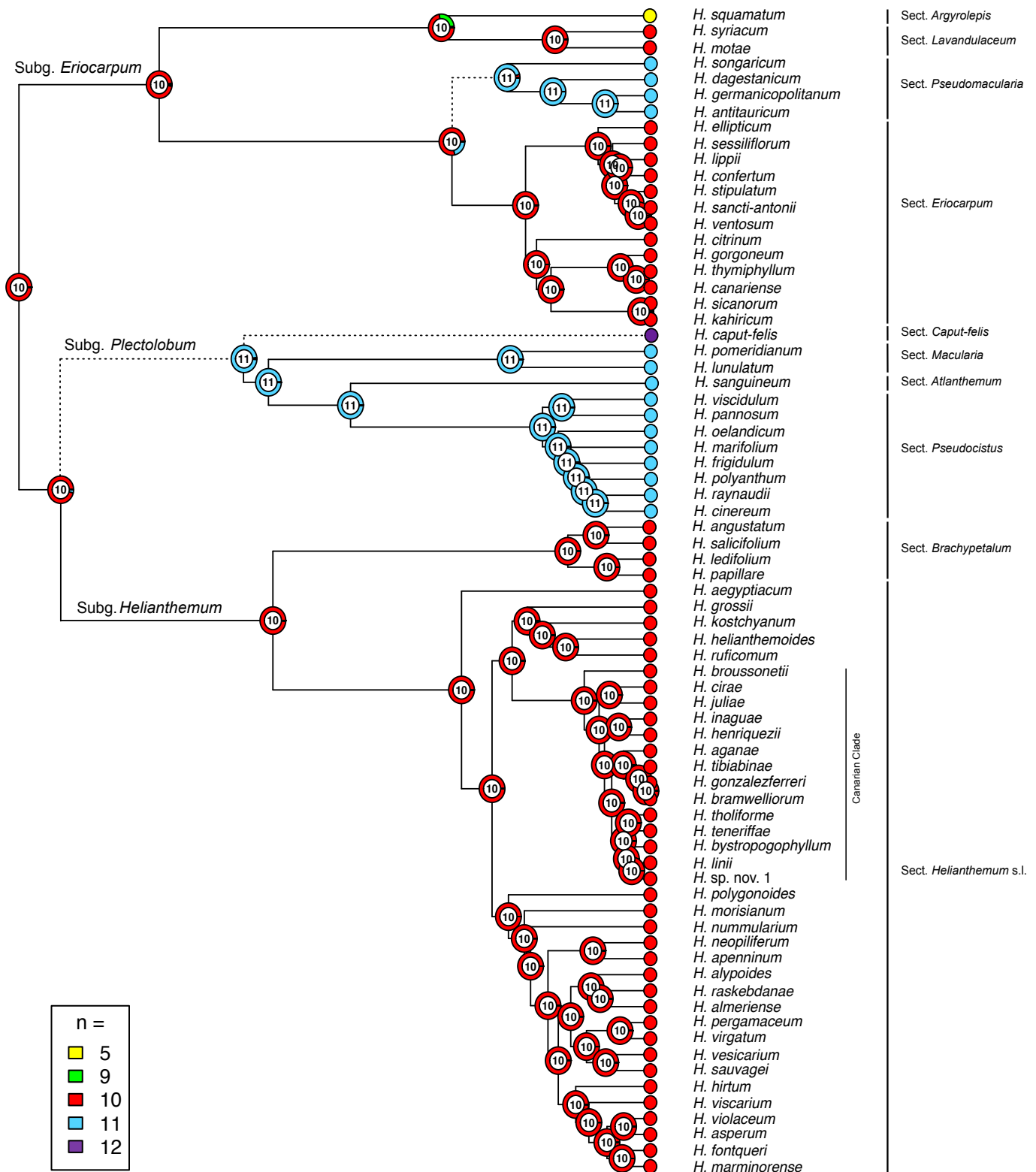


Fig. 5. Ancestral state reconstruction of chromosome number in *Helianthemum* based on the updated time-calibrated phylogeny using GBS (genotyping-by-sequencing) data (Martín-Hernanz & al. 2019). Pie charts represent the inferred probabilities, with the haploid chromosome number within the pie-section having the highest probability. Dotted lines represent cases of chromosome gain.

THL values increase from 22.58 μm in *H. subg. Eriocarpum* to 33.99 μm in *H. subg. Helianthemum* and 38.27 μm in *H. subg. Plectolobum*, with high coefficients of variation ($> 10\%$). This means that karyotype length is not a conserved cytogenetic trait neither among subgenera nor among sections (see Fig. 4). For example, in *H. subg. Eriocarpum* most species have low or medium THL values except those in *H. sect. Lavandulaceum*, and species of *H. sect. Brachypetalum* and *H. sect. Helianthemum* Canarian clade have clearly lower values of THL than the rest of *H. sect. Helianthemum* (i.e., *H. sect. Helianthemum* p.p.). In *H. subg. Plectolobum* the two species that integrate *H. sect. Macularia* have disparate THL values with *H. pomeridianum* having considerably lower THL values than *H. lunulatum* (All.) DC. (22.2 and 39.37 μm , respectively). In this case, both sister species diverged in a Pliocene vicariance now attested by an intercontinental disjunction, and their divergence also entailed an outstanding environmental niche shift (Aparicio & Albaladejo 2017; Martín-Hernanz & al. 2021b). The therophyte *H. sanguineum* (Lag.) Lag. ex Dunal also had a low THL value (24.09 μm). Sections *Pseudomacularia* and *Helianthemum* p.p. also showed high coefficients of variation, due to large variability among the four species of *Pseudomacularia*, and due to the existence of the therophyte *H. aegyptiacum* in *H. sect. Helianthemum* p.p. The THL values of the non-monospecific *H. sects. Lavandulaceum*, *Brachypetalum*, *Pseudocistus* were conserved among species (i.e., $\text{CV} < 10\%$).

Chromosome number evolution

The pattern of chromosome number evolution in *Helianthemum* remains invariant even after the inclusion of some species and the entire *H. sect. Pseudomacularia* (with the unexpected somatic number $2n = 22$): chromosome number shifts have not been a major driver in the evolution of *Helianthemum*, in which a constant rate of single chromosome increase or decrease is predominant. If $n = 10$ is the ancestral base chromosome number, *Helianthemum* has then evolved three independent instances of chromosome gain (see Fig. 5): (1) the ancestors of *H. sect. Pseudomacularia*, (2) the whole *H. subg. Plectolobum* and (3) the lineage of *H. caput-felis*. It is worth mentioning the rate of chromosome losses in the lineage of *H. squamatum*, which increased by several orders of magnitude compared to the whole phylogenetic tree. Although whole genome duplications have been considered a primary source of variation and evolution, dysploid changes can, indeed, be even more persistent than those achieved by polyploidy (Escudero & al. 2014).

Concluding remarks

The wealth of comparative cytogenetic information gathered in this study allows us to present a compelling pic-

ture of *Helianthemum* as a genus with stable chromosome numbers, whose evolution involved only three instances of slow chromosome gain. Nevertheless, imprints of large genome reorganizations in this genus are quite evident, such as in *H. squamatum*, and in the high variation in karyotype (i.e., genome) size that we have found at subgenus, section and species levels. Indeed, in *Helianthemum*, regardless of chromosome number, karyotype size contains very relevant systematic and evolutionary information which we summarize in Figure 6. Note that when the mean THL values are averaged across sections, ‘small’ and ‘large’ karyotypes appear to be separated by a gap between 28 and 37 μm . On the one hand, small karyotypes below 28 μm are found in most sections of *H. subg. Eriocarpum*, all the therophyte species regardless their taxonomic position, all the species *H. sect. Helianthemum* Canarian clade plus *H. pomeridianum*. On the other hand, large karyotypes over 37 μm are found in *H. sect. Helianthemum* p.p. (except the therophyte *H. aegyptiacum*) and the whole of *H. subg. Plectolobum* (except *H. pomeridianum*). In other words, ‘small’ karyotypes are present in desert specialists, therophytes and the recently diversified species of the Canary Islands (Albaladejo & al. 2021), whose breeding system is also predominantly autogamous (Martín-Hernanz & al. 2023). On the other hand, ‘large’ karyotypes characterise mostly xenogamous chamaephyte species of Mediterranean and Eurosiberian distribution (Martín-Hernanz & al. 2021b).

In future analyses we will use the power of a high-resolution phylogenomic reconstruction based on target capture data (Martín-Hernanz & al. unpublished) and 2C values of nuclear DNA amount (Pellicer & al. 2018) to trace the direction and strength of genome size evolution and its potential relationships with shifts in extrinsic (i.e., environmental niche) and intrinsic (i.e., breeding systems and habit) characteristics in the genus *Helianthemum*. Cytotaxonomy has always gone hand in hand with phylogenetics for a better understanding of chromosome and species evolution (Guerra 2012).

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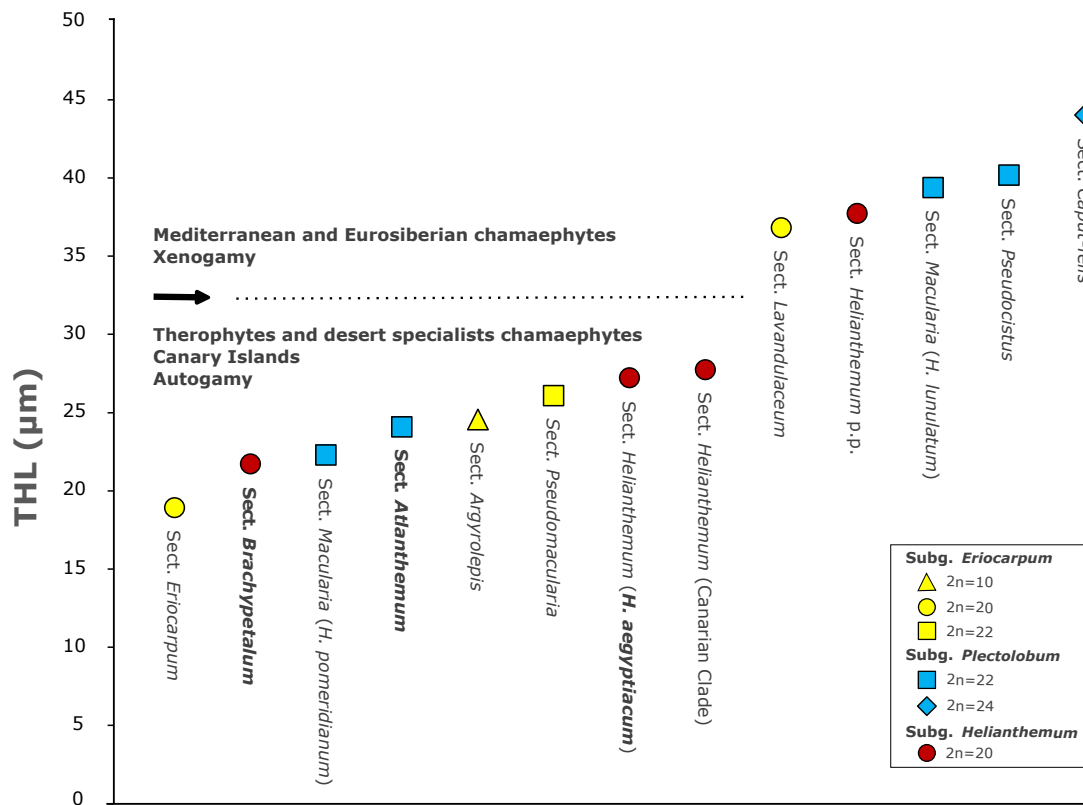


Fig. 6. Mean total haploid (monoploid) length of chromosome set (THL) averaged per section in *Helianthemum*. Note that the two species of sect. *Macularia* [*H. lunulatum* (All.) DC. and *H. pomeridianum* Dunal] are shown separately due to their divergence in mean THL value. *Helianthemum aegyptiacum* (L.) Mill. is the only therophyte species in sect. *Helianthemum* and is also shown separately. Subgenera are identified by colour and chromosome number by shape. Names of therophyte species and sections are shown in bold. The assignment of the geographic distribution, environmental niches and breeding systems of taxonomic categories is based on Martín-Hernanz & al. (2021b, 2023).

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AUTHOR CONTRIBUTIONS

Conceptualization: AA, SM-H; Data curation: AA, ER, SM-H; Resources: AA, RGA, ER, SM-H, PV, MDM, DU, SM; Investigation: AA, SM-H; Analysis: AA, RGA, SM-H; Funding acquisition: AA, RGA, MS; Writing original draft: AA, SM-H; Writing, review and editing: SM-H, AA, RGA, ER.

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Appendix 1. Source populations of the *Helianthemum* seeds studied in this paper ordered by population number (#). Voucher specimen numbers are given in brackets; s.n.: missing voucher.

#17. SPAIN. **Cádiz:** Olvera, Sierra de Lijar, 11 Jul. 2017, A. Aparicio & RG Albaladejo. *H. syriacum* (Jacq.) Dum.Cours. (SEV286515). **#41.** SPAIN. **Murcia:** Águilas, Cabo Cope, 12 May 2018, A. Aparicio & E. Rubio. *H. fontqueri* Sennen (SEV289821); *H. viscarium* Boiss. & Reut. (SEV286551). **#45.** SPAIN. **Almería:** Sorbas, Rio Aguas, 11 May 2018, A. Aparicio & E. Rubio. *H. alypoides* Losa & Rivas Goday (SEV286553). **#47.** SPAIN. **Almería:** Tabernas, 11 May 2018, A. Aparicio & E. Rubio. *H. almeriense* Pau (SEV289813). **#49.** SPAIN. **Málaga:** Parauta, Sierra de las Nieves, Conejeras, 20 Jul. 2021, RG Albaladejo, C. de Vega & E. Rubio. *H. marifolium* subsp. *andalusicum* (Font Quer & Rothm.) G.López (SEV289830). **#70.** SPAIN. **Granada:**

Quéntar, Puerto de la Mora, towards El Pozuelo, 22 Jun. 2022, A. Aparicio & E. Rubio. *H. raynaudii* Ortega Oliv., Romero García & C. Morales (SEV286570). #81. SPAIN. **Granada**: Albuñuelas, towards Venta del Fraile, Cortijo de los Lagartos, 22 Jun. 2022, A. Aparicio & E. Rubio. *H. violaceum* (Cav.) Pers. (SEV289843); *H. viscidulum* Boiss. (SEV289847). #82. SPAIN. **Granada**: Alhama de Granada, El Navazo, 26 May 2011, A. Aparicio & RG Albaladejo. *H. angustatum* Pomel (SEV289848). #111. MOROCCO. **El-Aïoum**: May Taieb, towards reservoir Mohamed V, 02 Jul. 2018, E. Rubio. *H. ruficomum* (Viv.) Spreng. (SEV287196). #114. MOROCCO. **Aknoul**: 18 km towards Taza, 05 Jun 2018, A. Aparicio, S. Martín-Hernanz & E. Rubio. *H. pergameum* Pomel (SEV286531). #117. MOROCCO. **Al-Hoceima**: 6 km towards Izemmourèn, 04 Jun. 2018, A. Aparicio, J. Aparicio, S. Martín-Hernanz & E. Rubio. *H. polyanthum* (Desf.) Pers. (SEV286532). #118. MOROCCO. **Al-Hoceima**: close to Izemmourèn, 04 Jun. 2018, A. Aparicio, S. Martín-Hernanz & E. Rubio. *H. grosii* Pau & Font Quer (SEV286534). #123. SPAIN. **Sevilla**: La Puebla del Río, pinares de Aznalcazar, 22 May 2022, A. Aparicio. *H. aegyptiacum* (L.) Mill. (SEV289812). #236. SPAIN. **Huelva**: Almonte, Matalascañas, 08 May 2022, A. Aparicio. *H. apenninum* subsp. *stoechadifolium* (Brot.) Samp. (SEV286539). #239. SPAIN. **Granada**: Escúzar, 10 Jul. 2018, A. Aparicio & E. Rubio. *H. apenninum* (L.) Mill. subsp. *apenninum* (SEV287171); *H. squamatum* (L.) Dum.Cours. (SEV289839). #242. SPAIN. **Guadalajara**: Tendilla, barranco Valdeandrés, 01 Aug. 2018, S. Martín-Hernanz. *H. oelandicum* subsp. *conquense* (Borja & Rivas Goday ex G.López) Martín-Hernanz, Velayos, Albaladejo & Aparicio (SEV289833). #262. SPAIN. **Málaga**: Parauta, Sierra de las Nieves, towards Puerto de los Pilones, 21 May 2015, A. Aparicio. *H. papillare* Boiss. (SEV289834). #274. MOROCCO. **Nador**: Ras-el-Ma, 2 km to Saidia, 01 Apr. 2016, A. Aparicio, J. Aparicio, S. Martín-Hernanz & E. Rubio. *H. raskebdanae* Alonso, Crespo, Juan & Sáez (SEV286763). #275. SPAIN. **Alicante**: Orihuela, Punta de Glea, 21 Apr. 2022, Aparicio, RG Albaladejo & E. Rubio. *H. caput-felis* Boiss. (SEV287178); *H. viscarium* Boiss. & Reut. (SEV289846). #276. SPAIN. **Murcia**: San Pedro del Pinatar, dune of San Pedro, 12 May 2018, A. Aparicio & E. Rubio. *H. marminorensis* Alcaraz, Peinado & Mart.Parras (SEV286758). #277. SPAIN. **Murcia**: Águilas, Cocedores' beach, 08 Sep. 2022, A. Aparicio & E. Rubio. *H. motae* Sánchez-Gómez, Jiménez & Vera (SEV286759). #282. MOROCCO. **Agadir**: Amerskroud towards Talaint, 29 Apr. 2022, A. Aparicio, R.G. Albaladejo & E. Rubio. *H. sauvagei* Raynaud (SEV286765). #283. MOROCCO. **Taroudant**: Ville Noughaylle, 30 Mar. 2016, A. Aparicio, J. Aparicio, S. Martín-Hernanz & E. Rubio. *H. lippii* (L.) Dum.Cours. (SEV289828). #288. CYPRUS. **Larnaca**: Xylotimou, Dhekelia, 12 Apr. 2016, A. Aparicio, M.A. Carrasco & S. Martín-Hernanz. *H. salicifolium* (L.) Mill. (SEV287202). #295. CYPRUS. **Ayia Eirini**: Ayia Erini, 14 Apr. 2016, A. Aparicio, M.A. Carrasco & S. Martín-Hernanz. *H. sanguineum* (Lag.) Lag. ex Dunal (SEV286764). #297. ITALY. **Sicily**: Gela, Torre Manfreda, 11 Apr. 2022, A. Aparicio, RG Albaladejo & E. Rubio. *H. salicifolium* (L.) Mill. (SEV289836); *H. sicanorum* Brullo, Giusso & Sciandr. (SEV286766). #309. SPAIN. **Málaga**: Parauta, La Ventilla, 30 Jun. 2018, A. Aparicio. *H. neopiliferum* Muñoz Garm. & Navarro (SEV289832). #348. CAPE VERDE. **Fogo**: Chã das Caldeiras, 22 Oct. 2016, I. Hernanz, R. Martín & S. Martín-Hernanz. *H. gorgoneum* Webb (SEV286753). #349. CAPE VERDE. **Fogo**: Chã das Caldeiras, 22 Oct. 2016, I. Hernanz, R. Martín & S. Martín-Hernanz. *H. gorgoneum* Webb (SEV289822). #352. MARRUECOS. **Taroudant**: Sidi Abdellah Oussaid-Alegiane, 01 May 2022, Aparicio, RG Albaladejo & E. Rubio. *H. pomeridianum* Dunal (SEV289835). #364. SPAIN. **Albacete**: Vicorto, towards Solama de la Obeja, 15 Apr. 2017, E. Rubio. *H. violaceum* (Cav.) Pers. (SEV289844). #369. SPAIN. **Oviedo**: Tineo, La Castañera, 13 Jun. 2017, A. Aparicio, RG Albaladejo, S. Martín-Hernanz & E. Rubio. *H. nummularium* subsp. *tinense* (M.Mayor & Fern.Benito) Martín-Hernanz, Velayos, Albaladejo & Aparicio (SEV287203). #373. SPAIN. **Orense**: Biobra, rute das Galegas, 13 Jun. 2017, A. Aparicio, RG Albaladejo, S. Martín-Hernanz & E. Rubio. *H. nummularium* subsp. *cantabricum* (M.Laínz) Martín-Hernanz, Velayos, Albaladejo & Aparicio (SEV289814). #374. MOROCCO. **Agadir**: Souss-Massa, Draâ: Afella Ighir, between Ait Mansour and Afella Ighir, 30 Apr. 2022, Aparicio, RG Albaladejo & E. Rubio. *H. ellipticum* (Desf.) Pers. (SEV289820); *H. kahiricum* Delile (SEV289826). #382. ISRAEL. **Ascalón**: Gvar'am, 02 Mar. 2018, A. Aparicio & RG Albaladejo, O. Fragman-Sapir & S. Martín-Hernanz. *H. stipulatum* (Forssk.) C.Ch. (SEV288162). #384. ISRAEL. **Nir Yitzhak**: Nir Yitzhak, 02 Mar. 2018, A. Aparicio & RG Albaladejo, O. Fragman-Sapir & S. Martín-Hernanz. *H. sessiliflorum* (Desf.) Pers. (SEV289837). #385. ISRAEL. **Bne Netsarim**: Bne Netsarim, 02 Mar. 2018, A. Aparicio, RG Albaladejo, O. Fragman-Sapir & S. Martín-Hernanz. *H. sessiliflorum* (Desf.) Pers. (SEV289838). #399. SPAIN. **La Palma**: Tijarafe, Torre del Time, 18 Jul. 2018, RG Albaladejo, S. Martín-Hernanz & M. Olangua. *H. linii* A.Santos (s.n.). #401. SPAIN. **Tenerife**: Ladera de Güimar, Canal de los Mil, 16 Jul. 2018, RG Albaladejo, S. Martín-Hernanz, M. Olangua & A. Santos. *H. teneriffae* Coss. (s.n.). #403. SPAIN. **Tenerife**: La Tejita, Montaña Roja, 09 May 2018, RG Albaladejo, S. Martín-Hernanz & M. Olangua. *H. canariense* (Jacq.) Pers. (SEV289815). #405. SPAIN. **Tenerife**: Chirche, Barranco Bermejo, 17 Jul. 2018, RG Albaladejo, S. Martín-Hernanz, M. Olangua & A. Santos. *H. sp. nov.* Tenerife (s.n.). #409. SPAIN. **Lanzarote**: La Caleta, Playa de Famara, 13 May 2018, S. Martín-Hernanz, M. Marín-Rodulfo & M. Olangua. *H. thymiphyllum* Svent. (SEV289842). #411. SPAIN. **Lanzarote**: Haría, El Bosquecillo, 14 May 2018, M. Díaz-Bertrana, S. Martín-Hernanz, M. Marín-Rodulfo, M. Olangua & A. Reyes. *H. gonzalezferreri* Marrero Rodr. (s.n.). #412. SPAIN. **Lanzarote**: Haría, Riscos de Guinate, Fuente de las Ovejas, 14 May 2018, S. Martín-Hernanz, M. Marín-Rodulfo, M. Olangua & A. Reyes. *H. bramwelliorum* Marrero Rodr. (s.n.). #418. SPAIN. **Gran Canaria**: Inagua, Andén de Tasarte, 17 May 2018, N. Cabrera, I. Guillerme, S. Martín-Hernanz, M. Marín-Rodulfo, J.E. Naranjo & M. Olangua. *H. inaguae* Marrero Rodr., González-Mart. & González-Art. (s.n.). #419. SPAIN. **Gran Canaria**: Pajonales, 17 May 2018, N. Cabrera, I. Guillerme, S. Martín-Hernanz, M. Marín-Rodulfo, J.E. Naranjo & M. Olangua. *H. bystropogophyllum* Svent. (SEV288156). #420. SPAIN. **Gran Canaria**: Montaña del Tauro, 18 May 2018, S. Martín-Hernanz, M. Marín-Rodulfo & M. Olangua. *H. tholiforme* Bramwell, Ortega & Navarro (SEV289841). #423. MOROCCO. **Immuouzer Du Kandar**: Ain Chifa, Gite Karine, 03 Jun. 2018, A. Aparicio, S. Martín-Hernanz & E. Rubio. *H. ledifolium* (L.) Mill. (SEV289827). #424. MOROCCO. **Gareb**: Mechra Hommadi, towards Hassi Berkane, 04 Jun. 2018, A. Aparicio, S. Martín-Hernanz & E. Rubio. *H. virgatum* (Desf.) Pers. (SEV289845). #425. MOROCCO. **Al Hoceimas**: Rouadi, Dchar Maya, itineraire Tikkit, 05 Jun. 2018, A. Aparicio, S. Martín-Hernanz & E. Rubio. *H. grosii* Pau & Font Quer (SEV287635). #426. MOROCCO. **Immuouzer Du Kandar**: towards Ifrane, 05 Jun. 2018, A. Aparicio, S. Martín-Hernanz & E. Rubio. *H. helianthemoides* (Desf.) Grosser (SEV287636). #429. MOROCCO. **Ifrane**: towards Ras el Ma, 05 Jun. 2018, A. Aparicio, S. Martín-Hernanz & E. Rubio. *H. cinereum* subsp. *rotundifolium* (Dunal) Greuter & Burdet. (SEV289816). #431. SPAIN. **Sevilla**: Alcalá de Guadaira, Cortijo El Maestre, 31 May 2018, A. Aparicio. *H. hirtum* (L.) Mill. (SEV289824). #432. SPAIN. **La Palma**: Andén de la Cañada, 15 Jun. 2018, A. Palomares. *H. broussonetii* Dunal (s.n.). *H. cirae* A. Santos (s.n.); *H. henriquezii* A.Rebolé, A.Acevedo & A.García (s.n.). #434. SPAIN. **Tenerife**: PN Cañadas del Teide, Mesa del Obispo, 17 Jul. 2018, RG Albaladejo, S. Martín-Hernanz, M. Olangua, A. Reyes, M. Suárez & A. Santos. *H. juliae* Wildpret (s.n.). #435. SPAIN. **La Gomera**: ex horto at Güimar, Tenerife, 09 May 2011, R. Mesa. *H. aganae* Marrero Rodr. & R.Mesa (s.n.). #436. SPAIN. **La Gomera**: ex horto at Centro de Juego de Bolas, 08 Jul. 2018, R. Mesa, R. Chine & J.L. Silva. *H. aguloi* Marrero Rodr. & R.Mesa (s.n.). #437. SPAIN. **Fuerteventura**: Jandia, Morro Cavadero, 05 Jun. 2018, M. Díaz-Bertrana. *H. tibiabinae* Marrero Rodr., Díaz Bertrana & S. Scholz (s.n.). #448. RUSSIA. **Dagestan**: 3 km south from Khunzakh, 18 Sep. 2021, P. Volkova & M. Ivanova. *H. dagestanicum* Rupr. (SEV289819). #500. FRANCE. **Tende**: summit of Covin, 11 Sep. 2013, L. Dixon, & K. Diadema. *H. lumulatum* (All.) DC. (s.n.). #525. MOROCCO. **Tiznit**: 15 km towards Agadir, 01 May 2022, Aparicio, RG Albaladejo & E. Rubio. *H. confertum* Dunal in DC. (SEV289817). #534. GREECE. **Vilia**: towards Aspropyrgos, 14 May 2022, Aparicio, RG Albaladejo, S. Martín-Hernanz & E. Rubio. *H. hymettium* Boiss. & Heldr. (SEV289825). #573. ITALY. **Sardinia**: Siniscola, Monte Albo, Gurturgius, 27 May 2022, A. Aparicio, RG Albaladejo & E. Rubio. *H. croceum* (Desf.) Pers. (SEV289818). #574. ITALY. **Sardinia**: Laconi, Borgata Pirastera, 28 Jun. 2022, A. Aparicio, RG Albaladejo & E. Rubio. *H. morisianum* Bertol. (SEV289831). #575. SWEDEN. **Vena**: Vena, 18 Aug. 2020, B. Widen. *H. nummu-*

larium (L.) Mill. subsp. *nummularium* (s.n.). #576. SWEEDEN. **Segerstad**: Öland, 14 Jul. 2019, B. Widén. *H. oelandicum* (L.) DC. subsp. *oelandicum* (s.n.). #615. SPAIN. **Granada**: Monachil, Trevenque, 22 Jun. 2022, J. Algarra. *H. pannosum* Boiss. (s.n.). #619. SPAIN. **Jaen**: Sierra Mágina, ex horto at Torre del Vinagre, 15 May 2022, S. García de Lucas. *H. frigidulum* Cuatrecasas (s.n.). #621. ALGERIA. **Aïnt Temuchent**: Madagh, 25 May 2022, M.D. Miara. *H. origanifolium* subsp. *africanum* B.Crespo, M.A.Alonso, A.Vicente & J.L.Villar (SEV289829). #641. ISRAEL. **Judea**: Judean Desert, 29 Apr. 2012, O. Friedmann. *H. vesicarium* Boiss. (ISRAEL GENE BANK 24853). #642. ISRAEL. **Eilat**: Upper Atek wadi, Golanot gorge, 01 Jun. 2015, D. Carmeli. *H. sancti-antoni* Schweinf. (ISRAEL GENE BANK 26757). #643. KYRGYZSTAN. **Krasnyy Most**: Boom Gorge, western side of A365, between road and railway line and alongside track up side valley, 20 Jun. 2017, G.A. Lazkov. *H. songaricum* Zhao, Zhu & Cao (Millennium Seed Bank, Royal Herbarium 1042902). #644. JORDAN. **Amman**: Rumman Hill, the Botanic Garden site 1 km after the main entrance to the dam water, 18 Jun. 2005, J. Abilalia, Z. Tehabshem & M. van Slageren. *H. ventosum* Boiss. (Millennium Seed Bank, Royal Herbarium 278216). #645. TURKEY. **Çankırı**: upper parts of Çakmaklıdere valley, 18 Jul. 2022, M. Sezgin. *H. germanicopolitanum* Bornm. (s.n.). #646. TURKEY. **Konya**: Hadim, Alanözü road, Bademli Village, roadside, 17 Jul. 2022, D. Ulukuş. *H. kostchyanum* Boiss. (s.n.). #647. TURKEY. **Adana**: Arslantaş-Ayvat Village, mountain steppe, stony slopes, 11 Aug. 2022, D. Ulukuş. *H. antitauricum* Davis & Coode (s.n.). #648. TURKEY. **Çankırı**: İnandık-Çankırı, 45 km from Çankırı, gypsum slopes, 30 Jul. 2022, D. Ulukuş. *H. germanicopolitanum* Bornm. (s.n.).