RESEARCH ARTICLE

Species delimitation in *Xanthium* sect. *Acanthoxanthium* (Heliantheae, Asteraceae) and the neglected species *Xanthium argenteum*

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Abstract *Xanthium* is a genus of annual herbaceous plants that stands out within Asteraceae for being wind-pollinated, diclinous monoecious, and bearing solitary pistillate flowers in peculiar spiny structures (burs). *Xanthium* sect. *Acanthoxanthium* is native to South America and characterized by the presence of trifurcate spines at the base of the leaves. Past taxonomic treatments of the section have been contradictory, some recognising up to six species, others reducing all to a single polymorphic species. Altogether, 42 samples of *X.* sect. *Acanthoxanthium* were analysed, the vast majority taken from herbarium specimens between 20 and 160 years old. We sampled multiple specimens of *X. spinosum* from throughout its broad range as well as those taxa with narrower distributions, covering the whole range of morphological variation in the section. When possible, we included types and original material. We used Hyb-Seq techniques to obtain information from about 1000 single-copy nuclear genes and complete plastomes. Phylogenomic data were submitted to coalescent-based species delimitation approaches (SPEEDEMON). Additionally, we performed geometric morphometric analysis of leaf outlines. The results strongly support the identification of four lineages in the section favouring the acceptance of four of the hitherto described species, i.e., *X. ambrosioides*, *X. argenteum*, *X. catharticum*, and *X. spinosum*. These results were to some extent corroborated by morphometric analyses. While *X. ambrosioides* was well distinct from *X. spinosum* based on leaf morphology, such difference was not observed between *X. spinosum* and *X. catharticum*. However, *X. catharticum* differs from *X. spinosum* in its ecological requirements, being a species rather adapted to high-mountain environments of the Neotropics. Intriguingly, *X. argenteum* – a taxon described from a single herbarium collection – was also inferred as a species.

Keywords *Acanthoxanthium*; coalescent model; integrative taxonomy; museomics; phylogenomics; *Xanthium argenteum*

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

The broad term species delimitation encompasses the act of identifying the boundaries between species. In the past, it was primarily based on morphometric traits. However, this approach led to an underestimation of species, for example, in the presence of cryptic (morphologically indistinguishable) or pseudocryptic species (Jörger & Schrödl, 2013), or an overestimation in case of extremely variable traits that are usually under the influence of many different factors (e.g., genetic, environmental, ecological). In the past decades, DNA sequencing has become widely employed for phylogenetic studies and species delimitation (Rannala & Yang, 2020). Even when using DNA-based approaches, species delimitation remains challenging in taxonomic groups where the speciation process is in its early stages, groups perfused by the presence of incomplete lineage sorting (ILS), and in which labile reproductive barriers result in introgression and gene flow (Degnan & Rosenberg, 2009).

The multispecies coalescent (MSC) model provides a statistical tool for assigning individuals to species based on sequence data. Methods based on this model can accommodate ILS, do not require reciprocal monophyly of species in gene trees (Knowles & Carstens, 2007), and can take into account the statistical uncertainty of gene trees (Yang & Rannala, 2010). Although several methods have been described in the last decades (see Rannala & Yang, 2020; Karbstein & al., 2024, for comprehensive reviews), model-based

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approaches, possibly on multilocus sequence data, should be preferred (Rannala, 2015). The novel software SPEEDEMON (Douglas & Bouckaert, 2022) can infer species delimitation under the MSC using the multiple loci retrieved from next generation sequencing (NGS) techniques.

The genus *Xanthium* L. (Asteraceae) is an ideal model to test these methodologies in taxonomically complex groups, for which infrageneric delimitation is notoriously difficult and has been highly controversial in the past. The genus, commonly known as cocklebur, comprises annual herbaceous plants that typically thrive in open environments, such as moist sandy pits, riverbanks, and coastal dunes, as well as ruderal places (Weaver & Lechowicz, 1983; Tomasello, 2018). Together with other genera of subtribe Ambrosiinae (Robinson, 1981; Tomasello & al., 2019), *Xanthium* stands out among Asteraceae for being wind-pollinated, and for carrying pistillate flowers in peculiar spiny structures (burs). Burs attach easily to animal fur, coats, and clothing (e.g., Weaver & Lechowicz, 1983; Lechowicz, 1984), which makes zoochory, particularly epizoochory, the most important dispersal mechanism for species of *Xanthium* (Benvenuti, 2007; Liehrmann & al., 2017; Gorb & al., 2019). Burs can also be found as a contaminant in goods of human trade. In the last few centuries, the trade of textile fibers has facilitated the dispersal of several lineages of the genus outside their native distribution range (Tomasello, 2018; Müller-Kiefer & Tomasello, 2022).

Past taxonomic treatments of the genus have been inconsistent, some recognising several taxa (Wallroth, 1844; Millspaugh & Sherff, 1919; Widder, 1923), others merging all in very few, polymorphic species (Löve & Dansereau, 1959). However, the genus can be subdivided into two sections: *Xanthium* sect. *Acanthoxanthium* DC., characterized by bearing trifurcate spines at the base of leaves, whereas species of *X.* sect. *Xanthium* have unarmed stems. *Xanthium* sect. *Acanthoxanthium* is known to be native to South America (Löve, 1975). Its distinctiveness has also been proven in sequence-based studies, where it always resulted as monophyletic (Tomasello & Heubl, 2017; Tomasello, 2018; Noedoost & al., 2021). The most common and widespread species is *X. spinosum* L. (Fig. 1), which was introduced to Europe and other continents soon after the first European expeditions to South America. The other taxa appear to have remained confined to their native range and only exceptionally have been found elsewhere as casual aliens (Van Kleunen & al., 2019).

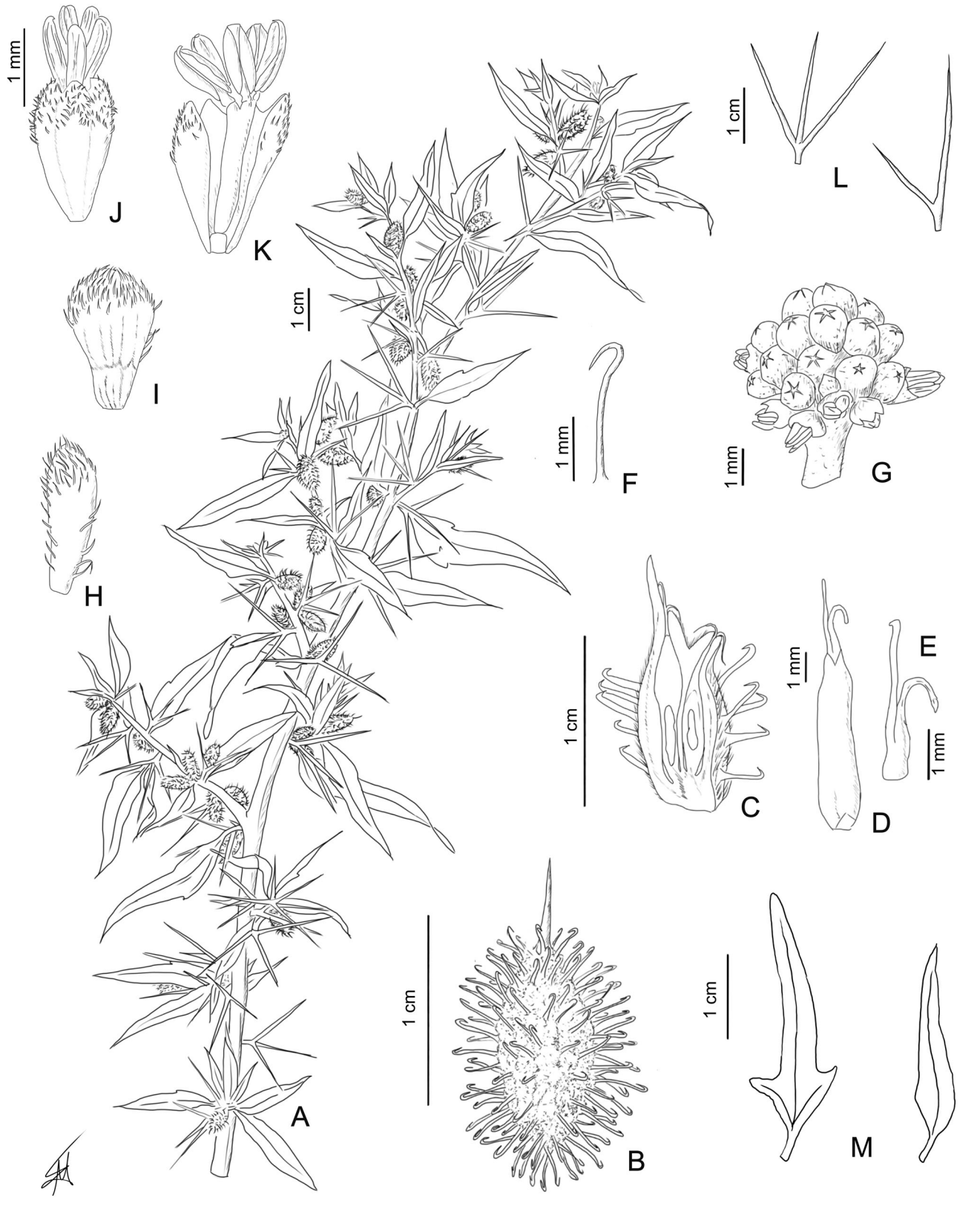
Within the section, species are distinguished mainly based on overall size and habitus, indumentum, and leaf shape and size. Leaf laminas are particularly variable and can be entire, tripartite, pinnatifid, and bipinnatifid. The number of species recognised has been subject of extensive debate. Widder (1923, 1964) recognised six species, along with six infraspecific taxa. Apart from *Xanthium spinosum*, he accepted *X. ambrosioides* Hook. & Arn. (Fig. 2) from central Argentina (southern South America), *X. catharticum* Kunth (Fig. 3), typical element of High Andes, and *X. medium* Nossotovsky, a species described based on a single unusual specimen from south-western Russia (characterized by a very strong taproot with tall, multibranched stems and simple thorns developed on one side of the leaf axil; Widder, 1923). He also raised *X. spinosum* var. *canescens* Costa (collected only few times in north-eastern Spain and south-western France) to the rank of species (*X. canescens* (Costa) Widder) and described *X. argenteum* Widder (Fig. 4) based on a single collection with a very peculiar phenotype (long, finely divided leaves covered by silvery hairs on both sides) as new to science (Widder, 1923). Years later, Löve (1975) recognised only *X. spinosum* and *X. ambrosioides* as species, whereas *X. catharticum* was placed as subspecies of the widespread *X. spinosum*. All other species and infraspecific taxa were merged into *X. spinosum*. A recent DNA-based species delimitation analysis identified two species in the section, namely *X. ambrosioides* and *X. spinosum* (Tomasello, 2018). However, only four samples of *X.* sect. *Acanthoxanthium* were included in the study, and most of the taxa described by Widder (1923, 1964) were missing.

In the present study, we aim to robustly identify species boundaries in *Xanthium* sect. *Acanthoxanthium*. For the scope, we used an exhaustive sampling, including taxa described by different authors (Appendix 1), and covering the whole range of morphological variation observable in the section. Where possible, we included nomenclatural types, original material, and/or samples from *loci classici* in the analyses, in order to unambiguously link the inferred lineages to available scientific names. We applied an integrative approach, taking advantage of genomic data (target enrichment of nuclear genes + plastomes), and geometric morphometric analyses of leaf shapes.

■ MATERIALS AND METHODS

Plant material. — In total 43 samples were analysed from herbaria and field collections. We used herbarium samples from a total of 39 specimens belonging to *Xanthium* sect. *Acanthoxanthium*. The specimens, including five types, were collected between 1840 and 2003 and were acquired from different herbaria: B, BA, BC, GOET, M, P, PR, TEX, and WU. The herbaria provided us with both leaf material (5 to 15 mg), and high-resolution images of the herbarium sheets. Additionally, three samples were collected, and silica-gel dried from field locations in Italy, Germany, and Argentina in 2018 and 2020 (Appendix 1). A specimen belonging to *X. strumarium* L. (*X.* sect. *Xanthium*) was included as an outgroup in some of the phylogenetic analyses.

DNA extraction, yield, and quality measurements. — For each sample, 5–10 mg of leaf material was transferred into a 2 ml Eppendorf tube containing a sterilised steel ball. Subsequently, the material was pulverised using a TissueLyser II (Qiagen, Hilden, Germany). We used two distinct extraction methods; for samples aged over 100 years, we employed a specific aDNA extraction method (PTB-DTT, Gutaker & al., 2017), whereas for more recent samples, we used the



Xanthium spinosum L.

Fig. 1. *Xanthium spinosum* L. (redrawn and modified by E. Manzo from Cabrera, 1978: 331: fig. 191). **A**, Floriferous branch; **B**, Bur (female capitulum); **C**, Longitudinal section of a young female capitulum; **D**, Female flower; **E**, Style; **F**, Spine of a female capitulum; **G**, Male capitulum; **H**, Palea of the male capitulum; **I**, Male bud; **J**, Male flower; **K**, Opened male flower; **L**, Branch spines; **M**, Leaves.

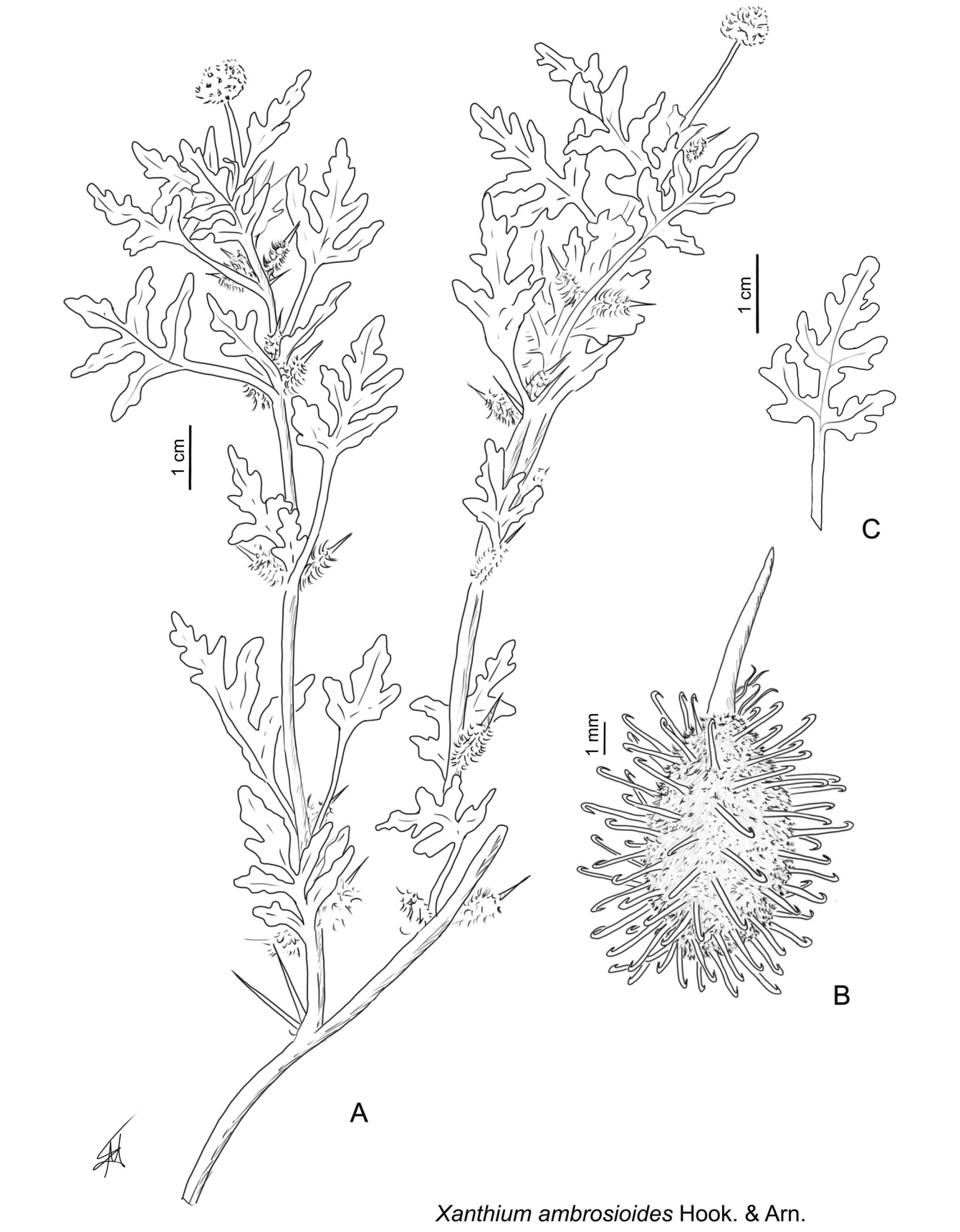


Fig. 2. *Xanthium ambrosioides* Hook. & Arn. (redrawn and modified by E. Manzo from Ariza Espinar, 2015: 266, fig. *Xanthium ambrosioides*). **A**, Floriferous branch; **B**, Bur (female capitulum); **C**, Leaf.

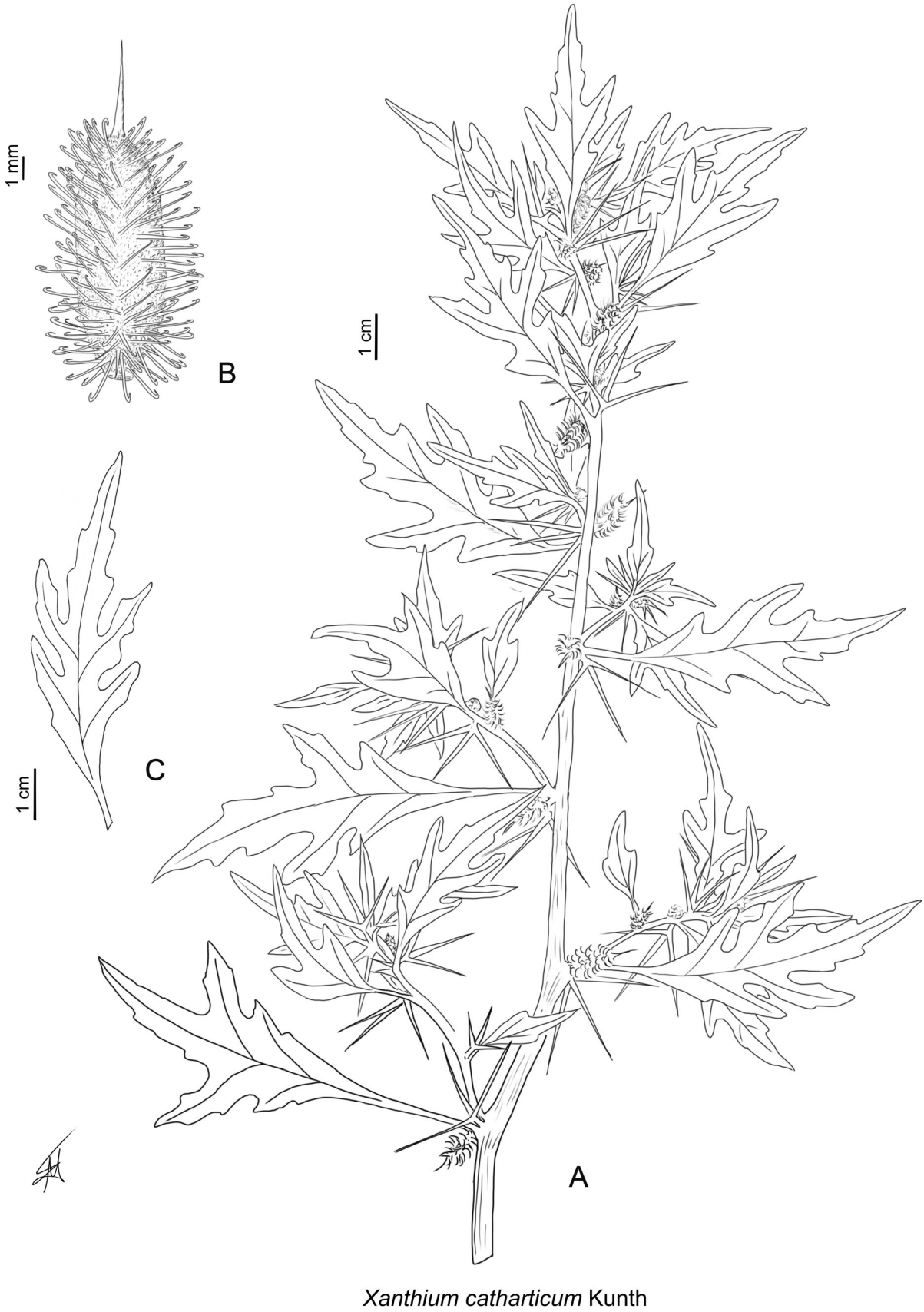


Fig. 3. *Xanthium catharticum* Kunth (redrawn and modified by E. Manzo from Cabrera, 1978: 323: fig. 135). **A**, Floriferous branch; **B**, Bur (female capitulum); **C**, Leaf.

Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). As for the PTB-DTT technique, we conducted extractions according to Dabney & al. (2013) with the specific modifications implemented by Gutaker & al. (2017). For the Qiagen kit, we adhered to the manufacturer's instructions and the modifications detailed in Marinček & al. (2022). We carried out the extractions under a laminar flow bench, using all precautions to prevent contaminations, sterilising the equipment with DNA Away (Thermo Fischer Scientific, Waltham, Massachusetts, U.S.A.) and utilizing an UVaClean UV Pipette Carousel (MTC Bio, Sayreville, New Jersey, U.S.A.) prior and after each extraction. Extracts were run in a 2% agarose gel to roughly estimate fragment lengths. Concentrations were measured using a Qubit 3 Fluorometer (Thermo Fisher Scientific) with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

Library preparation. — Library preparation was carried out either with the NEBNext Ultra II DNA Library Prep Kit

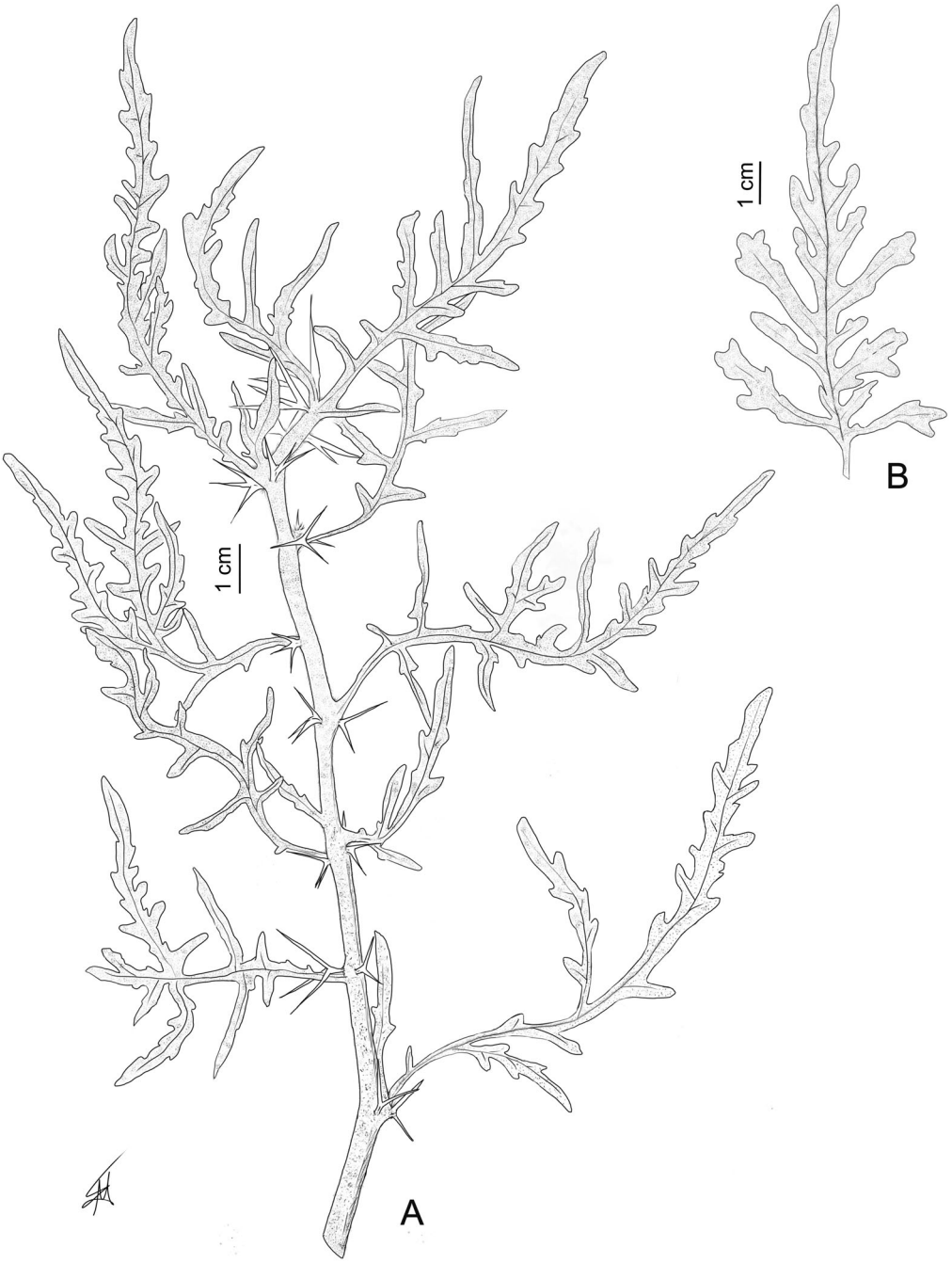


Fig. 4. *Xanthium argenteum* Widder (by E. Manzo, based on the herbarium specimen WU No. 0071716). **A**, Habitus; **B**, Leaf.

for Illumina or the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, Massachusetts, U.S.A.). The former was used for old herbarium samples, for which enzymatic fragmentation was not needed, the latter for more recent herbarium specimens and the few silica-gel dried material. In both cases, we followed the manufacturer's instructions. The sole modification was made by employing 1.5 volumes of HighPrep beads (MagBio Genomics, Gaithersburg, Maryland, U.S.A.), instead of the default 0.8 volumes, when purifying adapter-ligation products of very old herbarium specimens (see also Marinček & al., 2022). After adapter-ligation, samples underwent polymerase chain reaction (PCR) amplification for 14 cycles and were barcoded with the following sample-specific dual indices: NEBNext Multiplex Oligos for Illumina (Index Primers Set 1), NEBNext Multiplex Oligos for Illumina (Index Primers Set 2) and the NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) (New England BioLabs). The amplified reactions were then purified following the manufacturer's instructions. These libraries were partly sequenced directly (genome skimming), and partly used for the target enrichment (see below).

To acquire information on hundreds of single-copy nuclear genes, we used the myBaits COS Compositae 1Kv1 kit (Mandel & al., 2014; Daicel Arbor Biosciences, Ann Arbor, Michigan, U.S.A.). This kit has proved to be informative for phylogenetic studies at different taxonomic levels in members of the Asteraceae family (Jones & al., 2019). Six indexed samples were combined in equal concentrations, then dried in a Concentrator Plus (Eppendorf, Hamburg, Germany), followed by further dilution in 7 µl of distilled water. The hybridization step was carried out at 65°C for 20 hours. For the subsequent PCR amplification, P7 and P5 Illumina adapters were used as primers alongside the 2X KAPA HiFi HotStart Ready Mix (Roche, Basel, Switzerland).

Concentrations were measured using Qubit 3. Quality checks for both enriched and unenriched libraries were performed on a QIAxcel with the DNA High Resolution Kit 1200 (Qiagen), size marker QX Size Marker 50bp-800bp v.2.0, and alignment marker QX DNA Alignment Marker 15bp-5kb. If adapter-dimer peaks were detected (approximately 125 bp), the libraries were subjected to size selection of 140–600 bp using the BluePippin (Sage Science, Beverly, Massachusetts, U.S.A.), and 2% cassettes with the 2% DF Marker V2.

Most of the samples were then pooled equimolarly and sequenced (together with samples from another study) on an Illumina NovaSeq 6000 system (Illumina, San Diego, California, U.S.A.) using a SP P500 Xp (2 × 150 bp kit). One lane was used for the "unenriched libraries" (which did not go through the target enrichment procedure), and the other lane for the "targeted" ones. A few samples were sequenced on different runs of an Illumina MiSeq system using either the 2 × 250 or the 2 × 150 bp kit. All the sequencing was done at the NGS Integrative Genomics (NIG) Core Unit of the University of Göttingen.

Raw reads processing. — Raw reads were first processed in HybPhyloMaker v.1.8.2 (Fér & Schmickl, 2018). Adapters and low-quality reads were removed using Trimmomatic v.0.33 (Bolger & al., 2014) with the default settings specified in HybPhyloMaker. Duplicate reads were eliminated using FastUniq v.1.1 (Xu & al., 2012). The resulting quality-trimmed reads were submitted to different mapping strategies. To gather information on cpDNA, the quality-trimmed reads were mapped against a plastome of *Xanthium spinosum* available on the GenBank (MT668935; Raman & al., 2020) using BWA v.0.7.16a (Li & Durbin, 2009) with default options. Consensus sequences were produced in ConsensusFixer v.0.4 (Töpfer, 2018) with the "minimum relative abundance of the alternative base to call a wobble" set to 0.3, the "minimal coverage to call consensus" to 5, and the "minimal coverage to call insertion" to 20.

The nuclear regions were assembled primarily in HybPiper v.2.0 (Johnson & al., 2016) and following the strategy elucidated in Jones & al. (2019). In HybPiper, reads are first mapped using BWA and then de-novo assembled into contigs using SPAdes v.3.15.2 (Bankevich & al., 2012). This pipeline gives the possibility to retrieve flanking regions (and introns) of the targeted loci (using the flag –run_intronerate), and to examine the dataset for paralog sequences. A total of 342 regions of the initial 1061 were flagged as paralog in HybPiper and excluded from subsequent analyses. Alignments missing more than 25% of the samples were also excluded from further analyses. Since some of the selected alignments still showed evidence of paralogy, we decided to score the alignments further using the approach employed in Karbstein & al. (2022). Accordingly, we scored the alignments using the following parameters: (i) the R^2 of mutational saturation regression curves (Philippe & Forterre, 1999); (ii) the standard deviation of the sample-specific long-branch scores (LB scores; Struck & al., 2014); (iii) the clocklikeness; and (iv) average bootstrap (BT) support. We scored the respectively best performing 25% of loci with 1 and the remainder with 0. Finally, we selected the 50 loci with the highest overall score (suppl. Table S1).

HybPiper retrieves a single contig per locus per sample, and information on heterozygote sites, potentially present in some locus/sample, is lost. Therefore, and parallel to HybPiper, we assembled the nuclear loci in HybPhyloMaker, which can use IUPAC ambiguity codes in case of heterozygous sites. For the scope, we used only the 719 loci found to be paralogue-free in HybPiper. As (pseudo-)reference for mapping, we used a sequence consisting of the concatenated target sequences separated by 800 Ns each. Consensus sequences were produced (as above explained for the plastome assembly) with ConsensusFixer v.0.4. These were aligned with target exon using BLAT v.35 (Kent, 2002) to generate PSLX files. Afterward, they were combined through assembled_exons_to_fastas.py (Weitemier & al., 2014) to generate exon-based matrices. Matrices were aligned in MAFFT v.7.305b (Katoh & Standley, 2013), utilizing the software's default settings. We excluded further putative paralogues by employing the HybPhyloMaker4a2_selectNonHet.sh script and designating a maximum of 5 heterozygous sites per locus

("maxhet" in the HybPhyloMaker settings file). HybPhyloMaker conducts two consecutive rounds of missing data removal. First, samples with more than a certain percentage of missing data ("missingpercent" in the settings file) are removed from each alignment. We set this value to 40. Consequently, alignments with less than a certain percentage of samples (specified as "speciespresence" in the settings file) are excluded. We set this value to 75, in order to exclude alignments with more than 25% of missing sequences (as for the HybPiper dataset). Finally, 319 regions were retained after paralog and missing-data filtering. Alignment statistics were calculated utilizing amas (Borowiec, 2016), trimAl v.1.2 (Capella-Gutiérrez & al., 2009), and mstatx v.1.0 (Collet, 2012) as implemented in HybPhyloMaker.

Phylogenetic analyses. — Maximum likelihood (ML) phylogenetic trees were inferred in RAxML-ng v.1.1.0 (Kozlov & al., 2019). For nuclear data, the 391 single-copy genes selected in HybPhyloMaker were concatenated and used as input. The best-fitting substitution models were calculated using the Bayesian information criterion (BIC) in ModelTest-NG v.1.1.0 (Darriba & al., 2020) for each partition separately (suppl. Table S2). ML analyses were run with 100 bootstrap replicates.

The plastome alignment was partitioned following the annotation in MT668935 (Raman & al., 2020). As for the nuclear dataset, the best-fitting substitution model was identified using ModelTest-NG for each partition separately. Subsequently, we run RAxML-ng with 100 bootstrap replicates. In both nuclear and chloroplast analysis a sample belonging to the species *Xanthium strumarium* L. (X183; see Appendix 1) was used as an outgroup.

To examine the nuclear phylogenetic tree for conflicting or poorly informative branches, the quartet sampling (QS) method (Pease & al., 2018) was used on the nuclear tree with 100 replicates per branch. Quartet sampling results in branch-specific values that indicate how many QS replicates produced a quartet topology that is concordant with the input phylogeny (Quartet Concordance [QC]), estimate if the frequencies of the two possible discordant topologies are equal or skewed toward one discordant topology (Quartet Differential [QD]), and specify the percentage of informative QS replicates (Quartet Informativeness [QI]). Finally, a last score (Quartet Fidelity [QF]) displays the number of replicates in which a taxon produces congruent topologies with the input tree (Pease & al., 2018).

Species delimitation analyses. — We performed species delimitation analyses with the software package SPEEDEMON v.1.1.0 (Douglas & Bouckaert, 2022), implemented in BEAST2 (Bouckaert & al., 2019). SPEEDEMON is able to infer species delimitation on multilocus datasets and under the multispecies coalescent model. It outperforms similar approaches (e.g., STACEY; Jones, 2017) at achieving parameter convergence, especially on large molecular datasets (Douglas & Bouckaert, 2022).

Due to the high computational burden of such parameter-rich Bayesian analyses, we proceeded using the 50 nuclear loci selected in HybPiper. Input .xml files were produced in BEAUti v.2.7.6 (Bouckaert & al., 2019). Sequence substitution, clock- and gene-trees models were treated as unlinked across loci. Substitution models were selected *a priori* in ModelTest-NG v.1.1.0 for each locus separately and the strict clock was enforced for all loci.

As species tree prior, we used the Yule-Skyline Collapse, which is a combination of the Collapse model (employed in STACEY; Jones & al., 2015) and the Yule-Skyline model (Bouckaert, 2022). We set the "collapse weight" to a gamma distribution with default values ($\alpha = 2.0$; $\beta = 2.0$), while the Yule-skyline collapse birth rate was set to a normal distribution with the mean value centred in $1.0e^{-4}$. The epsilon value was left as default ($1.0e^{-4}$).

Two independent analyses were run for $10e^{9}$ Markov chain Monte Carlo (MCMC) iterations, sampling every 50,000 generations. We checked the convergence of the MCMC in Tracer v.1.7 (Rambaut & al., 2018), making sure that the two independent runs reached similar values for each parameter and ESS values above 200. The tree files from the two independent runs were eventually combined using LogCombiner v.2.7 (Bouckaert & al., 2019). The combined results were submitted to the ClusterTreeSetAnalyser tool with epsilon value of $1.0e^{-4}$. Additionally, we produced a similarity matrix, analysing the BEAST combined results with the SpeciesDelimitationAnalyzer (SDA; Jones & al., 2015) with "collapseheight" (i.e., the epsilon value of the Collapse model) of $1.0e^{-4}$ and cutoff of 0.9. Therefore, the similarity matrix was produced using a modified version of the R script provided by Jones & al. (2015). In both cases (i.e., the ClusterTreeSetAnalyser and the SpeciesDelimitationAnalyzer), we applied a 10% burn-in.

To test the consistency of species delimitation analyses on the phylogenomic dataset, we produced similar analyses using the 391 loci retrieved from HybPhyloMaker. Accordingly, we produced subsets of 50 loci randomly chosen from the 391 selected regions and performed SPEEDEMON analyses with the same settings as for the main analyses based on the HybPiper dataset. The outgroup accession was excluded from all coalescent-based species delimitation analyses.

Morphometrics. — The outline of leaves from high-resolution herbarium specimens was obtained using the lasso tool of the paint.net software (Brewster, 2023). The leaf images were transformed into binary (black-on-white) images. The Momocs v.1.4.1 (Bonhomme & al., 2014) package of R (R Core Team, 2021) was used to perform morphometric analyses. An elliptic Fourier transform (EFT; Giardina & Kuhl, 1977) was conducted on the digitised outlines using the *efourier*() function. The number of harmonics was set to 12, and the number of contour points was fixed at 1000 for each sample. Outlines of 43 leaves (one per sample) were imported into R for the final analysis, since the complexity of the leaf shapes resulted in unhelpful EFA results when using more outlines. Therefore, we performed principal component analysis (PCA) on the EFT measurements.

■ RESULTS

Sequencing and data filtering. — The data obtained from all sequencing runs amounted to 46 Gb. The average number of raw reads retained per sample was 6,979,105 (ranging from 666,798 to 18,195,434; see suppl. Table S3A). After quality filtering 10.4% of reads on average was excluded. Of the remaining reads, approximately 10% were removed after duplicate reads removal. On average 5,617,197 reads per samples were submitted to the different assembly strategies (see suppl. Table S3A). Raw reads data are deposited in the European Nucleotide Archive (ENA), under Bio-Project accession number PRJEB74768.

Detailed information about the efficiency of mapping during plastome and targeted regions assemblies, are reported in suppl. Table S3B,C. The 50 loci selected from HybPiper (and used for species delimitation purposes) resulted in a total amount of 16,169 bp, containing 186 parsimony-informative sites (suppl. Table S3B). The 391 (supposedly single copy) loci retrieved in the HybPhyloMaker dataset (used for ML tree inference and species delimitation purposes) resulted in a total length of 125,739 bp with 1074 parsimony-informative sites. As for the plastome, complete sequences were retained for 38 samples, resulting in a total alignment length of 152,799 bp, and 107 parsimony-informative sites (Table S3C). Alignments of both chloroplast and nuclear data have been deposited in the Göttingen Research Online (GRO) repository (https://doi.org/10.25625/P73PGZ).

Phylogenetic analyses. — The ML analysis of both chloroplast and nuclear alignments revealed four well-supported clades. Concerning the chloroplast tree (Fig. 5), samples initially ascribable to *Xanthium ambrosioides* and *X. spinosum* var. *laciniatum* (Scheuerm. & Thell. ex Widder) Widder form the early-diverging clade (bootstrap support: 82) well distinct from the remaining samples. The next split separates samples ascribable to *X. catharticum* (bootstrap support: 100) from two other clades: one formed by lectotype material of *X. argenteum* and an additional sample with similar morphology, the other including *X. spinosum* with all remaining taxa (bootstrap supports of 96 and 77, respectively).

The overall topology of the nuclear tree is similar to the chloroplast one, with the only difference that the clade containing *Xanthium catharticum* samples is sister to the *X. spinosum* clade, instead of *X. argenteum* (Fig. 6). *Xanthium spinosum* var. *laciniatum* (X157) is sister (with a bootstrap support of 81) to the clade comprising samples attributable to *X. ambrosioides* (bootstrap support of 97). Furthermore, the clade containing the lectotype of *X. argenteum*

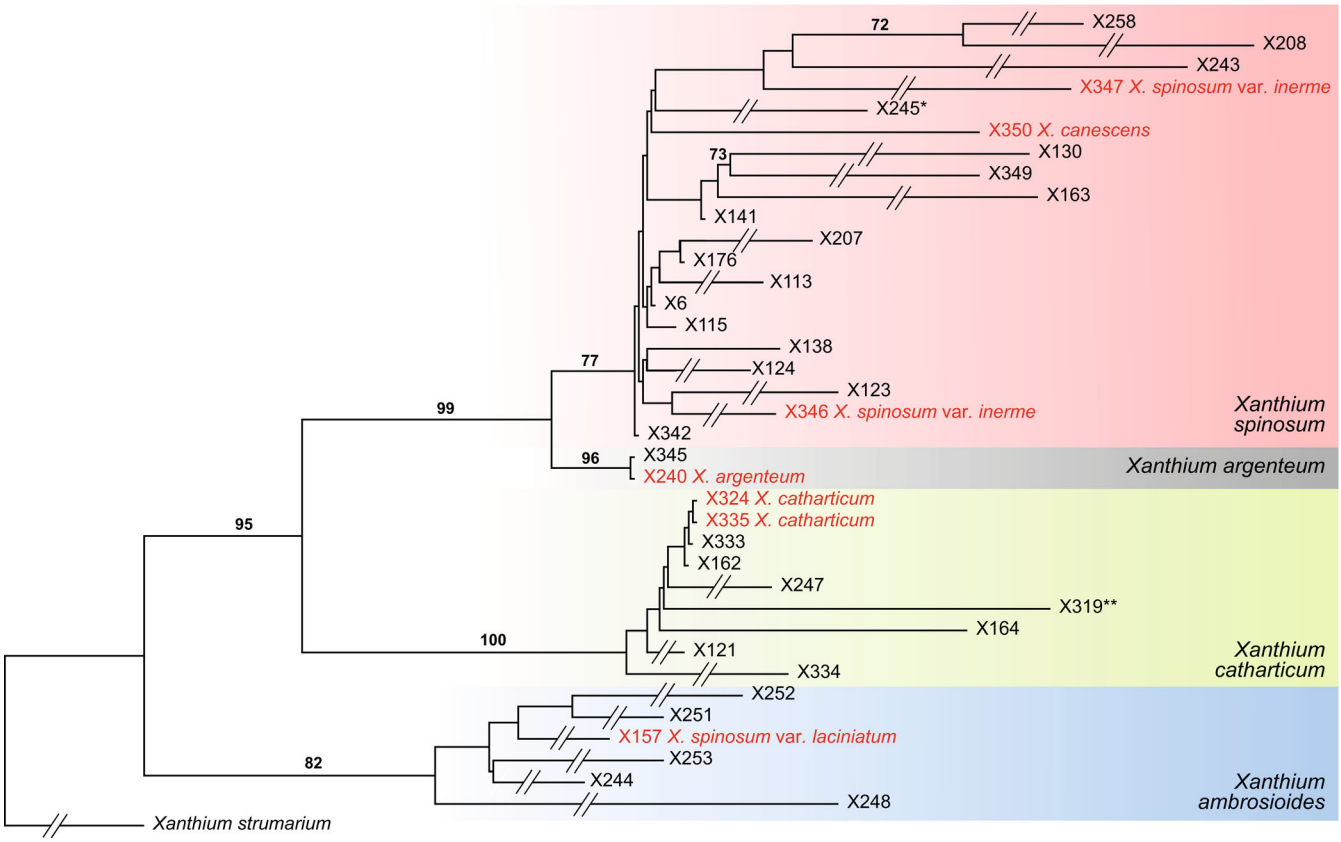


Fig. 5. Maximum likelihood (ML) tree based on the chloroplast data and including 38 individuals from *Xanthium* sect. *Acanthoxanthium*, along with a *X. strumarium* sample (outgroup). Bootstrap values over 70 are shown on branches. Boxes around clades are according to the new specific assignment: red for *X. spinosum*, grey for *X. argenteum*, green for *X. catharticum,* and blue for *X. ambrosioides*. Samples in red are types or original material included in the analysis, samples marked * and ** are specimens of probable hybrid origin.

and the one including samples ascribable to *X. catharticum* were inferred as fully supported. The remaining clade, including samples attributable to *X. spinosum* and the further taxa, received a bootstrap support of 99. Sample X245, initially identified as *X. catharticum*, has an ambiguous position here, being inferred as sister to *X. spinosum* (sister relationship however not highly supported), whereas in the chloroplast tree it was reconstructed as part of the *X. spinosum* clade. The quartet sampling analysis (Fig. 6) showed low values of QC and QD, while still having high values of QI in the nodes concerning the samples X157, X245, and X319.

Species delimitation analyses. — The SPEEDEMON species delimitation analysis based on the HybPiper 50 single-copy regions identified six genetic clusters within the section (Fig. 7). The best scenario of joint species assignment and species tree topology received a posterior probability of 7.26, while the posterior probability of the best species delimitation scenario was equal to 12.6. The main inferred species corresponded to the clades found in both the nuclear and the chloroplast phylogenetic trees; i.e., a cluster formed by lectotype material of *X. argenteum* and sample X345 (posterior probability 96.2), one formed by the samples ascribable to *X. ambrosioides* (posterior probability 90.7), the cluster of samples identified as *X. catharticum* (posterior probability 32.5) and the clade with *X. spinosum* and all remaining taxa (posterior probability 42.2). Additionally, the specimens X157 (*X. spinosum* var. *laciniatum*) and X245 (initially determined as *X. catharticum*) formed their own clusters with a posterior probability of 99.8 and 78.5, respectively. The less probable results in most cases presented a shift in the position of one of these two samples or of the sample X319 (*X. catharticum*), with the other clusters being unchanged.

The additional analyses done with the subsets of the 391 loci selected in HybPhyloMaker retraced the results of the main analysis, with the four main genetic clusters always recognisable (i.e., *X. ambrosioides*, *X. argenteum*, *X. catharticum,* and *X. spinosum*; suppl. Fig. S1). Main differences concern the position of sample X157 (*X. spinosum* var. *laciniatum*), most of the times reconstructed as part of the *X. ambrosioides* cluster, and the samples X245 and X319 (initially identified as *X. catharticum*), which were assigned to the remaining three main clusters in the different analyses. All the .xml input files and clustering results from

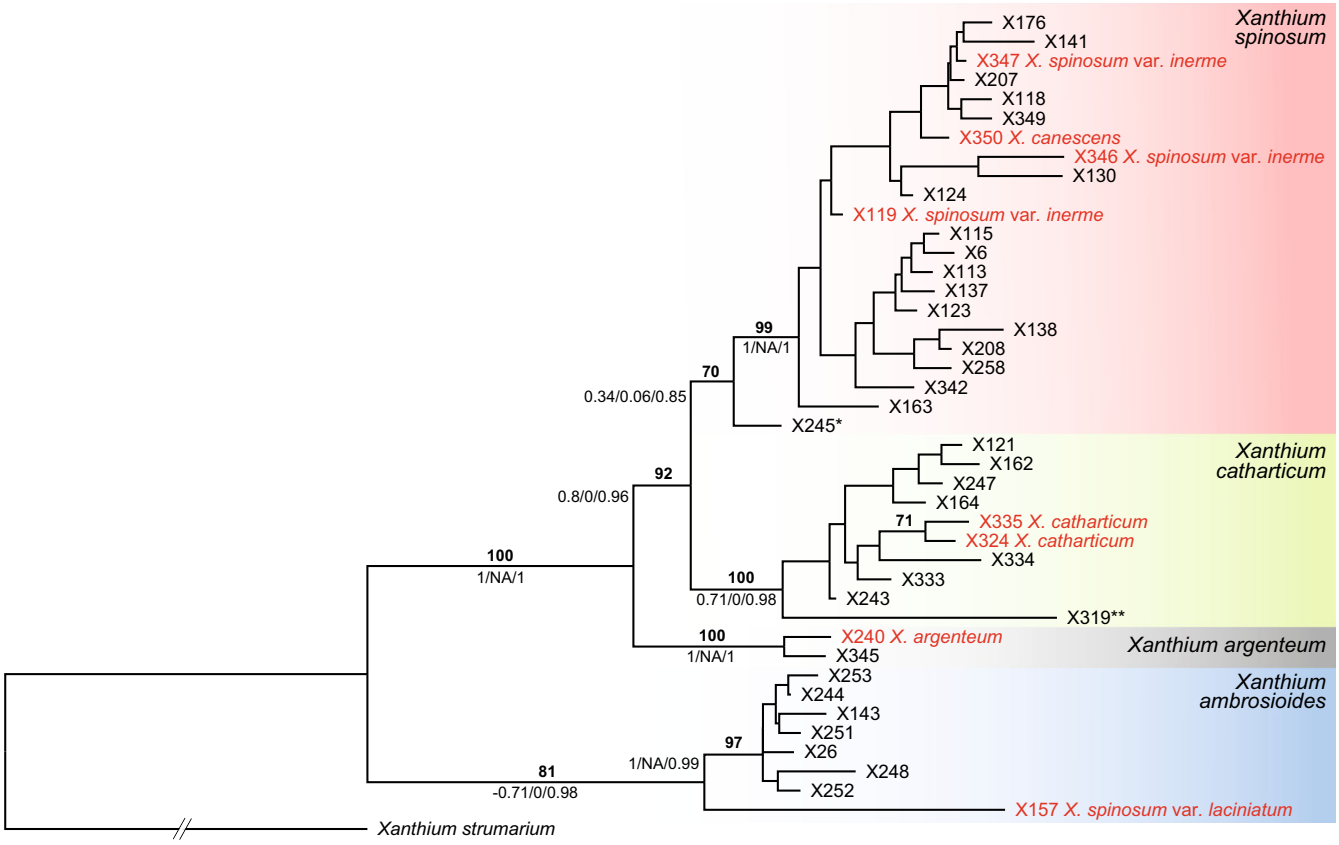


Fig. 6. Maximum likelihood (ML) tree based on 391 nuclear loci and including 43 individuals from *Xanthium* sect. *Acanthoxanthium*, along with a *X. strumarium* sample (outgroup). Bootstrap values of 70 and above are shown on branches. Quartet sampling values are shown under or to the side of backbone branches. The values represent, in order, Quartet Concordance / Quartet Differential / Quartet Informativeness. Boxes around clades are according to the new specific assignment: red for *X. spinosum*, grey for *X. argenteum*, green for *X. catharticum,* and blue for *X. ambrosioides*. Samples in red are types or original material included in the analysis, samples marked * and ** are specimens of probable hybrid origin.

SPEEDEMON have been deposited in the GRO repository under (https://doi.org/10.25625/P73PGZ).

Geometric morphometrics. — The geometric morphometric analyses, based on the shape extracted from leaves of herbarium specimens, produced a less resolved differentiation among the samples included in the study. The first three axes of the PCA jointly explain 73.5% of the total variance (43.3%, 17.9%, and 12.3% for axes PC1, PC2, and PC3, respectively). The analysis (Fig. 8) supported the deep morphological separation between *Xanthium ambrosioides* and the other taxa, whereas samples found in the *X. spinosum* and *X. catharticum* clusters of the DNA-based analyses were not distinguishable based on leaf outline. Any conclusion on *X. argenteum* is difficult to be drawn, due to the few samples attributable to this taxon and included in the analyses, and to the challenge of obtaining complete and well-preserved leaves from their respective herbarium specimens. The R-script file has been deposited in the GRO repository (https://doi.org/10.25625/P73PGZ).

■ DISCUSSION

Our study aimed at delimiting species in *Xanthium* sect. *Acanthoxanthium*. For the scope, we utilized an integrative approach, including DNA-based species delimitation analyses and geometric morphometrics. In comparison with previous studies (Tomasello & Heubl, 2017; Tomasello, 2018), we used a more comprehensive sampling, including as much as possible, all taxa described in the past, type material, and other relevant specimens. Only a few samples from *X.* sect. *Acanthoxanthium* were included in Tomasello (2018), i.e., two samples belonging to *X. spinosum*, and two *X. ambrosioides* collected in Leipzig within one year, all specimens collected outside the native distribution range of the section. In that study, both taxa were confirmed as independent species in the coalescent-based species delimitation analyses. Indeed, *X. ambrosioides* is quite distinct from *X. spinosum* by its prostrate habitus, and somewhat smaller and more divided leaves (pinnatifid). Not surprisingly, it was the only taxon that Löve (1975) recognised as species besides *X. spinosum*. In light of these considerations, we decided not to sample from the type specimens of the aforementioned two taxa. Instead, we concentrated our efforts on including type material from the other taxa described in the section. From the six species accepted in Widder (1923), we were not able to include any material for *X. medium*. This species was described based on a single specimen collected in Russia and showed an atypical growth and simple spines (instead of trifurcate). Most probably, it represents an aberrant form as already suspected by Rostowzew (1890), Widder (1923), and Löve (1975).

The taxonomy of *Xanthium* sect. *Acanthoxanthium* has undergone many taxonomic revisions in the past two centuries (e.g., Hooker & Arnott, 1841; Costa y Cuxart, 1864;

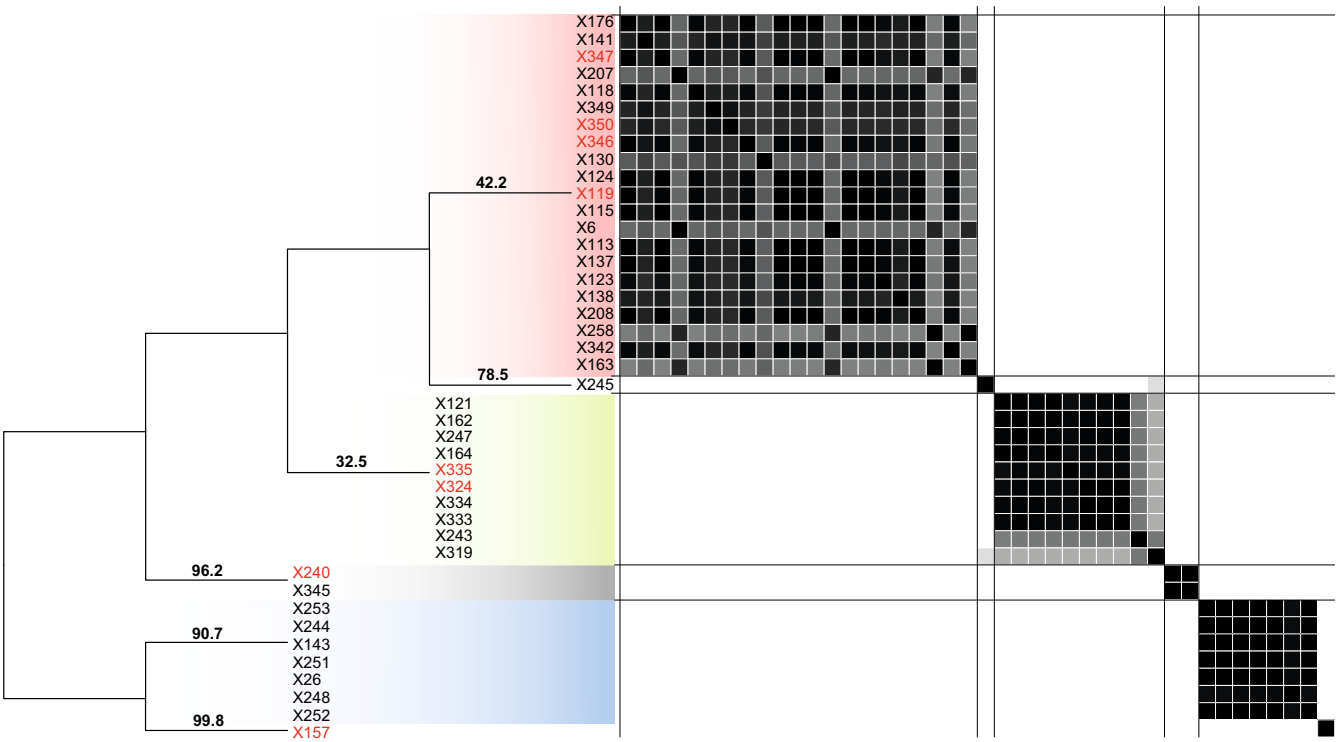


Fig. 7. Coalescent-based species clustering tree from SPEEDEMON (**left**). Posterior probability values for species assignments are shown on tree branches. Coloured boxes correspond to the new species classification (as in Figs. 5 and 6): red for *X. spinosum*, grey for *X. argenteum*, green for *X. catharticum,* and blue for *X. ambrosioides*. In red are shown types/original materials used in the analysis. Similarity matrix (**right**) shows posterior probabilities for pairs of individuals to belong to the same cluster (species). Black represents a posterior probability of 1.0, white 0.0.

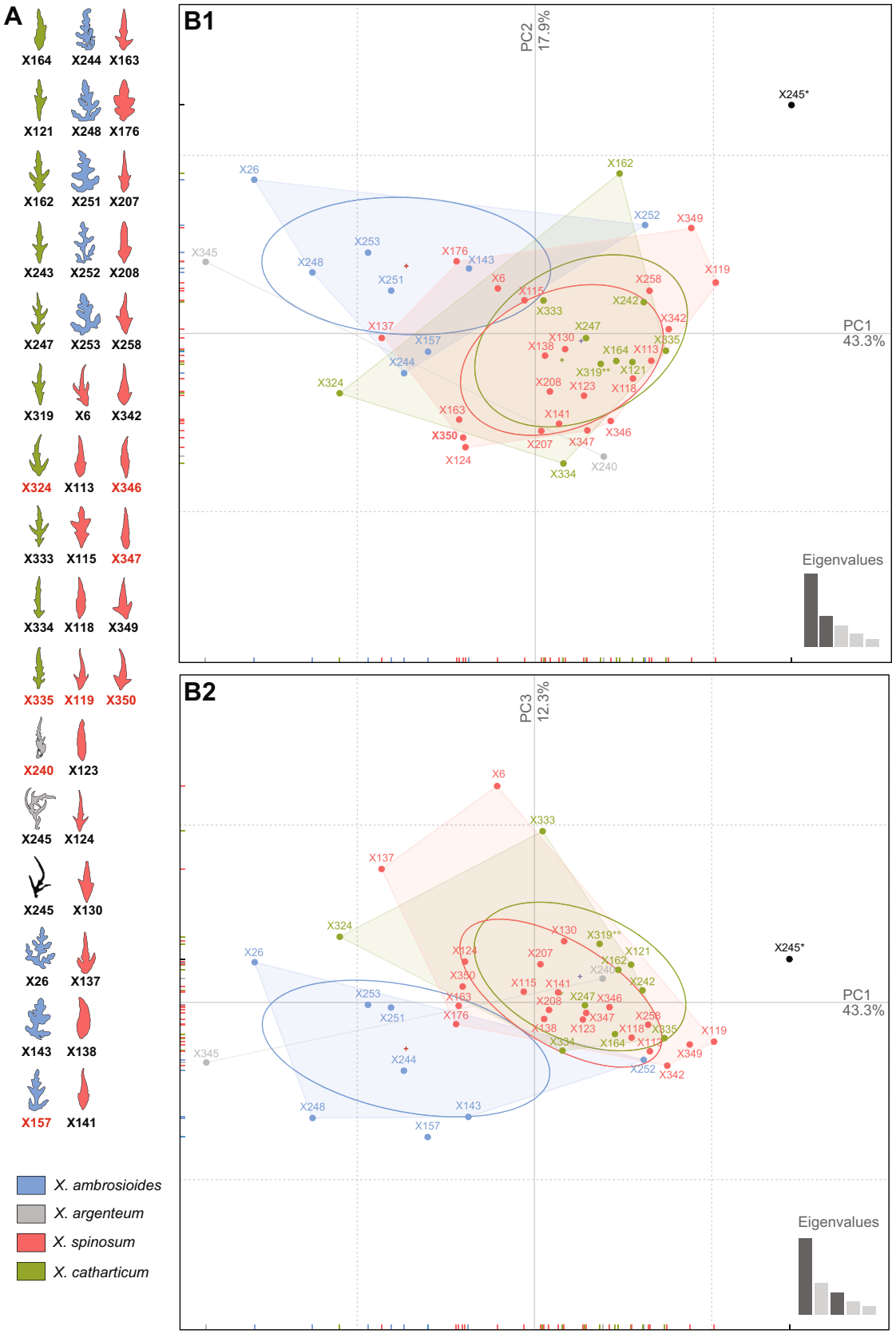


Fig. 8. A, Key showing leaf outline and species groups by colour (as in Figs. 5–7). Samples in red represent types/original material; **B**, PCA of elliptical Fourier data from leaf outlines (PC1 vs. PC2 and PC1 vs. PC3). Samples are coloured by species clusters (Figs. 5–7). Percent of variance explained by each PC are indicated on the axes. Ellipses show quantile confidence intervals of 0.75.

Fourreau, 1869; Bitter, 1908; Widder, 1923, 1964; Löve & Dansereau, 1959; Löve, 1975). Previous genetic studies on the genus did not provide sufficient data to conduct a thorough species delimitation of the section. Therefore, our results represent the first complete analysis of the section based on different sources of evidence. The ML phylogenetic trees (Figs. 5, 6) and the species delimitation analyses (Fig. 7; see also suppl. Fig. S1) produced consistent results. Four main genetic lineages were identified: (i) a clade containing mainly samples attributable to *X. ambrosioides*; (ii) the type specimen of *X. argenteum* and an additional specimen; (iii) samples ascribable to *X. catharticum*; and (iv) a major clade including *X. spinosum* and a few remaining taxa. These results are partially corroborated by the morphometric analyses. *Xanthium ambrosioides* resulted well-separated from the other genetic lineages, while no clear separation is observable between *X. spinosum* and *X. catharticum* in the PCA (Fig. 8). The latter should have slightly bigger leaves (the plants should have a bigger overall size), often more divided than those in *X. spinosum* (which are mostly simple or trilobed). However, the outline approach followed in our study does not seem to perceive these differences. On the other hand, *X. catharticum* seems to be quite distinct from *X. spinosum* from an ecological point of view. When plotting the latitude (± grades from the equator) against the elevation of the sampling locality of the accessions included in the study, the majority of those found to be in the *X. catharticum* genetic cluster are found well-separated from all the others (Fig. 9). In this respect, *X. catharticum* seems to be the vicariant of *X. spinosum* at high elevations of tropical mountain ranges of South America. Following Widder (1923), it does not seem to ever have been found outside its native range. We included samples collected at high elevations in the Paleotropics (e.g., X6), which turned out to belong to *X. spinosum*, corroborating therefore Widder's statement. Löve (1975) considered this taxon a subspecies of *X. spinosum*.

The fourth species resulting from our analyses is *Xanthium argenteum*. This taxon was described by Widder (1923) based on two specimens housed in B and WU. The specimens were collected in the Chilean province Ñuble, and were incomplete specimens, missing important characters such as the burs. The leaves, however, are big, deeply bi-pinnatipartite, with very thin and long segments and thus very distinct (Widder, 1923). In contrast to *X. catharticum*, which is probably the closest taxon with respect to leaf morphology, leaves appeared to have a different indumentum, being covered with silvery hairs on both surfaces. Löve (1975) did not give to this taxon any taxonomic recognition,

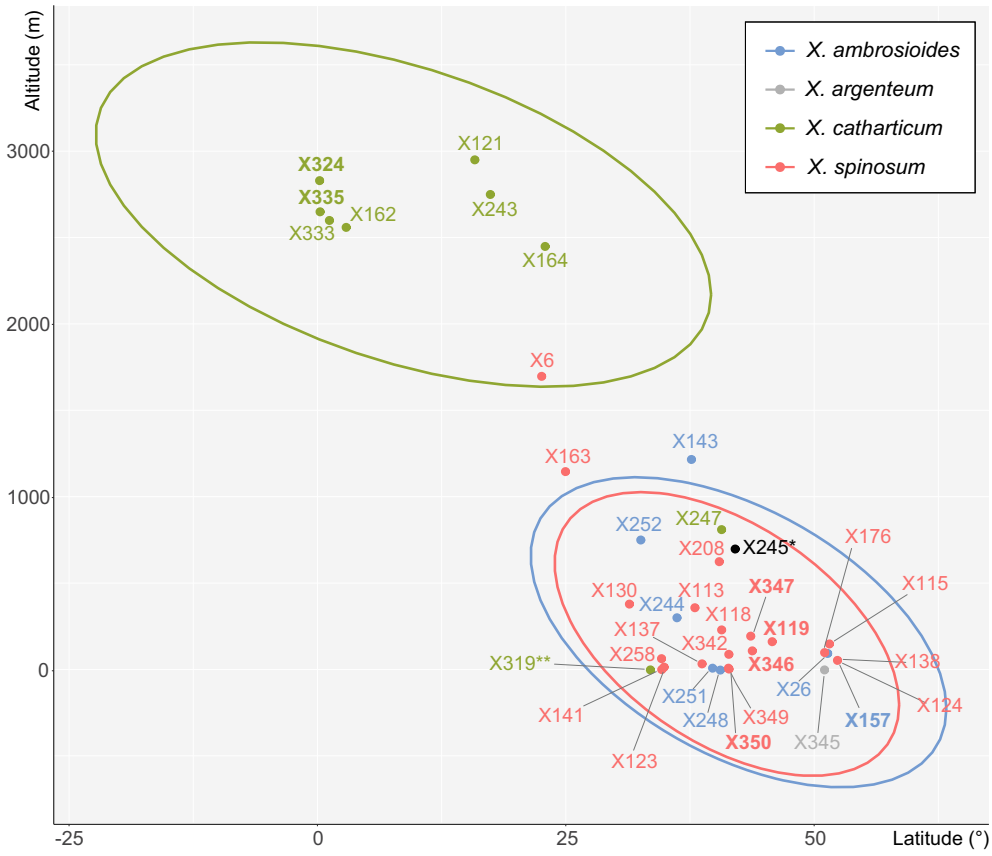


Fig. 9. Elevation of the collection localities of the samples included in the study plotted against their latitude (intended as absolute distance from the equator expressed in °; see Appendix 1). Colours are for species (as in Figs. 5–7). *Xanthium catharticum* is the only species capable of growing at elevations higher than 2000 m. Samples indicated with * and ** are specimens of probable hybrid origin.

considering it rather an "ephemeral extreme type of *X. catharticum* in respect to hairiness". We were able to sample the only extant syntype at WU and managed to find an additional specimen at P with a similar phenotype, collected in the port of Dunkerque (France) in 1950 (X345; P04380863), and identified by the collector as *X.* cf. *argenteum*. Astonishing enough, these two samples clustered together in all phylogenetic trees and species delimitation analyses (Figs. 5–7; suppl. Fig. S1). The preliminary ID of the P specimen thus could be confirmed and the hypothesis of these plants representing an abnormality of another taxon could be rejected. All our efforts to find other records potentially attributable to this species have resulted in the above-mentioned herbarium specimen and a single, dubious field observation (https://www.inaturalist.org/observations/191626931). In this circumscription, *X. argenteum* most probably represents a very rare species, native to Central Chile, observed and collected very few times until present. Further studies are needed to assess the distribution range and conservation status of this long-time neglected species.

A few samples showed inconsistent placement in the different phylogenomic analyses. Sample X157 is an original collection of *Xanthium spinosum* var. *laciniatum*, sampled by Scheuermann in the wool factory of Döhren (Hannover, Germany). It presents deeper divided leaves than those in *X. spinosum*, resembling somewhat *X. ambrosioides*. Thellung gave it the variety rank (in sched.) and Widder provided a formal description first as form (Widder, 1923), then as variety (Widder, 1964) of *X. spinosum*. In our phylogenomic analyses, this sample either clustered in the *X. ambrosioides* clade (Fig. 5; suppl. Fig. S1A–D,H) or it is found as sister to the aforementioned clade (Figs. 6, 7; suppl. Fig. S1E–G). In the morphometric analyses, it falls also into the group with all the other *X. ambrosioides* (blue in Fig. 8). Therefore, we conclude that these specimens collected by Scheuermann in the wool factory of Döhren must be attributed to *X. ambrosioides*. The topological inconsistency among analyses could be due to the high level of missing data for this sample (e.g., in the species delimitation analyses in Fig. 7 this sample was present only in 12 of the 50 loci used; see suppl. Table S3B). Alternatively, these specimens could be introgressed plants, having as maternal contributor *X. ambrosioides* (see position in Fig. 5), hybridizing with the (likely) several plants of *X. spinosum* growing at that time in the same locality. The low QD value of the quartet sampling analysis (QD = 0; Fig. 6) seems to support the latter hypothesis. Wool factories were preferential places for the introduction in Europe and elsewhere of members of *X.* sect. *Acanthoxanthium*, which arrived in such places as a contaminant of wool imported from South America. In our sampling, we have another accession (X138) collected in the same place and ascribable to *X. spinosum*.

The sample X245, collected at El Maiten (Argentina) and initially identified as *Xanthium catharticum*, clustered with *X. spinosum* in the chloroplast phylogenetic tree (Fig. 5) and in some of the species delimitation analyses (suppl. Fig. S1A–D,G–H), whereas in the nuclear tree and in the species delimitation analyses, it formed its own cluster in a sister position to *X. spinosum* (Fig. 7, suppl. Fig. S1E,F). As for sample X157, we are probably dealing with a hybrid plant that, although it shows a phenotype closer to *X. catharticum*, seems to be genetically closer to *X. spinosum*. Also in this case, QC and QD values of the quartet sampling analysis (0.34 and 0.061, respectively) confirm this possibility. Interestingly, this sample was placed apart in the morphometric analyses, distant from either the *X. catharticum* or *X. spinosum* samples (Fig. 8). However, examples of transgressive phenotypes in plant hybrids are relatively common (Schwarzbach & al., 2001; Johnston & al., 2004; Gallego-Tévar & al., 2018; Hodač & al., 2023).

To a minor extent, sample X319 was found to be either sister to *Xanthium catharticum* (Fig. 6) or as part of the same (Fig. 5). The species delimitation analyses placed it as part of the *X. catharticum* cluster, even if with lower posterior probability compared to other samples (Fig. 7). It resembles morphologically *X. catharticum* but was collected at sea level (not a suitable environment for the species). Gene-flow with *X. spinosum*, growing abundantly in such environments, might be an explanation. In the QS analysis, its position was relatively highly supported (QC = 0.71), even though the very low QD value (QD = 0) suggests gene-flow involving this sample. However, this sample shows high levels of missing data (suppl. Table S3A,B) and any conclusion should be taken carefully. All the other taxa included in the study, i.e., *X. canescens*, *X. spinosum* var. *inerme,* and "*X. spinosum* f. *integrifolium*" ined., resulted as part of *X. spinosum*. In most of the cases, they have to be considered mutants showing an abnormal development of certain traits, such as stem spines and leaves. As an example, *X. spinosum* var. *inerme* was described based on plants missing the typical tricuspidate spines at the base of the leaves. Offsprings of this plant have been cultivated in various botanical gardens in Europe in the early twentieth century. Instead of the typical spines, these plants were developing side branches and/or female capitula (Widder, 1923). According to Widder (1931), this was the product of a recessive mutation. These mutants have arisen multiple times independently of each other (Widder, 1964), as corroborated by the non-monophyletic position indicated for the samples of this variety in our phylogenetic analyses (Figs. 5, 6). The same is plausible for the other taxa showing an abnormal spine development (e.g., *X. brachyacanthum* DC., *X. spinosum* var. *synacanthum* Widder, *X. spinosum* var. *pseudinerme* Widder), as already discussed in Löve (1975). Since these plants have been observed growing sympatrically with "normal" *X. spinosum*, showing single, obvious morphological differences in comparison with the "typical" plants (probably controlled a single or few genes; Widder, 1931), if any, the rank of forma should be more appropriate for these taxa (Stuessy & al., 2014).

■ TAXONOMIC TREATMENT

Four species belonging to *Xanthium* sect. *Acanthoxanthium*, are recognised, including two forms for *X. spinosum*. We

refer to Widder (1923, 1964), Ariza Espinar (2015), and Alves (2020, 2024) for a complete synonymy and morphological description of taxa.

1. ***Xanthium ambrosioides*** Hook. & Arn. in J. Bot. (Hooker) 3: 310. 1841 ≡ *Acanthoxanthium ambrosioides* (Hook. & Arn.) D.Löve in Lagascalia 5(1): 66. 1975 – **Lectotype (designated here):** Argentina, Buenos Ayres [now Buenos Aires city and surroundings], *J. Tweedie 738* (K barcode K000373061 [digital image!], well prepared individual in the centre; isolectotypes: E barcode E00249291 [digital image!], K barcode K000373061 [digital image!], individual right-hand centre).

= *Xanthium spinosum* f. *laciniatum* Scheuerm. & Thell. ex Widder in Repert. Spec. Nov. Regni Veg. Beih. 20: 135. 1923 ≡ *Xanthium spinosum* var. *laciniatum* (Scheuerm. & Thell. ex Widder) Widder in Phyton (Horn) 11: 76. 1964 – Holotype: Germany, Hannover, Döhrener Wollwäscherei, 2 Sep 1913, *Scheuermann s.n*. [Herb. Thellung] (BAS barcode BAS-00019329 [digital image!]).

Xanthium ambrosioides is illustrated in Fig. 2.

Note. – The herbarium of W.J. Hooker is at K (Stafleu & Cowan, 1979), whereas Arnott's herbarium was in GL, now on permanent loan to E (Stafleu & Cowan, 1976), and consequently specimens of both syntype collections of *Xanthium ambrosioides* are present in both herbaria. In E both are mounted on one sheet, and it is difficult to tell which part of the plant belongs to which collection. The sheet at K contains at least four collections: K000373059 (*Beechey*) and K000373060 (*Chamisso*) are not types for *X. ambrosioides*, whereas K000373061 (*Tweedie 738*) and K000373062 (*Gillies 96*) are. The plants on the right and left margins in the centre are somewhat difficult to attribute to either collector, and the two lower specimens (both *Gillies 96*) are poorly prepared. The most complete and unambiguous plant in the centre (*Tweedie 738*) is selected here as the lectotype.

Although Stafleu & Cowan (1988) cite Thellung's herbarium from Z, a large part is in BAS, including the type of *Xanthium spinosum* f. *laciniatum*. The second collection cited, but only as a literature reference and explicitly not seen by Widder (France, Dep. Loiret, Feulardes, *J. Benoist* [Oct.] 1913), may be preserved at ORM and represents a paratype collection.

2. ***Xanthium argenteum*** Widder in Repert. Spec. Nov. Regni Veg. Beih. 20: 118. 1923 – **Lectotype (designated here):** Chile, [Region of] Ñuble [formerly province of Ñuble; most likely collected by Philippi prior to 1888] (WU No. 0071716 [digital image!]).

Xanthium argenteum is illustrated in Fig. 4.

Note. – The second syntype cited by Widder (1923: 118) from B ("Chile, Ñuble, ded. Philippi 1888") was destroyed in World War II: no collector was given for the specimen at WU, and none is mentioned on the specimen itself. However, there is a stamp "Acquis. Journ. No 852" and the corresponding entry in the acquisition book at WU indicates that the specimen was part of a consignment received from Philippi in February 1888 in exchange for duplicates of the "Flora Austro-Hungarica" issued by WU.

3. ***Xanthium catharticum*** Kunth in Humboldt & al., Nov. Gen. Sp., ed. fol., 4: 216. 1818 ≡ *Xanthium armatum* Humb. ex Wallr., Beitr. Bot. 1: 242. 1842, nom. illeg. ≡ *Acanthoxanthium spinosum* subsp. *catharticum* (Kunth) D.Löve in Lagascalia 5(1): 64. 1975 – **Lectotype (designated here):** Quito, *Bonpland 3006* (P 2D-barcode P00320266 [digital image!]).

Xanthium catharticum is illustrated in Fig. 3.

Note. – Additional original material: Ecuador, Pichincha province, "Chillo [Los Chillos], Quito, [F.W.H.A. Humboldt &] A.[J.A.] Bonpland 3006" (P barcode P02537136 [digital image!]); habitat in America meridionali [A.J.A. Bonpland & F.W.H.A.] Humboldt 3006 (B-W barcode BW1747200 [digital image!]). As the protologue does not mention a repository or a single collection, there is no holotype. The only material that was certainly available to Kunth is the one that was donated to P shortly after Humboldt and Bonpland's return. It is therefore the only element that unambiguously represents a syntype and is selected here as a lectotype. The exact nomenclatural status of the remaining two specimens of the original material at B-W and P (identified as such by the unifying number *3006*, representing the number from Humboldt & Bonpland's *Journal de botanique*) cannot be assessed, as given the fate of Humboldt & Bonpland's collections (see Tkach & al., 2016), it remains doubtful whether they were actually accessible and used by Kunth when preparing the description. Wallroth's name, although treated differently in the literature, is illegitimate because he cites Kunth's name synonymously. Also, Wallroth was probably wrong in attributing the name *Xanthium armatum* to Humboldt instead of (more likely) Willdenow.

4. ***Xanthium spinosum*** L., Sp. Pl. 2: 987. 1753 – Lectotype (designated by Widder in Phyton (Horn): 11: 72. 1964, as 'Holotypus'): "Habitat in Lusitania" [Portugal], Herb. Linnaeus No. 1113.3 (LINN [digital image!]).

= *Xanthium spinosum* var. canescens Costa, Introd. Fl. Cataluña: 160–161. 1864 ≡ *Xanthium canescens* (Costa) Widder in Repert. Spec. Nov. Regni Veg. Beih. 20: 121. 1923 – Type: Spain, Catalonia: "ad oras fluvii Besòs [Besòs River] raro, versus Badalona 1860, [A.C.] *Costa* [y Cuxart] *s.n*." (LE n.v.).

Xanthium spinosum is illustrated in Fig. 1.

Note. – Widder (1964: 72) did not cite a number under *Xanthium spinosum*, but clearly associated the term "TYPE" with the single specimen of the species in LINN (LINN No. 1113.3), thus effecting typification and rendering superfluous the later one by Wijnands (1983: 87). Widder (1923) cited the type for the name from LE, which we have not seen. No duplicate was found in the *Xanthium* collections at BC.

Xanthium spinosum f. ***inerme*** (Bel) O.Bolòs & Vigo in Collect. Bot. (Barcelona) 17(1): 90. 1988 ≡ *Xanthium*

spinosum var. *inerme* Bel in Rev. Bot. Bull. Mens. 11: 481. 1893 – Neotype (designated by Widder in Phyton (Horn): 11: 77. 1964): France, Occitania: Tarn department, Albi. "Cult. [cultured], de graines recues del'auteur [J. Bel], Albi, Tarn, July 1897, ex herb. H. Sudre" *s.n.* (Z [digital image!]).

= *Xanthium spinosum* var. *pseudinerme* Widder ex Parodi in Physis (Buenos Aires) 8: 480. 1927 – Holotype: Argentina, city of Buenos Aires, [Villa] Pueyrredón (F.C.C.A. [Spanish abbreviation for Argentine Railway]), 12 Apr 1926, *A. Burkart 393* (SI barcode 020519 n.v.).

Note. – The original type material was not located by Widder (1964: 77 – "Der von dem Entdecker der auffälligen Sippe, J. Bel, im Jahre 1892 am Ufer des Flusses Tarn in Frankreich gesammelte und an E. Malinvaud übermittelte Beleg war mir nicht zugänglich" (The specimen collected by the finder of this conspicuous taxon, J. Bel, on the banks of the river Tarn in France in 1892 and sent to E. Malinvaud was not available to me") and is not among the digitised specimens in P, where according to Stafleu & Cowan (1981) Malinvaud's herbarium is kept. We were also unable to locate the neotype at Z. There is a collection by H. Sudre with the same details as the neotype but collected in 1896 (P04130610) and numerous other specimens grown between 1894 and 1897 from the same source (P04114960 – included in our analyses; P04114968, P04322116, P04322118, P04322119, P04403066, P04403067, P04454999 [digital images!]). Any of these could serve as a neotype if the original material and the previously designated neotype are lost.

Key to taxa of *Xanthium* sect. *Acanthoxanthium*

1. Leaves pinnatifid to bipinnatifid, covered on both sides by silvery thrichomes 2
1. Leaves simple, trilobed, or pinnatipartite, with silvery hairs abaxially and with strigose pubescence adaxially..3
2. Plant decumbent, with weak stems and branches; leaves up to 5 cm long, lobed with stocky segments..... ..... ***X. ambrosioides***
2. Plant erect; with stronger stems; leaves more than 5 cm long, deeply lobed with very thin segments..***X. argenteum***
3. Plants >50 cm tall; leaves trilobed or pinnatipartite; strigose hairs dispersed over the adaxial surface of the leaves .....***X. catharticum***
3. Plants up to 50 cm tall; leaves simple or trilobed; adaxial leaf surface strigose, mainly on the midrib ....***X. spinosum***
4. Stems spiny at the base of the leaves .... ***X. spinosum*** f. ***spinosum***
4. Stems spineless ..... ***X. spinosum*** f. ***inerme***

■ AUTHOR CONTRIBUTIONS

EM and ST designed the research; DGG, JLP, and ST sampled plant materials; EM and ST performed laboratory work; EM, ST, and SN analysed the data; EM wrote the manuscript with contributions from DGG, JLP, CB, and ST.

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Appendix 1. List of samples used in the study, along with information on the herbarium voucher, European Nucleotide Archive (ENA) accession number, identification according to Widder (1923, 1964), collection place, collection date (day/month/year), collector name(s) and number, inferred coordinates (latitude and longitude) and elevation, and the new species assignment (samples marked * and ** are specimens of probable hybrid origin). An estimate of the precision (Prec.) of the inferred coordinates is expressed in numbers from 1 to 5: 1) exact coordinates; 2) within 1–3 km; 3) within 5–10 km (e.g., name of a city); 4) only province/region known; 5) only state or nothing known. Inferred localities with a precision of 1–3 were used to build the scatterplot in Fig. 9.

Lab ID	Herbarium voucher	Accession	Identification (Widder, 1923, 1964)	Collection place	Collection date	Collector(s)	Coll. Number	Notes	Latitude	Longitude	Elevation (m)	Prec.	New species assignment
X6	M-0158771	ERS 18958779	*Xanthium spinosum* L.	Avis Dam; Windhuk (Namibia)	14/03/1963	*R. Seydel*	*3435*		−22.56993883	17.12921017	1700	1	*X. spinosum* L.
X26	B100467880	ERS 18958740	*X. ambrosioides* Hook. & Arn.	Leipzig; Saxony (Germany)	23/07/1940	*V. Fiedler*	*s.n.*		51.35787624	12.33982911	100	1	*X. ambrosioides* Hook. & Arn.
X113	GOET061876	ERS 18958769	*X. spinosum* L.	Ventimiglia; Sicily (Italy)	21/10/2020	*S. Tomasello*	*TS1288*	field collected	37.99334069	13.5288985	364	1	*X. spinosum* L.
X115	GOET061879	ERS 18958770	*X. spinosum* L.	Cult. Bot. Gard. Göttingen (Germany)	13/11/2020	*S. Tomasello*	*TS1292*	field collected	51.53813637	9.935432823	155	1	*X. spinosum* L.
X118	GOET061880	ERS 18958771	*X. spinosum* L.	Salerno; Campania (Italy)	11/09/2018	*S. Tomasello*	*TS1091*	field collected	40.687042	14.7553004	236	1	*X. spinosum* L.
X119	GOET042990	ERS 18958759	*X. spinosum* var. *inerme* Bel	Cult. Bot. Gard. Münster (orig Bot. Gard. Lyon) (Germany)	1903		*s.n.*	cult. from original material	45.77425567	4.853897774	168	1	*X. spinosum* L.
X121	GOET042660	ERS 18958758	*X. catharticum* Kunth	Chuquibamba (Perú)	18/03/1957	*H. Ellenberg*	*204*		−15.83939182	−72.65113537	2950	1	*X. catharticum* Kunth
X123	GOET042994	ERS 18958762	*X. spinosum* L.	Miguelete; Dep. Montevideo (Uruguay)	04/1934	*W.G. Herter*	*379*	ined. "*integrifolium*"	−34.8947091	−56.17944145	21	2	*X. spinosum* L.
X124	GOET043085	ERS 18958764	*X. spinosum* L.	Hannover Döhren (Germany)	14/08/1924		*1097*		52.34789359	9.759297943	60	2	*X. spinosum* L.
X130	GOET043095	ERS 18958772	*X. spinosum* L.	Cordoba (Argentina)	12/1870	*T.G. Lovenh*	*259*		−31.40535366	−64.14356717	385	3	*X. spinosum* L.
X137	GOET043090	ERS 18958773	*X. spinosum* L.	Lisbon (Portugal)	07/1840	*F. Welwitsch*	*s.n.*		38.71211117	−9.136050311	40	3	*X. spinosum* L.
X138	GOET043082	ERS 18958763	*X. spinosum* L.	Hannover Döhren (Germany)		*R. Scheuermann*	*s.n.*		52.3328543	9.759743455	60	1	*X. spinosum* L.
X141	BA62708	ERS 18958774	*X. spinosum* L.	Avellaneda; Buenos Aires (Argentina)	25/03/1913	*A. Rodriguez*	*202*		−34.65798349	−58.36704238	7	1	*X. spinosum* L.
X143	BA89510	ERS 18958741	*X. ambrosioides* Hook. & Arn.	El Huecú; Neuquén (Argentina)	1963	*Dimitri, Correa Luna & Amorín*	*5105*		−37.64541792	−70.58179142	1220	2	*X. ambrosioides* Hook. & Arn.
X157	B101067779	ERS 18958747	*X. spinosum* var. *laciniatum* Scheuern. & Thell. apud Widder	Hannover Döhren (Germany)	1912–1917	*R. Scheuermann*	*499*	original material	52.3328543	9.759743455	60	1	*X. ambrosioides* Hook. & Arn.

(Continues)

pendix 1. Continued.

ab)	Herbarium voucher	Accession	Identification (Widder, 1923, 1964)	Collection place	Collection date	Collector(s)	Coll. Number	Notes	Latitude	Longitude	Elevation (m)	Prec.	New species assignment
162	B101067796	ERS 18958751	*X. catharticum* Kunth	Cuenca; Azuay (Ecuador)	05/01/1968	*W. Schwabe*	*68077*		−2.893272413	−79.01092296	2560	2	*X. catharticum* Kunth
163	B100261444	ERS 18958778	*X. catharticum* Kunth	San Agustín; Dep. Salta (Argentina)	05/03/1988	*L.J. Novara*	*7752*		−24.96772057	−65.42164497	1150	1	*X. spinosum* L.
X164	B100349123	ERS 18958752	*X. catharticum* Kunth	San Pedro de Atacama (Chile)	11/03/2001	*M. Ackermann*	*235*		−22.91928849	−68.20846741	2450	1	*X. catharticum* Kunth
X176	B101095040	ERS 18958765	*X. spinosum* L.	Dresden; Saxony (Germany)	05/06/1918	*H. Stiefelhagen*	*s.n.*		51.05315946	13.81161048	105	1	*X. spinosum* L.
X207	PR86412	ERS 18958775	*X. spinosum* L.	Rheinland (Germany)		*H. Krüger*	*s.n.*		51.54267618	7.210415828	50	4	*X. spinosum* L.
X208	PR240794	ERS 18958766	*X. spinosum* L.	Madrid (Spain)	01/10/1947	*A. Rodriguez*	*s.n.*		40.44678573	−3.725395961	630	1	*X. spinosum* L.
X240	WU0071716	ERS 18958748	*X. argenteum* Widder	Ñuble (Chile)		*Philippi*	*s.n.*	type				4	*X. argenteum* Widder
X243	BA302808	ERS 18958753	*X. catharticum* Kunth	Valle; Cochabamba (Bolivia)	1929	*J. Steinbach*	*9708*		−17.40466323	−66.10657246	2750	3	*X. catharticum* Kunth
X244	BA33212	ERS 18958742	*X. ambrosioides* Hook. & Arn.	Loventuel (Argentina)	1939	*Fortuna*	*85*		−36.18899864	−65.28726164	306	1	*X. ambrosioides* Hook. & Arn.
X245	BA89509	ERS 18958780	*X. catharticum* Kunth	El Maiten (Argentina)	1961	*Roguero*	*137*		−42.04932409	−71.16200526	703	2	*
X247	BA12474	ERS 18958754	*X. catharticum* Kunth	Correntoso; Neuquen (Argentina)	1934	*A. Burkart*	*6403*		−40.68139979	−71.6487589	815	3	*X. catharticum* Kunth
X248	BA6660	ERS 18958743	*X. ambrosioides* Hook. & Arn.	Bahia San Blas (Argentina)	1932	*Laguerre*	*s.n.*		−40.55218196	−62.23382761	4	1	*X. ambrosioides* Hook. & Arn.
X251	BA60567	ERS 18958744	*X. ambrosioides* Hook. & Arn.	Igarzabal (Argentina)	1963	*W. Partridge*	*s.n.*		−39.77331726	−62.61455794	15	2	*X. ambrosioides* Hook. & Arn.
X252	BA251015	ERS 18958745	*X. ambrosioides* Hook. & Arn.	San Francisco del Monte de Oro; Mina del Pilón (Argentina)	1925	*Castellanos*	*s.n.*		−32.5481607	−66.16287703	755	1	*X. ambrosioides* Hook. & Arn.
X253	BA46475	ERS 18958746	*X. ambrosioides* Hook. & Arn.	Agua de la Chilena; Dep. San Rafael (Argentina)	1942	*R. Leal*	*7654*		−35.26180337	−68.27365243	1059	4	*X. ambrosioides* Hook. & Arn.
X258	BA19172	ERS 18958776	*X. spinosum* L.	Chacabuco; Buenos Aires (Argentina)	1936	*M.P. Urulay*	*s.n.*		−34.63516448	−60.46751695	70	1	*X. spinosum* L.
X319	TEX00561301	ERS 18958781	*X. catharticum* Kunth	San Carlos; Cartagena (Chile)	16/03/1931	*H. Gunckel*	*4830*		−33.51746202	−71.60974715	5	1	**
X324	P00320266	ERS 18958755	*X. catharticum* Kunth	Quito (Ecuador)		*A.J.A. Bonpland*	*3006*	type	−0.218737078	−78.51218914	2830	2	*X. catharticum* Kunth

(Continues)

Appendix 1. Continued.

Lab ID	Herbarium voucher	Accession	Identification (Widder, 1923, 1964)	Collection place	Collection date	Collector(s)	Coll. Number	Notes	Latitude	Longitude	Elevation (m)	Prec.	New species assignment
X333	P02537131	ERS 18958756	*X. catharticum* Kunth	Prov. de Pasto (Colombia)	1851–1857	*J. Triana*	*1334*		1.205491952	−77.26598572	2600	2	*X. catharticum* Kunth
X334	P02537124	ERS 18958750	*X. catharticum* Kunth	Chile	03/1896	*Philippi*	*s.n.*					5	*X. catharticum* Kunth
X335	P02537136	ERS 18958757	*X. catharticum* Kunth	Chillo; Quito (Ecuador)		*A.J.A. Bonpland*	*3006*	type	−0.26640951	−78.48328473	2650	2	*X. catharticum* Kunth
X342	P04115576	ERS 18958767	*X. spinosum* L.	Gràcia; Barcelona (Spain)	10/1886	*D. Luizet*	*s.n.*		41.40977285	2.154370591	95	2	*X. spinosum* L.
X345	P04380863	ERS 18958749	*X. argenteum* Widder	Dunkerque (France)	26/10/1930	*M. Bouly de Lesdain*	*s.n.*		51.04494694	2.351472762	5	1	*X. argenteum* Widder
X346	P04114960	ERS 18958760	*X. spinosum* var. *inerme* Bel	Saint-Sulpice la Pointe; Toulouse (France)	09/1897	*M. Bel.*	*4115*	locus classicus	43.77329957	1.685363603	115	1	*X. spinosum* L.
X347	P04108168	ERS 18958761	*X. spinosum* var. *inerme* Bel	Bédarieux; Hérault (France)	16/08/1902	*De Rey, Paillade*	*s.n.*	cited in Thellung (1912)	43.61283074	3.155534693	200	1	*X. spinosum* L.
X349	BC135959	ERS 18958777	*X. spinosum* L.	La Bordeta; Barcelona (Spain)	23/09/1955	*O. de Bolós, F. Masclans*	*s.n.*		41.37012943	2.131641444	15	1	*X. spinosum* L.
X350	BC39008	ERS 18958768	*X. canescens* (Costa) Widder	Lit du Besòs; Barcelona; (Spain)	16/08/1921	*F. Sennen*	*4200*	locus classicus	41.42507641	2.221785157	10	1	*X. spinosum* L.
X183	B100618346	ERS 18958782	*X. strumarium* L.	Strasbourg (France)	27/08/1868		*s.n.*	outgroup					