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Recent Long-Distance-Dispersal Explains the Range Disjunction of the Old-Word Cocklebures (*Xanthium strumarium*)

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ABSTRACT

Aim: In the present study, we focused in addressing questions concerning the biogeographic history of *Xanthium strumarium*, an Old World native species whose close relatives are generally all native to the American continent.

Location: The species distribution covers the Eurasian continent and some African regions and close islands.

Methods: We employed herbarium material and target enrichment (herbariomics) sequence data of over 700 single copy loci to estimate the divergence times of the species. Ancestral range reconstruction was employed to test different hypotheses on the events that determined the arrival, differentiation and dispersal of the ancestor of *X. strumarium* in the Old World.

Results: The crown age of *X. strumarium* was estimated to be ~156.58 ka. In the phylogenomic analysis, the individuals from the different geographic areas grouped mostly congruently based on their collection origin, the earliest diverging clade comprising samples from India, and then progressively clades including samples from Asia, Europe and Africa. The same pattern was observed in the biogeographic analysis, with a movement of the ancestral ranges going from east to west. The ancestral range of the species was inferred to be the Indian subcontinent.

Main Conclusions: Both the use of herbarium specimens as old as 240 years and the use of modern sequencing techniques clarified the phylogenetic relationships, divergence time and biogeography of *Xanthium strumarium*. Most probably, the ancestor of the species reached the Old World by way of a trans-Pacific long-distance dispersal from the Americas to Southeastern Asia, followed by westward colonisation of the Old World.

1 | Introduction

The existence of morphologically similar plants in different continents has fascinated botanists and biogeographers since the early 18th century. Probably, this phenomenon was first noticed by the French Jesuit Joseph-François Lafitau, who found American ginseng near Montreal in 1716, a genus otherwise known from eastern Asia (Wen 1999). Since then, intercontinental disjunctions in the natural geographic ranges of many plant groups have fascinated scientists like Linnaeus (see Graham 1972), Darwin (see Wen et al. 2010), and Wallace (1880). Most intriguing to them were the possible explanations for such distributional gaps (Thorne 1972). The first who systematically analysed such disjunctions was Asa Gray, who made floristic comparisons among eastern North America, western North America, Japan, and Europe (Gray 1840, 1846, 1856, 1857), finding that the eastern rather than western North America showed higher floristic affinity with eastern

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Asia (the eastern Asian-eastern North American disjunction, also known as the ASA GRAY disjunction; Krutzsch 1989). Thorne (1972) provided a systematic classification of the major intercontinental disjunction patterns, recognising 16 main categories. In the last decades, many attempts have been made to understand if vicariance or long-distance dispersal (LDD) events could explain these main intercontinental disjunctions (e.g., Xiang and Soltis 2001; Milne 2006; Villaverde et al. 2017; Harris et al. 2018 for reviews).

Intercontinental disjunctions between eastern Asia and North America have been studied extensively in the last two decades (see reviews by Wen 1999, 2001; Donoghue and Smith 2004; Wen et al. 2010). Most studies to date have focused on temperate plant groups and have highlighted the importance of the Bering land bridge for floristic exchanges between the two continents (Li and Wen 2013). Less effort has been devoted to tropical or subtropical regions (but see, e.g., Wang et al. 2004; Wen et al. 2008; Li and Wen 2013). The amphi-Pacific Tropical disjunction was one of the major intercontinental disjunction classes recognised by Thorne (1972). Plant taxa exhibiting such distribution occur in both the Neotropics and the subtropical and tropical regions on the western borders of the Pacific Ocean. Eighty-nine genera of flowering plants that exhibit the amphi-Pacific tropical distribution were enumerated by Thorne (1972), and van Steenis (1962) presented a list of "amphi-transpacific" genera organised in latitudinal groups. How these taxa achieved their present amphi-Pacific disjunct ranges has long been speculated (van Steenis 1962; Raven and Axelrod 1974).

The cocklebur, *Xanthium* L. (Asteraceae), is a genus of annual herbaceous plants of the Heliantheae tribe. It is a peculiar genus among the Asteraceae, known for being wind-pollinated and for having burs, peculiar inflorescences enclosing two female flowers and completely covered with spines and two beaks (Figure 1). These burs have been shown to have a clear dispersal function, being able to adhere to animal fur and/or float in water for several days and so being spread for hundreds of kilometres (Liddle and Elgar 1984). *Xanthium* is a cosmopolitan genus with various taxa dispersed outside their original distribution range by human activity, becoming in some cases widespread crop competitors (Bloomberg et al. 1982; Byrd and Coble 1991; Yuan et al. 2018; Jehlík et al. 2019).

Historically, there has been some confusion among the taxa described in the genus, which has resulted in frequent misidentification to the present day, particularly in the case of *X.* sect. *Xanthium* (Tomasello 2018; Müller-Kiefer and Tomasello 2022). In their respective works, Widder (1923) and Millspaugh and Sherff (1919) recognised over 20 species each, while Löve and Dansereau (1959) divided the genus into only three species, grouping the whole *X.* sect. *Xanthium* into a single species, *Xanthium strumarium* L. A recent study (Tomasello 2018) demonstrated that there is a clear genetic distinction between *X. strumarium*, and the other species complexes of the section (i.e., *X. orientale* L. and *X. chinense* Mill.). The focus of the present study is *X. strumarium* as defined in Tomasello (2018), which differs from the often-misidentified *X. orientale* (native to America and invasive in the Old World).

1.1 | *Xanthium strumarium*

It is a thermophilic complex that is commonly found in sandy habitats, such as ditches and riverbanks (Arcangeli 1882; Widder 1923). It is mainly distributed throughout Eurasia and Africa, and known to be native to these regions, with records in the literature dating back to the Dioscorides' *De Materia Medica* (First century BC) and fossil records from the last interglacial period (Löve 1975; Chauhan 1991). However, the species has suffered a progressive decline in the last two centuries (parallel to the spreading of the non-native *X. orientale*) and is nowadays difficult to find in nature in a great part of its native range (Müller-Kiefer and Tomasello 2022). Differently from its congenerics, the species did not manage to spread outside its native distribution range. The only putative stable populations found outside the Old World were in southern Brazil, initially described by Vellozo (1881) as a different species (*X. brasilicum* Vell.) and presumably originated from burs arrived from the Mediterranean basin (Widder 1923).

Xanthium strumarium distribution range is unique when compared to the other species of the genus, which are all native to

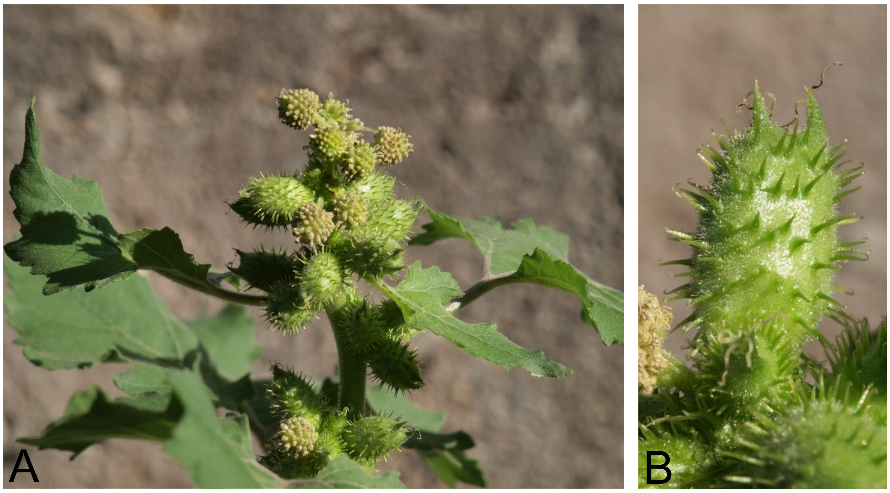

FIGURE 1 | (A) Flowering branch of a *Xanthium strumarium* individual cultivated at the Old Botanical Garden of the University of Göttingen and showing male and female inflorescences. (B) Close up of a bur (female capitulum).

America (Tomasello 2018). Even subtribe Ambrosiinae Less. and tribe Heliantheae Cass. to which *Xanthium* belongs are well-known to have an American origin and distribution centre (Baldwin 2009). The subtribe, for example, is composed by about thirteen genera and over 150 species (Tomasello et al. 2019), and the only other taxon non-native to America apart from *X. strumarium* is *Ambrosia maritima* L. (Martin et al. 2018). *Ambrosia maritima* is supposed to be native to the Mediterranean basin and the coast of central-western Africa, although its distinctiveness from other taxa of the genus with an American origin is questionable based on phylogenetic data (Martin et al. 2018). At the tribal level, only a few examples are known of genera including species with native ranges found outside America (Baldwin 2009), all of them from subtribe Ecliptinae Less. (*Blainvillea* Cass., *Eclipta* L., *Indocypraea* Orchard, *Exomiocarpon* Lawalrée, *Fenixia* Merr., *Hoffmanniella* Schltr. ex Lawalrée, *Pentalepis* F.Muell.).

The very distinctive distribution of *X. strumarium* in the Old World may be the results of a relatively recent long-distance dispersal event, as hypothesized also for *Ambrosia maritima* (Martin et al. 2018). This could be explained by means of the dispersal strategy of *Xanthium* burs, that include zoochory (as above mentioned), but also hydrochory and thalassochory. Takakura and Fujii (2010) demonstrated that burs of *Xanthium* can be soaked in salty water for weeks and still be viable, a feature that could have favoured the arrival of *Xanthium* to the Old World via sea. Also, mammal and bird migration routes from America to Eurasia (Backensto et al. 2016; Yong et al. 2021) might have facilitated the spread of *Xanthium* diaspores in Eurasia.

On the other hand, it is widely acknowledged that from the Miocene to the present, numerous alternations of cold and warm periods have impacted the palaeogeography of continents, leading to the cyclical connections and separations of landmasses through ice sheet formation and sea-level changes (Brikiatis 2014; Hosner et al. 2015). These shifts resulted in the formation of different land bridges that facilitated animal and plant dispersal (Hosner et al. 2015; Maguilla et al. 2018). When concerning connections between America and the Old World, a few important events need to be mentioned. The De Geer Land Bridge appeared during the Late Cretaceous and Early Palaeocene in two time windows around 69 million years ago (Ma) and 65.5 Ma, connecting North America with Eurasia through Greenland and Fennoscandia (Brikiatis 2014). The Thulean Route, present from the Early Palaeocene until the Early Eocene, also connected North America with Eurasia via the British Isles and Greenland (Brikiatis 2014). The Japan Land Bridges appeared multiple times due to sea level changes, connecting the Japanese archipelago to modern Russia during the Pleistocene (Millien-Parra and Jaeger 1999). Finally, The Beringia Land Bridge, perhaps the most well-known, connected North America to Eurasia through Russia, Alaska, and Canada and appeared multiple times during the Palaeocene and most recently during the Last Glacial Maximum circa 30–20 thousand years ago (Brikiatis 2014; Hoffecker et al. 2016).

With the present study, we want to test if the arrival of the diaspores of the ancestor of *X. strumarium* in the new continent was caused by a long-distance dispersal event or if alternatively, it was the result of a progressive dispersal through a land bridge (e.g., the Beringian Bridge). Additionally, we aim at addressing a few main questions concerning the biogeographic history of *Xanthium strumarium*. (a) What is the temporal framework in which the ancestor of the species arrived in the Old World? (b) What have been the possible pathways followed by the species to spread all-over the Old World? To answer these questions, we used herbarium specimens and applied target enrichment of nuclear genes to infer the phylogeography of the species. We used Bayesian methods to estimate its divergence time and ancestral range reconstruction to test different possible scenarios of the pathways followed by the genus *Xanthium* to reach and colonise the Old World.

2 | Materials and Methods

2.1 | Plant Material

To ascertain the biogeographic history of *X. strumarium*, we have sampled as much as possible trying to cover the whole distribution range of the species for a total of 53 samples (Table 1). In order to estimate the divergence time of the complex, we included 15 additional samples from other taxa of the genus *Xanthium*. A total of 68 samples were analysed, two of which collected, and silica-gel dried from plants cultivated at the Botanical Garden of the University of Göttingen (Germany; Figure 1), all the others retrieved from herbarium collections. The sampled herbarium specimens were collected between 1782 and 2018 and acquired from various herbaria: B, BA, BOLO, CAT, FI, G, GH, GOET, M, P, PR, TEX, and WU (acronyms following Thiers 2024).

2.2 | DNA Extraction

Genomic DNA was extracted from 5 to 10 mg of dried leaf material. The fragments were inserted into a 2 mL Eppendorf tube with a sterilised steel ball and pulverised with a TissueLyser II (Qiagen, Hilden, Germany). Two distinct extraction methodologies were employed to maximise the quantity and quality of extracted DNA. For samples exceeding 100 years of age, an ancient DNA (aDNA) extraction method was utilised (PTB-DTT, Dabney et al. 2013; Gutaker et al. 2017). For the more recent samples, the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was employed, in accordance with the manufacturer's instructions and the modifications detailed in Marinček et al. (2022).

The extractions were conducted under a laminar flow bench, and the equipment sterilised with DNA Away (Thermo Fisher Scientific, Waltham, USA) and UVaClean UV Pipette Carousel (MTC Bio, Sayreville, USA). All precautions to prevent contamination were employed. The extracts were then run in a 2% agarose gel to roughly estimate fragment lengths. Concentrations were measured using a Qubit 3 Fluorometer (Thermo Fisher Scientific) and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

2.3 | Library Preparation

Two distinct kits were employed for library preparation in accordance with the manufacturer's instructions. The first was

TABLE 1 | List of samples used in the ancestral area reconstruction and phylogenetic analyses, along with information on the herbarium voucher, ENA accession numbers, identification at species level, collection place and date. Notes indicate outgroups and the analysis for which samples were employed, whether Ancestral Range Reconstruction (ARR) or Dating Analysis (DA). Biogeographic areas to which the samples were assigned in the BioGeoBEARS analyses are also indicated with Latin capital letters as also in Figure 2: American (A), Indian (B), Sino-Japanese (C), Malaysian (D), Indochinese (E), Madagascan (F), Siberian (G), African (H), Mediterranean (I) and European (J).

Lab ID	Herbarium voucher	Accession number	Identification	Collection place	Collection date	Notes	Biogeographic area
X8	M-0158773	ERS20849905	*Xanthium strumarium* L.	Nara (Japan)	16/09/1996	ARR	C
X10	M-0158774	ERS20849859	*Xanthium strumarium* L.	Zeilitzheim (Germany)	15/08/1993	ARR	J
X114	GOET061878	ERS20849860	*Xanthium strumarium* L.	Cult. Göttingen (Germany)	13/11/2020	ARR	J
X134	GOET043118	ERS20849861	*Xanthium strumarium* L.	Hannover (Germany)	1821	ARR	J
X147	GOET042881	ERS20849862	*Xanthium strumarium* L.	Jakarta (Indonesia)	1879	ARR	D
X149	GOET043138	ERS20849863	*Xanthium strumarium* L.	Vipiteno (Italy)	09/1883	ARR	J
X150	GOET035727	ERS20849864	*Xanthium strumarium* L.	Germany	1818	ARR	J
X151	GOET035728	ERS20849865	*Xanthium strumarium* L.	India	1818	ARR	B
X160	B101067785	ERS20849866	*Xanthium strumarium* L.	Azaza (Sudan)	14/05/1951	ARR	H
X177	B100754514	ERS20849867	*Xanthium strumarium* L.	Cologne (Germany)	09/09/1956	ARR/DA	J
X178	B100766855	ERS20849868	*Xanthium strumarium* L.	Caccamo (Italy)	08/1888	ARR	I
X179	B101096387	ERS20849869	*Xanthium strumarium* L.	Skopje (North Mazedonia)	18/08/1917	ARR	J
X180	B100549302	ERS20849870	*Xanthium strumarium* L.	Baumgarten (Austria)	05/08/2008	ARR	J
X181	B100552436	ERS20849871	*Xanthium strumarium* L.	Tulln an der Donau (Austria)	25/07/2007	ARR	J
X182	B100618287	ERS20849872	*Xanthium strumarium* L.	Celje (Slovenia)	08/1908	ARR	J
X183	B100618346	ERS20849873	*Xanthium strumarium* L.	Strasburg (France)	27/08/1868	ARR	J
X184	B100667848	ERS20849874	*Xanthium strumarium* L.	Qingdao (China)	1899–1903	ARR	C
X185	B100356226	ERS20849875	*Xanthium strumarium* L.	Geumgang Mt. (North Korea)	28/09/1997	ARR/DA	C
X188	B101122207	ERS20849876	*Xanthium strumarium* L.	Karaj (Iran)	11/08/1933	ARR/DA	J
X198	PR901932	ERS20849877	*Xanthium strumarium*	Hořice (Czech Republic)	26/08/2018	ARR/DA	J
X199	PR564904	ERS20849857	*Xanthium strumarium* L.	Sturovo (Slovakia)	24/08/1956	ARR	J
X200	PR101903	ERS20849879	*Xanthium strumarium* L.	Koskovce (Slovakia)	08/1898	ARR	J
X203	PR969921	ERS20849880	*Xanthium strumarium* L.	Sharon Plain (Israel)	05/09/1951	ARR	I
X206	PR969832	ERS20849881	*Xanthium strumarium* L.	Thuringia (Germany)	?	ARR	J
X211	PR969988	ERS20849882	*Xanthium strumarium* L.	Java (Indonesia)	1842–1844	ARR/DA	D
X215	PR969989	ERS20849883	*Xanthium strumarium* L.	East India	1837–1838	ARR	B

(Continues)

TABLE 1 | (Continued)

Lab ID	Herbarium voucher	Accession number	Identification	Collection place	Collection date	Notes	Biogeographic area
X222	PR311228	ERS20849884	*Xanthium strumarium* L.	Kunashir (Russia)	22/09/1968	ARR	C
X226	sn BOLO	ERS20849885	*Xanthium strumarium* L.	Bolzano (Italy)	1842	ARR	J
X230	FI064241	ERS20849886	*Xanthium strumarium* L.	Algiers (Algeria)	08/1869	ARR	I
X232	FI064242	ERS20849887	*Xanthium strumarium* L.	Hong Kong	1858	ARR	E
X233	FI064240	ERS20849888	*Xanthium strumarium* L.	El Kantara (Algeria)	18/09/1902	ARR	I
X235	FI064236	ERS20849889	*Xanthium strumarium* L.	Java (Indonesia)	1842–1844	ARR	D
X237	CAT020884	ERS20849890	*Xanthium strumarium* L.	Mazzara del Vallo (Italy)	09/06/1973	ARR	I
X322	GOET002242	ERS20849891	*Xanthium strumarium* L.	Nagasaki (Japan)	1862	ARR	C
X323	GOET043112	ERS20849892	*Xanthium strumarium* L.	Lower Saxony (Germany)	22/10/1998	ARR	J
X325	P00069185	ERS20849893	*Xanthium strumarium* L.	Ethiopia	1844	ARR	H
X326	P02823998	ERS20849894	*Xanthium strumarium* L.	Khartum (Sudan)	04/03/1840	ARR	H
X327	P02511185	ERS20849895	*Xanthium strumarium* L.	Yunnan (China)	11/08/1908	ARR	C
X328	P02511187	ERS20849896	*Xanthium strumarium* L.	Vĩnh Long (Laos)	20/07/1929	ARR	E
X329	P02511177	ERS20849897	*Xanthium strumarium* L.	Puducherry (India)	05/1837	ARR/DA	B
X330	P02511343	ERS20849898	*Xanthium strumarium* L.	Taiwan (Republic of China)	09/06/1933	ARR	E
X331	P02537065	ERS20849899	*Xanthium strumarium* L.	Brasil	08/1967	ARR	I
X332	P02537059	ERS20849900	*Xanthium strumarium* L.	Cachambu (Brasil)	26/01/1886	ARR	I
X337	P02398528	ERS20849901	*Xanthium strumarium* L.	Mauritius (Republic of Mauritius)	?	ARR	F
X353	G00305683	ERS20849902	*Xanthium strumarium* L.	Öskemen (Kazakistan)	12/07/1782	ARR	G
X354	GOET042883	ERS20849903	*Xanthium strumarium* L.	Sri Lanka	1863	ARR/DA	B
X355	GOET042616	ERS20849904	*Xanthium strumarium* L.	Sabra (Ethiopia)	1871	ARR	H
X187	B100528156	ERS20849855	*Xanthium chinense* Mill.	Babil (Iraq)	30/11/1973	ARR outgroup	A
X197	B101067808	ERS20849856	*Xanthium chinense* Mill.	St. Thomas (Virgin Islands; USA)	1827–1840	ARR outgroup/DA	A
X207	PR86412	ERS18958775	*Xanthium spinosum* L.	Herne (Germany)	10/1931	ARR outgroup/DA	A

(Continues)

TABLE 1 | (Continued)

Lab ID	Herbarium voucher	Accession number	Identification	Collection place	Collection date	Notes	Biogeographic area
X210	PR070007	ERS20849857	*Xanthium chinense* Mill.	Rahway (New Jersey; USA)	?	ARR outgroup/ DA	A
X240	WU0071716	ERS18958748	*X. argenteum* Widder	Ñuble (Chile)	?	ARR outgroup	A
X305	TEX00407690	ERS20849858	*Xanthium orientale* L.	Greenville (Texas; USA)	21/09/1995	ARR outgroup/ DA	A
X115	GOET061879	ERS18958770	*Xanthium spinosum* L.	Cult. Göttingen (Germany).	13/11/2020		DA
X124	GOET043085	ERS18958764	*Xanthium spinosum* L.	Hannover (Germany)	14/08/1924		DA
X141	BA62708	ERS18958774	*Xanthium spinosum* L.	Avellaneda (Argentina)	25/03/1913		DA
X164	B100349123	ERS18958752	*X. catharticum* Kunth	San Pedro de Atacama (Chile)	11/03/2001		DA
X173	B101094951	ERS20849907	*Xanthium orientale* L.	Agen (France)	September ?		DA
X175	B100549529	ERS20849906	*Xanthium chinense* Mill.	East Peoria (Illinois; USA)	15/09/1945		DA
X190	B100535623	ERS20849908	*Xanthium chinense* Mill.	Lesperance (Missouri; USA)	05/10/1969		DA
X242	BA31/1400	ERS20849909	*Xanthium orientale* L.	Ciudad de Concordia (Argentina)	31/01/1931		DA
X251	BA60567	ERS18958744	*Xanthium ambrosioides* Hook. & Arn.	Igarzabal (Argentina)	17/04/1963		DA
X253	BA46475	ERS18958746	*Xanthium ambrosioides* Hook. & Arn.	Agua de la Chilena (Argentina)	27/01/1942		DA
X261	GH00014158	ERS20849910	*Xanthium orientale* L.	Vermont (USA)	23/09/1899		DA
X292	TEX00561321	ERS20849911	*Xanthium orientale* L.	Sierra Nevada (California; USA)	28/09/2008		DA
X309	TEX00456487	ERS20849912	*Xanthium chinense* Mill.	Burkeveille (Texas; USA)	18/09/2010		DA
X333	P02537131	ERS18958756	*Xanthium catharticum* Kunth	Prov. de Pasto (Colombia)	1851–1857		DA
X345	P04380863	ERS18958749	*X. argenteum* Widder	Dunkerque (France)	26/10/1930		DA

the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, USA), which was utilised for herbarium samples that did not require enzymatic fragmentation. The second was the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England BioLabs), which was employed for more recent specimens. For old herbarium samples, 1.5 volumes of HighPrep beads (MagBio Genomics, Gaithersburg, USA) was utilised instead of the default 0.8 volumes during the purification step of adapter-ligation products (see also Marinček et al. 2022).

Following adapter-ligation, a PCR amplification was conducted for 14 cycles and samples were barcoded using sample-specific dual indices (NEBNext Multiplex Oligos for Illumina (Index Primers Set 1), NEBNext Multiplex Oligos for Illumina (Index Primers Set 2), NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs); New England BioLabs), and subsequently purified with the HighPrep magnetic beads.

Target enrichment was performed using the myBaits COS Compositae 1Kv1 kit (Mandel et al. 2014; Daicel Arbor Biosciences, Ann Arbor, USA). Samples were pooled in equal concentrations in a group of six, dried in a Concentrator Plus (Eppendorf, Hamburg, Germany) and rehydrated in 7 μL of distilled water. This solution was subjected to the hybridisation incubation, which was performed at 65°C for 20 h. Finally, the hybridization products were PCR amplified using P7 and P5 Illumina library primers and the 2X KAPA HiFi HotStart Ready Mix (Roche, Basel, Switzerland) and purified with the HighPrep magnetic beads.

Concentrations were measured using the Qubit 3, and quality control was performed on a QIAxcel (Qiagen) using the DNA High Resolution Kit 1200, the QX Size Marker 50 bp-800 bp v2.0 and the QX DNA Alignment Marker 15 bp-5 kb. When present, adapter dimer peaks (approximately 125 bp) were removed using the BluePippin (Sage Science, Beverly, USA) and 2% cassettes with the 2% DF Marker V2.

Samples were then pooled equimolarly and sequenced on an Illumina NovaSeq 6000 system (Illumina, San Diego, California, USA) using an SP P300 Xp (2 × 150bp kit). Some samples were sequenced on different runs of an Illumina MiSeq system using either a 2 × 250bp (500 circles) or 2 × 150 bp (300 circles) v2 kit. Sequencing was performed at the NGS Integrative Genomics (NIG) Core Unit at the University of Göttingen.

2.4 | Raw Reads Processing

Raw reads were initially processed in HybPhyloMaker v.1.8.2 (Fér and Schmickl 2018). The trimming of the adapters and removal of low-quality reads were performed using Trimmomatic v.0.33 (Bolger et al. 2014) whereas duplicate reads were eliminated using FastUniq v.1.1 (Xu et al. 2012). Filtered reads were mapped using BWA v.0.7.16a (Li and Durbin 2009) with standard settings. As a reference sequence we used the target loci of *Helianthus annuus* L., which were concatenated and separated by batches of 800 Ns. Consensus sequences were produced with ConsensusFixer v.0.4 (Töpfer 2018) with "plurality" set to 0.3 and "mincov" to 5.

The mapped reads were aligned with the target exons using BLAT v.35 (Kent 2002) to generate PSLX files. Subsequently, locus-wise *fasta files were generated and aligned using MAFFT v.7.305b (Katoh and Standley 2013) with default settings. Missing data filtering was conducted first removing sequences with more than 40% of missing data ("missingpercent" in HybPhyloMaker), then removing alignments including less than 75% of samples ("speciespresence" in HybPhyloMaker). Finally, 732 regions were retained (Table S1a).

2.5 | *Xanthium strumarium* Divergence Time

We used the concatenated dataset with the 732 regions obtained in the previous step and 26 samples from all recognised species of the genus (Table 1). Input files for Beast2 (Bouckaert et al. 2019) were prepared using BEAUti v.2.7.6 (Bouckaert et al. 2019) and the "beast" template. We used the GTR + G as sequence substitution model, letting Beast2 optimise model parameters. The "Random Local Clock" was selected as clock model. In order to obtain absolute divergence times, we followed the approach used in Tomasello et al. (2020) and gave both an informative prior on the clock rate and a calibration point. Accordingly, we gave the "clockrates" a uniform distribution (min: $5.0e^{-5}$, max: $5.0e^{-7}$) with an initial value of $5.0e^{-6}$. Assuming a standard substitution rate of $5e^{-9}$ in plants (Wolfe et al. 1989), and since *Xanthium* plants are annuals, the clock rate will result in $5e^{-6}$ mutations per site per thousand years (± an order of magnitude). As for the calibration point, we based it on the oldest *Xanthium* fossil, consisting of bur fragments found in Indiana (USA) and dating back to the Upper Hemphillian/Blancan North American stages (Farlow et al. 2001). Therefore, we applied to the root of the tree a lognormal prior distribution with mean 3.0 and standard deviation 2.9 (95% highest prior density ranging between 3000 and 8910 thousand years ago (ka)).

We ran two analyses for 100,000,000 generations, sampling every 10,000 iterations. Convergence between different analyses and effective sample size (ESS) were checked in Tracer v.1.7 (Rambaut et al. 2018). The tree files from the two independent runs were combined using LogCombiner v.2.7 (Bouckaert et al. 2019). Finally, a maximum clade credibility tree was calculated in TreeAnnotator v.2.6 (Bouckaert et al. 2019), applying 10% burn-in, a posterior probability limit of 0.5, and "Mean Heights" for node heights. Additional sets of analyses were performed using only the clock rates prior or the fossil calibration. This was done to assess the effect of using both calibration and clock-rate priors in the same analysis.

2.6 | De-Novo Assembly and Generation of a Paralogs-Aware Reference

Some of the 732 loci retained from HybPhyloMaker showed signal of paralogy (shared polymorphic sites across all taxa). *Xanthium*, together with members of subtribe Ambrosiinae Less. (i.e., *Ambrosia* L., *Iva* L., *Parhenium* L.), has diploid chromosome number equal to 36, which is double the amount present in the related subtribes, and may suggest a (paleo-)tetraploid origin of the whole subtribe. The (paleo-)tetraploid nature of the genus was also corroborated based on isozyme data (Dinelli et al. 2003). Convinced that the presence of paralog signal would have more heavily affected paralog filtering in the recently diverged *X. strumarium*, and that not enough loci would have remained if paralogous loci were simply excluded (see e.g., Ufimov et al. 2022), we decided to process further the dataset and follow another strategy. Rather than excluding paralog loci, an attempt was made to separate orthologous sequences and include them as separate loci in the analyses.

Therefore, we re-assembled the target regions using the software CAPTUS (Ortiz et al. 2023). In contrast to HybPhyloMaker, CAPTUS implements both *de-novo* and reference-based assembly and provide a better paralog detection strategy. It gives the possibility to retrieve different contigs hits per locus (e.g., different paralogues). The raw reads were cleaned from low-quality reads and adapters using the "captus_assembly clean" step with standard settings. The resulting quality-trimmed reads were de novo assembled with MEGAHIT v.1.2.9 (Li et al. 2015) using the command "captus_assembly assemble" with standard settings. Contigs from target regions were extracted with "captus_assembly extract" and using the *H. annuus* sequences from the myBaits COS Compositae 1Kv1 kit as reference. The "-max_paralogs" flag was disabled, to retrieve all secondary hits for each locus. Therefore, we selected the sample with the highest number of retrieved loci (i.e., X114), and extracted all hits (paralogs) for all loci. Those sequences (2150 in total) were used as references in the subsequent analyses.

2.7 | Phylogeny of the *Xanthium strumarium* Species Complex

A total of 48 *X. strumarium* samples and six outgroups (Table 1) were employed. Data processing and assembly was done in HybPhyloMaker using the aforementioned 2150 sequences as reference. Read mapping was achieved using BWA v0.7.16a (Li and Durbin 2009), with the addition of a mismatch penalty of 8 to make reads more stringently map to the reference. Consensus sequences were generated in ConsensusFixer as it was done for the dating. BLAT was utilised for the alignment of the mapped reads with the targeted exons to generate PSLX files. The minimum sequence identity between the probe and the sample (termed 'minident') was set to 99. The subsequent steps were carried out as for the age estimation analyses. Further putative paralog sequences were excluded by employing the "HybPhyloMaker4a2_selectNonHet.sh" script and designating a maximum of 5 heterozygous sites per locus ("maxhet" in the HybPhyloMaker settings file). Missing data filtering was again performed as for the age estimation analyses.

A total of 744 loci (Table S1b) were retrieved, concatenated, and used to generate an ultrametric Bayesian phylogenetic tree. The analyses were conducted using the Beast2 software, employing the "Optimized Relaxed Clock", the GTR+G as substitution model, and the "Birth-Death Model" tree model. To obtain absolute divergence times, the crown age of *X. strumarium* was calibrated using the age obtained in the above analysis (i.e., it was fixed to the estimate obtained in the age estimation analyses). Two independent analyses were run for 100,000,000 generations, sampling every 10,000 iterations. Convergence and effective sample size (ESS) were checked in Tracer v.1.7. The tree files from the two independent runs were combined using LogCombiner v.2.7 (Bouckaert et al. 2019), and a maximum clade credibility tree was calculated in TreeAnnotator v.2.6 applying 10% burn-in, a posterior probability limit of 0.5, and "Mean Heights" for node heights.

2.8 | Ancestral Range Reconstruction

The BioGeoBEARS (Matzke 2018) R package was employed to infer the biogeographic history of *Xanthium strumarium*. This package is used to reconstruct the ancestral geographic distribution on phylogenies using different models and testing the best fitting one in a maximum likelihood framework. The three models employed were DEC (Dispersal-Extinction-Cladogenesis; Ree and Smith 2008), DIVA (Dispersal-Vicariance Analysis; Ronquist 1997) and BayArea (Bayesian Inference of Historical Biogeography for Discrete Areas; Landis et al. 2013). These can be combined with a founder-event ("jump") speciation model specified with the parameter "j" (Matzke 2018), or with a distance-dependent dispersal probability model specified with the parameter "x" and with a distance matrix (van Dam and Matzke 2016). A series of tests were conducted to assess the performance of the twelve models, both with and without the "j" or "x" parameter and the combination of both on the ultrametric tree obtained in Beast2. The biogeographic areas were set following Groves (2022). Samples were assigned to areas as shown in Figure 2 (see also Table 1 and Table S3). The ancestral area of the internal nodes above the crown of *X. strumarium* was fixed, using the "fixnodes" setting in the "define_BioGeoBEARS_run" command, to the Americas only, since (as mentioned above) all the close relatives of the species are confined to this continent and the oldest fossils of any *Xanthium* species were also found there. A likelihood ratio test (LRT) was employed to compare the performance of the different models (e.g., DEC+j against DEC) and to select the best-fitting model using the Akaike Information Criterion (AIC).

3 | Results

3.1 | Sequencing and Data Filtering

A total of circa 110 Gb was the amount of data obtained through sequencing. On average, 8,517,649 raw reads per sample (ranging from 639,500 to 23,676,866) were obtained, of which 12.2% were excluded on average after quality filtering. An additional 13.4% was excluded after duplicate removal, ending up with a mean of 6,456,042 reads per sample that were submitted to subsequent analysis. Further information on the raw data filtering and mapping can be found in Table S1a,b. The 714 loci selected for the dating analysis resulted in a total of 228,946 bp with 5582 parsimony-informative sites, whereas the 744 single-copy loci selected for the in group phylogenetic analyses and the ancestral area reconstruction, had a total of 181,154 bp with 591 parsimony-informative sites.

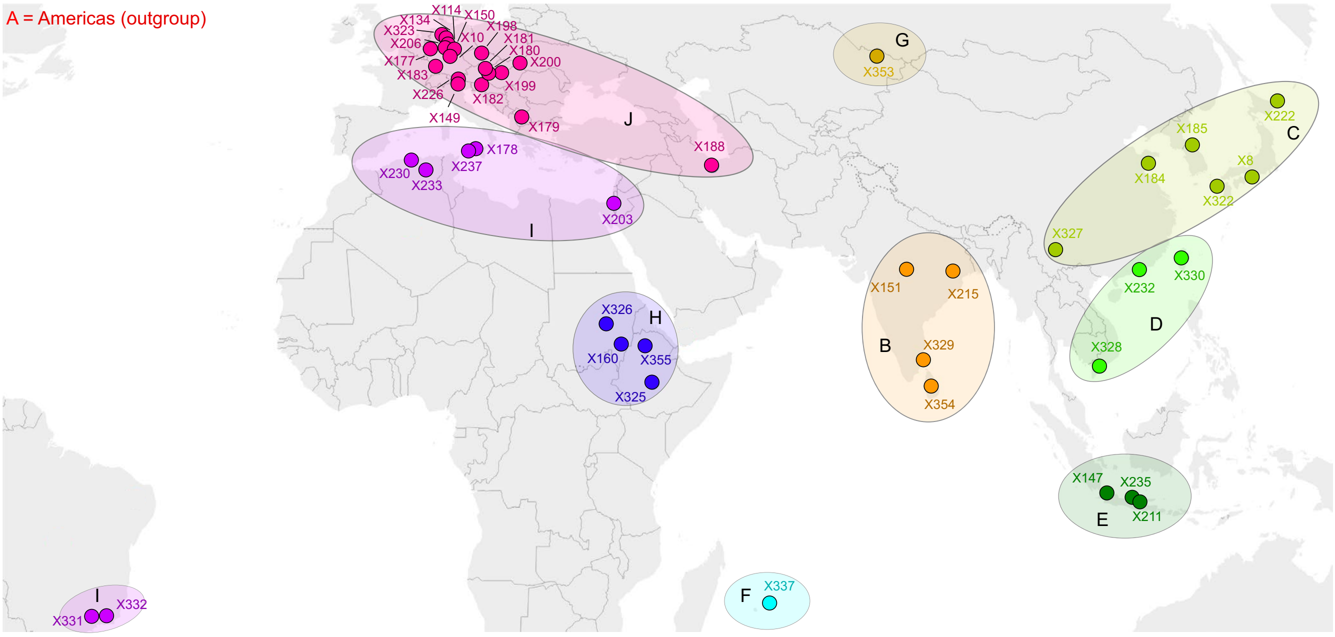

FIGURE 2 | Distribution map of the specimens of *X. strumarium* analysed (outgroups are excluded). Numbers correspond to the samples IDs, coloured ellipses and letters refer to the area defined for the ancestral range reconstruction analysis as in Table 1, Table S3 and Figure 4: American (A), Indian (B), Sino-Japanese (C), Malaysian (D), Indochinese (E), Madagascan (F), Siberian (G), African (H), Mediterranean (I) and European (J).

3.2 | Dating

The results from the Beast2 analyses were mostly congruent among the three methods (Figures 3 and S1.1–1.2). The crown age of *X. strumarium* is estimated to be ~156.58 ka (95%HPD: 133.78–187.61 ka). The results from the analyses including only the calibration point or the informative prior on the clock rates were mostly congruent to those of the main analyses. The most different was the one using only clock rates, where the crown age of *X. strumarium* was estimated to be ~135.17 ka (95%HPD = 120.31–149.18; Table 2).

3.3 | Phylogeny of *X. strumarium*

The Bayesian analysis (Figure 4A), revealed various well supported clades and showed a clear geographic pattern. The earliest diverging clade included samples from India and Sri Lanka, followed by a split in which samples from East Asia and Mauritius separated from other clades (posterior probability of 0.99). In the latter clades, sample X353, from Siberia, is found to be sister to the rest of the samples, which are divided into three other clades: a first including samples collected in the Horn of Africa (posterior probability of 1.0); a second counting specimens originated in the Mediterranean (along with the two Brazilian samples; posterior probability of 0.99) and a third including specimens from the continental Europe, and X355, collected in Ethiopia (posterior probability of 1.00).

3.4 | Ancestral Range Reconstruction

Results showed that the models including the "j" parameters were more likely in most cases. The best-fitting model resulted to be the DIVALIKE+j (Table S3; Figure 4). It is noteworthy that all the analyses generated with the jump dispersal parameter recovered the same ancestral range for *X. strumarium*, therefore converging on the same results.

These support a dispersal scenario. The ancestral area of the crown node of the species is reconstructed to be the Indian subcontinent. Then a clear dispersal pattern is depicted by the analyses, with progressive dispersal of the species to eastern Asia (from where it reached the Malay Archipelago, South-Eastern Asia, and Mauritius), then westward to Siberia and the continental Europe (as defined in Figure 2; but see also uncertainty in the ancestral area estimation for this node, Figure 4B) to then arrive to the Horn of Africa and the Mediterranean on one side, and to Europe on the other (Figure 4).

4 | Discussion

With the present contribution, we aimed to reconstruct the biogeographic history of *Xanthium strumarium* and shed light on the reasons for its current disjunct distribution range. *Xanthium strumarium* is a recently diverged species distributed throughout the entire Eurasian continent, Africa, and nearby islands. In the present study, we used herbarium material as old as 240 years and target enrichment of nuclear genes, which has proven to be extremely useful for degraded DNA and short-length fragments (e.g., Hart et al. 2016; Fortes and Paijmans et al. 2015; Brewer et al. 2019; Marinček et al. 2022; Manzo et al. 2024). The use of herbarium material has been of primary importance, as it greatly facilitated sampling throughout the whole distribution range of the species. Moreover, as mentioned above in the paper, the species has become extremely rare and/or even locally extinct in various parts of its distribution range (Müller-Kiefer and Tomasello 2022), and with field sampling alone would not have been possible to get a geographically complete and sufficiently

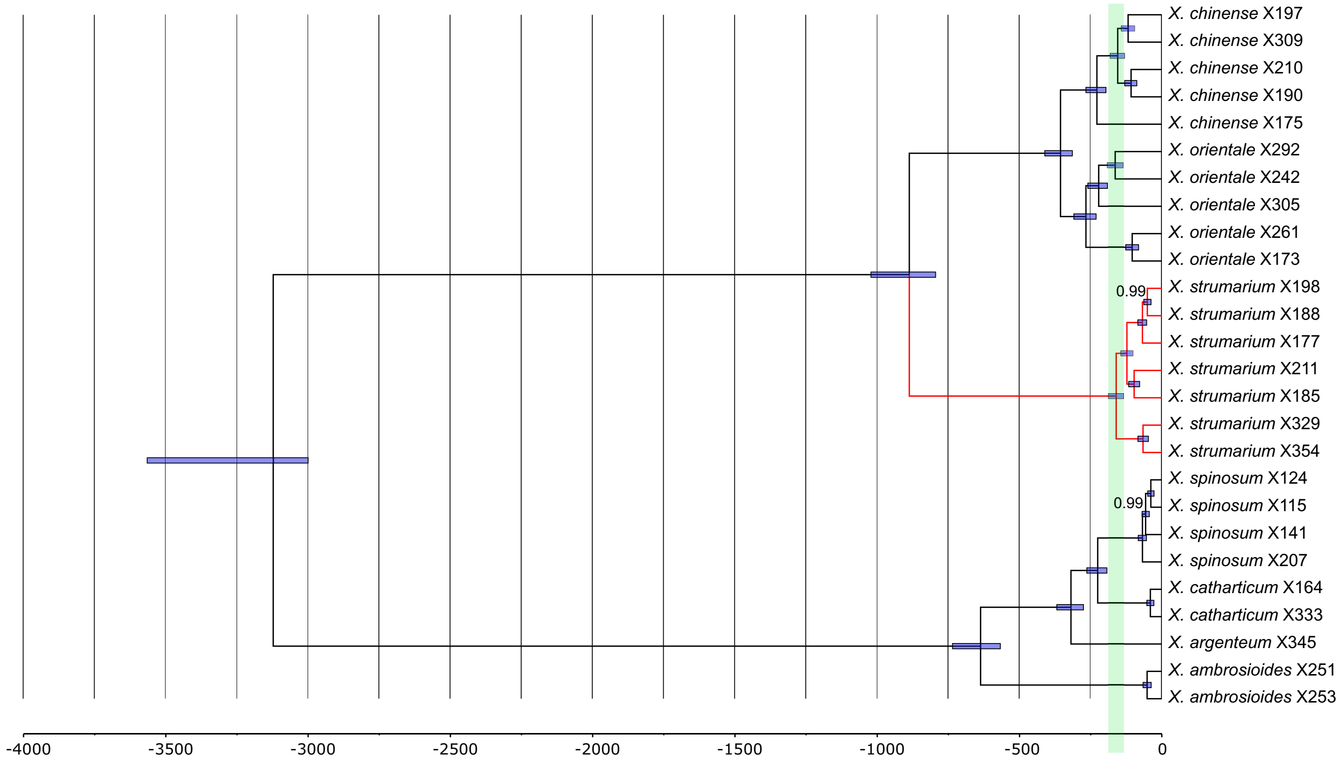

FIGURE 3 | Maximum clade credibility tree estimated in Beast2 based on the nuclear alignment including 26 individuals from the genus *Xanthium*, using both a calibrated root and an informative prior for the clock rate. Blue bars represent 95% highest posterior density (HPD) intervals of the estimated age. Only posterior probabilities lower than 1.0 are shown above branches. The focus group is indicated with red branches, and the light green region indicates the time frame for the crown age of *X. strumarium*.

TABLE 2 | Results of the Beast2 analyses when using separately calibration point and clock rate, or when using both.

	Crown age of *X.* sect. *Xanthium*	Crown age of *X. strumarium*	Root prior	Root age estimates
Calibration and clock rate	**864.07 (794.84–1022.09)**	**156.58 (133.78–187.61)**	**3000.00–8190.00**	**3013.38 (3000.00–3565.54)**
Only calibration	861.37 (654.2–1172.53)	152.03 (107.45–207.43)	3000.00–8190.00	3012.18 (3000.00–3476.35)
Only clock rate	748.25 (712.45–783.46)	135.17 (120.31–149.18)		2625.89 (2557.05–2700.99)

Note: In bold are the estimates of the main analyses presented in Figure 3. Estimates are expressed in thousands of years (ka) and number in parentheses refer to 95% HPD (high posterior density).

dense dataset. Our study highlights the importance of herbarium collections for the study of widespread taxa (Hedenäs 2019; Muñoz-Rodríguez et al. 2022) and/or extinct (or very rare) entities (Hardion et al. 2021; Zedane et al. 2016; Manzo et al. 2024).

Upon examination of the nuclear phylogenetic tree, a clear geographical pattern starting from India, through East Asia and then westwards to Europe and Africa is revealed. In particular, the Indian specimens form an early diverging clade well separated from the other sample. We included two specimens collected in Brazil (in the area nearby Rio de Janeiro) in the early 19th century (X331, X332). Those *Xanthium* growing there were already known to Vellozo, who described them as a separated species (i.e., "*Xanthium brasilicum*"; Vellozo 1881). Widder (1923) considered these plants to belong to *X.* subsect. *Orthorrhyncha* (i.e., coinciding with *X. strumarium* according to Tomasello (2018)) and being similar to the plants of the subsection growing in the Mediterranean basin (back then also known as *X. antiquorum* Wallr. *X. priscorum* Wallr., *X. anatolicum* Boiss. & Heldr., among other names). He hypothesized that those plants were likely originated from burs brought accidentally by the Portuguese from the Mediterranean basin to Southern Brazil. Alternatively, they might be related to American representatives of the genus, as for example *X. chinense* Mill., which is found in the Caribbean archipelago and may resemble in some cases the morphology of *X. strumarium* (e.g., smaller burs, almost glabrous). However, our analyses corroborate Widders's hypothesis. The two Brazilian samples are found nested in *X. strumarium* in the phylogenetic analysis, within the clade with samples from the Mediterranean basin (Figure 4).

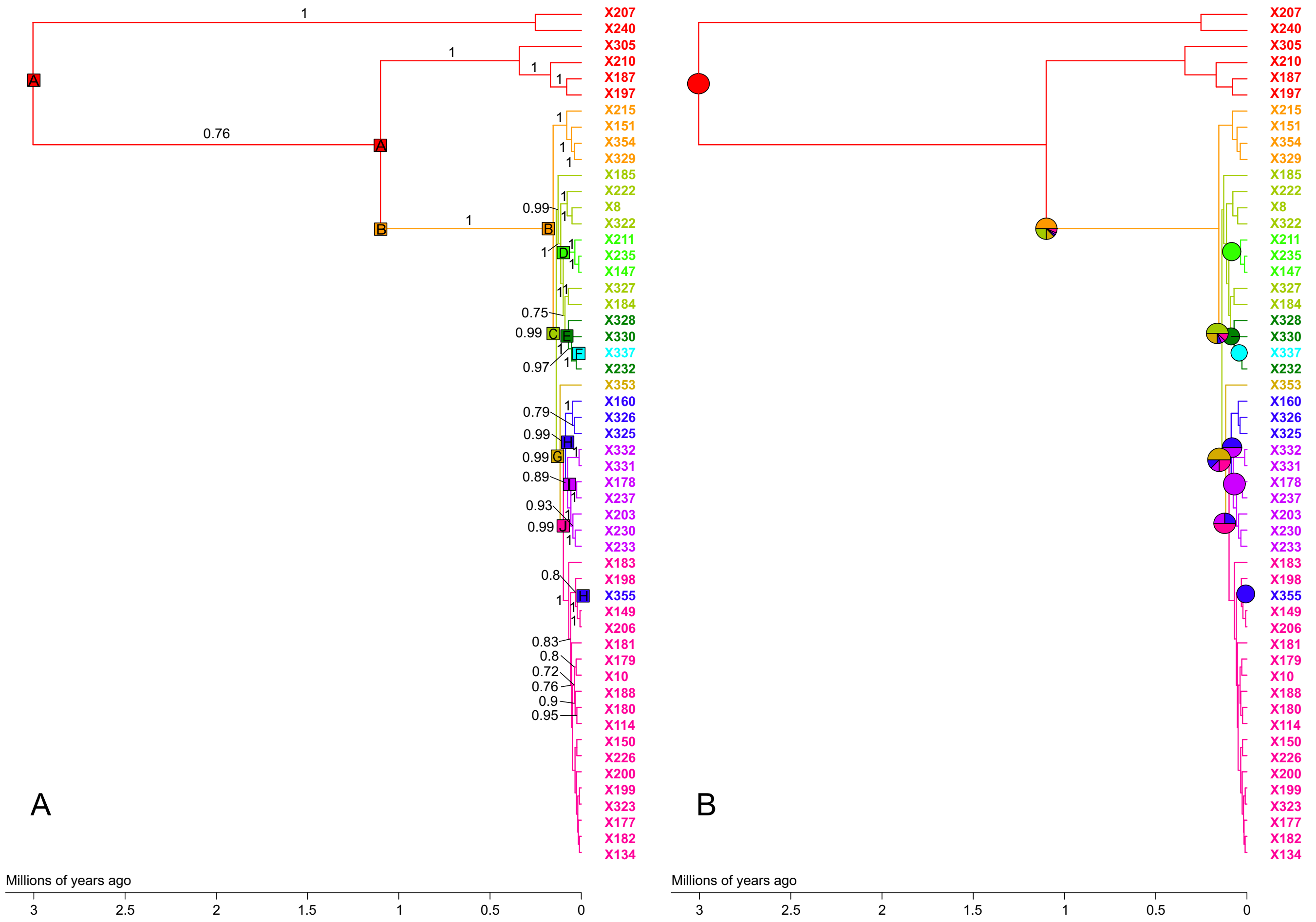

FIGURE 4 | (a) Ancestral range reconstruction of the *X. strumarium* complex as estimated in BioGeoBEARS using the DIVALIKE+j model. For visualisation convenience, the most-likely range is shown only when a change in the reconstructed area occurs. Results are shown on the ultrametric Bayesian tree estimated in Beast2, based on 744 nuclear loci and including 54 individuals from *Xanthium strumarium*, along with outgroup samples. Posterior probability support values above 0.7 are shown under or beside branches. Capital letters and colours indicate the areas defined for the analysis as in Table 1, Table S3 and Figure 2. Accordingly, red is for American, orange for Indian, acid-green for Sino-Japanese, green for Malaysian, dark green for Indochinese, light blue for Madagascan, ochre for Siberian, blue for African, violet for Mediterranean and pink for European. (b) Plot of the most-likely geographical range at each node (just before speciation) and post-split (just after speciation). Pie charts represent the probabilities of each possible geographical range for a given node. For representation convenience, only pie at every node where a change in the ancestral area occurred are shown.

4.1 | Time Frame of Dispersal Events and the Colonisation of the Old World

The complex is estimated to have initiated its diversification recently, between ~135 and ~157 ka (Table 2, Figures 3 and S1.1–1.2). This places the arrival in the Old World of the ancestor of *X. strumarium* between the end of the Penultimate Glacial Period (PGP) and the Eemian (Last Interglacial; Velichko and Isavea 1992; Van Andel and Tzedakis 1996), period that probably provided optimal climatic conditions for a thermophilic species.

Following the Eemian period, the Last Glacial Period (LGP) commenced and lasted until 11,700 years ago. This period was characterised by alternating periods of advance and retreat of the ice sheet in the northern part of Eurasia (Adams 1993). The Last Glacial Maximum (LGM) is estimated to have occurred between 26,000 and 20,000 years ago during which temperatures were 8°–10° lower than the present in both summer and winter (Frenzel, 1992a, 1992b). Based on studies on the changes in ^{14}C abundance, fossil and sedimentological information, it was inferred that during this period, ice sheets were present at higher latitudes in the Old World, and that at lower latitudes the landscape was characterised by steppe-tundra vegetation, while southern Europe and China resembled a semi-desert or dry steppe region, and south-east Asia by grassland and dry forest (Ray and Adams 2001). The conditions in the central and northern part of the continent did not align with the known ecological requirements of *X. strumarium*, suggesting that during the LGM the species may have survived in refugia southwards in temperate, subtropical, and/or tropical habitats. These habitats were likely located in India, the Indonesian archipelago, southern East Asia, and/or Africa (Ray and Adams 2001). Survival in the Indian subcontinent and in Southeastern Asia is also plausible given that the oldest fossils of *Xanthium* in the Old World have been found in India, dating back to the Pleistocene (Chauhan 1991).

The colonisation of the westernmost part of the distribution range (Europe and the Mediterranean) most likely occurred during the last phases of the LGM and afterwards with the retreat of the ice sheet in the European continent (crown age of the European samples estimated to be around 60,000–70,000 years ago; Figure 3). This is in line with the fossil record in Europe, with fossils dating back to the LGM and prehistorical times (Járai-Komlodi 1968; Florin 1969; Šoštarić et al. 2009; Chichinadze and Kvavadze 2013).

4.2 | From America Into Southeastern Asia

The phylogenetic tree in Figure 4 shows largely a pectinate topology with samples from Southeastern and Eastern Asia diverging earlier, and then progressively those from Central Asia, East Africa, the Mediterranean basin, and Europe. The ancestral area of *Xanthium strumarium* in the Old World is estimated to be the Indian subcontinent (Figure 4). Samples from the westernmost part of the distribution range (Europe, the Mediterranean basin and the Horn of Africa) are of more recent origin. It therefore seems plausible that the genus reached the Old World from the east rather than from the west. The very recent age of the species would exclude an old vicariance explanation for the disjunct distribution observed in the genus (e.g., the West Gondwanan vicariance hypothesis, or the Boreotropic hypothesis). A long-distance dispersal from North America to Eastern Asia must be invoked for the arrival of the ancestor of *X. strumarium* in the Old World.

The question is still open whether a single trans-Pacific long-distance dispersal have managed to bring the ancestor of *X. strumarium* from America to India and South-Eastern Asia, or if it was rather a “progressive” migration across the Bering land bridge. The fact that India was inferred as the ancestral range of the species may support the former hypothesis. The importance of transoceanic LDD in explaining the trans-Pacific distribution of several subtropical and thermophilic temperate taxa has been highlighted in recent studies (De Queiroz 2005; Nathan 2006; Michalak et al. 2010; Nie et al. 2013; Wu et al. 2023).

Different vectors could have allowed the transport of *Xanthium* burs across the Pacific Ocean. *Xanthium* burs can survive floating on saline water for several weeks (Takakura and Fujii 2010). Dispersal via oceanic drift is an important mechanism for species growing in proximity of seashores or riverbanks, and/or with a wide distribution range (Heyligers 2007; Miryeganeh et al. 2014; Snak et al. 2016; Liu et al. 2021; Yamazaki et al. 2023). Trans-oceanic plant dispersal events have been observed to be predominantly east to west rather than *vice versa* (Parrish 2023). An important factor playing a role here may be the North Equatorial Current (NEC), which is a westward marine current that occurs in the Pacific, Atlantic and Indian Oceans at latitudes between the 5° and 20° north. Water movements driven by such currents can be relatively fast ($1\,\mathrm{m\,s^{-1}}$; Renner 2004) and can therefore transport plant propagules westwards across the oceans in a time span of weeks to few months. Transoceanic westward dispersal driven by ocean currents has been demonstrated in other plant groups (e.g., *Canavalia* Adans., Snak et al. 2016; *Cycas* L., Liu et al. 2021). The westward transport through sea currents of *Xanthium* burs might be responsible not only for the arrival of the ancestor of *X. strumarium* in the Old World (from the Americas to India or Southeastern Asia), but also for the further westward spreading of the species. For example, a sample collected in Mauritius (X337) is more closely related to those from the Malay Archipelago rather than to those from the much closer India, or Horn of Africa. Such patterns of westward dispersal across the Indian Ocean for plants with floating fruits have been corroborated using genomic data and ocean drift modelling (Liu et al. 2021).

An alternative mechanism allowing the LDD of *Xanthium* burs across the Pacific Ocean might have been avian migrations. Birds are recognised as important vectors for LDD events in plants, especially thanks to ingested seeds that survive gut passage and are egested elsewhere (i.e., endozoochory, Yang et al. 2018; Zhu et al. 2020; see also van Leeuwen et al. 2023), or, to a lesser extent, through externally transported propagules (i.e., epizoochory, Green et al. 2023). Epizoochorous LDD of plant seeds by birds has been invoked to explain the disjunct distribution range of a number plant groups (e.g., *Senecio mohavensis* A. Gray, Coleman et al. 2003; *Plantago ovata* Forssk., Meyers and Liston 2008; *Oligomeris* Cambess., Martín-Bravo et al. 2009; tribe Omphalodea Weigend, Otero et al. 2019). However, the burs of *Xanthium* are larger than the seeds in those plant groups, and even if they do adhere effectively to mammalian fur, there is little evidence of burs being found attached on bird plumage.

The fact that India was inferred as the ancestral area of *X. strumarium* does not support the hypothesis of a dispersal from America into Asia via the Beringia corridor. The Beringia land bridge has played an important role in facilitating floral and faunal exchange between Asia and North America during the last glacial periods (Waltari et al. 2007). Among plants, some examples of taxa that are thought to have crossed the Beringian land bridge are *Heracleum lanatum* W. Bartram (Harris 2007), some species of *Saxifraga* L. (DeChaine et al. 2013), *Juniperus* L. (Gutiérrez-Larruscain et al. 2024; Mao et al. 2012), *Carex* L. (Maguilla et al. 2018) and *Aphananthe*Planch. (Yang et al. 2017). To consider the Beringia land bridge as a dispersal route for the ancestor of *X. strumarium*, we have to assume the arrival during the penultimate (or even an earlier) glacial period, followed by extinction at high latitudes and survival in India during glacial periods, and subsequent dispersal out of India during the last interglacial. India and Southeastern Asia are known to have hosted refugia for thermophilic temperate Asian plant species (Ray and Adams 2001). However, most of the cases for which a dispersal through the Beringia corridor has been proposed are cold-adapted plant groups, and it is difficult to imagine that the climatic conditions in Beringia during glacial periods would have been suitable for the growth and fruiting of the ancestor of *X. stumarium*.

4.3 | Final Remarks

Our study highlights the importance of herbarium collections for the study of rare species and/or species with a wide distribution range. The utilisation of specimens as old as 240 years, together with the new sequencing techniques, has allowed us to better understand the phylogenetic relationships, divergence time and biogeography of the extremely young species *X. strumarium*.

Most probably, between the two last glacial periods an LDD event from the Americas to Southeastern Asia led to the arrival and spread of the ancestor of *X. strumarium* in the Old Word. Our findings once more corroborate the importance of long-distance-dispersal in explaining transoceanic disjunct distributions in plant groups.

Author Contributions

Eleonora Manzo, Salvatore Tomasello designed research. Salvatore Tomasello sampled plant materials. Eleonora Manzo and Salvatore Tomasello performed laboratory work. Eleonora Manzo and Salvatore Tomasello analysed the data. Eleonora Manzo wrote the manuscript with the contribution of Salvatore Tomasello.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw reads data of the studied samples are deposited in the European Nucleotide Archive (ENA), under Bio-Project accession number PRJEB74768. Single accession numbers are specified in Table 1. All the Beast2 tree results and results from the different BioGeoBEARS models have been deposited in the GRO repository under https://doi.org/10.25625/FJHGEC.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.