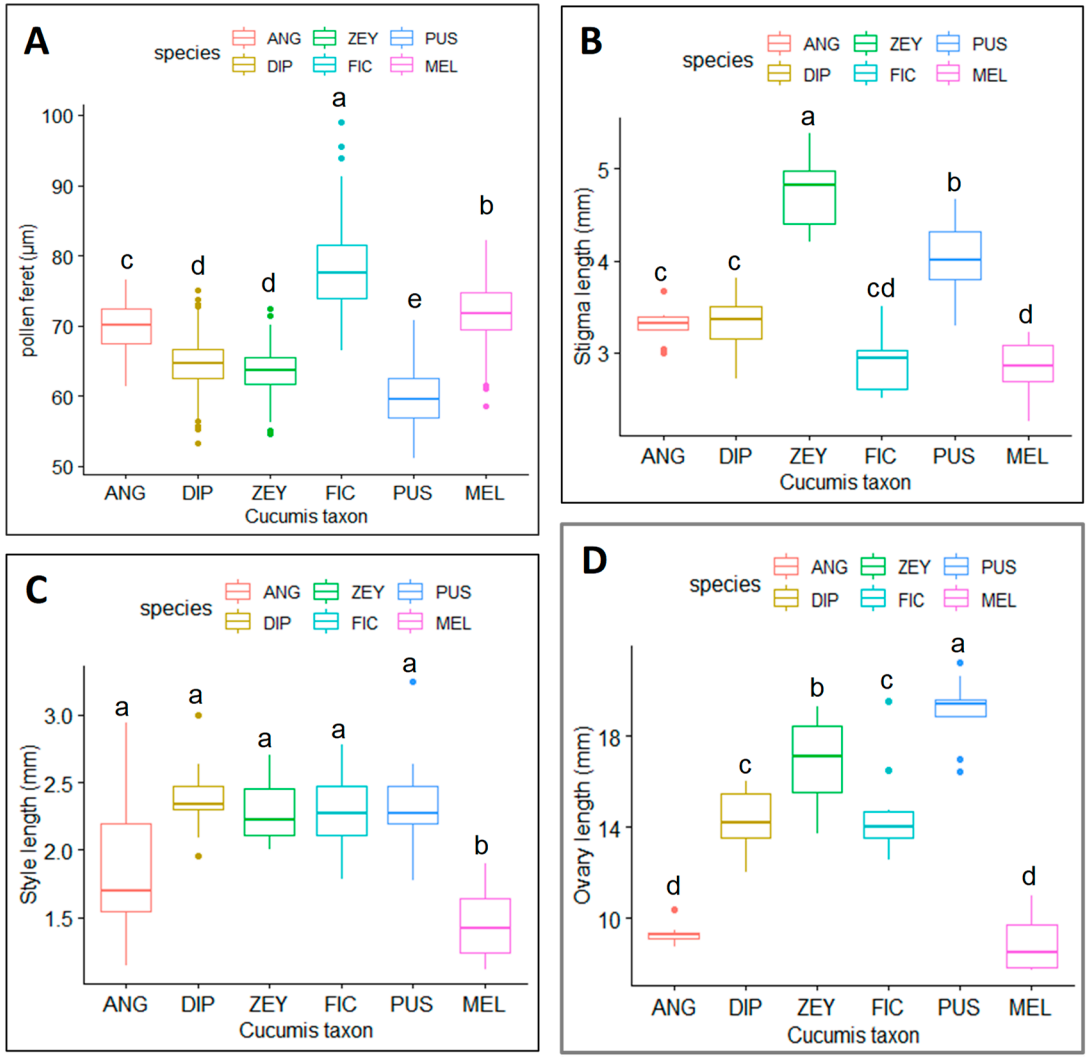
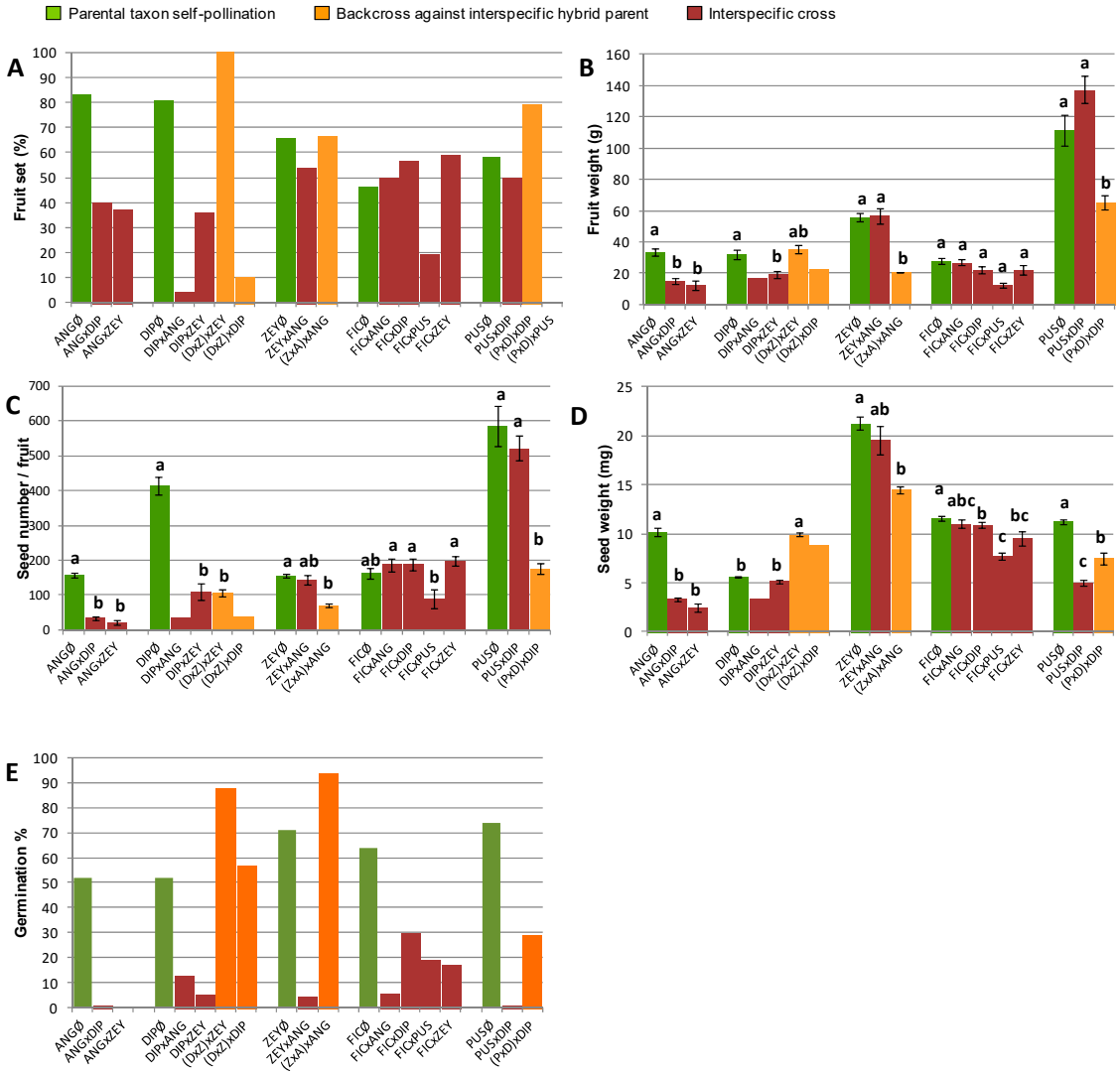
Supplementary Data

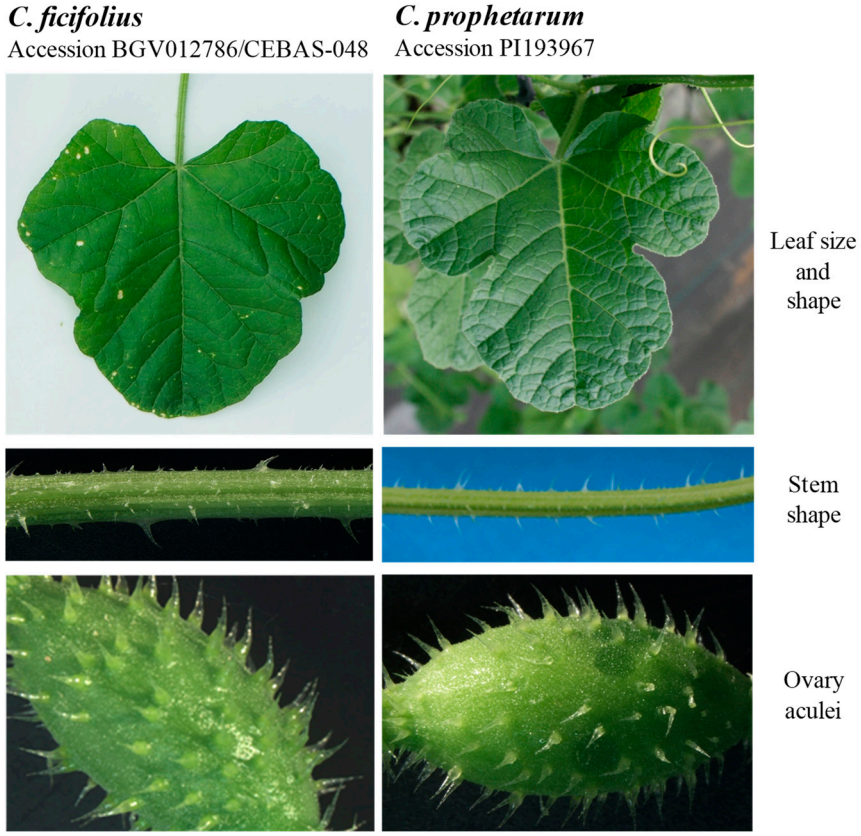
Supplementary Figure S1. Evaluation of pre-mating factors in *Cucumis spp*. Box-whisker plots show variability of pollen grain size (A) and stigma (B), style (C) and ovary (D) lengths in the six *Cucumis* species crossed in this work: ANG (*Cucumis anguria*); DIP (*C. dipsaceus*); ZEY (*C. zeyheri*); FIC (*C. ficifolius*); PUS (*C. pustulatus*) and MEL (*C. melo*). Means marked by the same non-capital letter are not statistically different (P>0.05; ANOVA test, see Methods). Feret´s diameter determined by ImageJ software (the longest distance between two points along the boundary of the projected area of pollen grains) was used to estimate pollen grain size. Stigma, style and ovary lengths were estimated by measuring 10 pistils. Pollen grain size was estimated by measuring ~200-300 individual grains.


A
species
ANG
DIP
ZEY
FIC
PUS
MEL
pollen feret (µm)
100
90
80
70
60
50
c
d
d
a
e
b
ANG
DIP
ZEY
FIC
PUS
MEL
Cucumis taxon
B
species
ANG
DIP
ZEY
FIC
PUS
MEL
Stigma length (mm)
5
4
3
c
c
a
cd
b
d
ANG
DIP
ZEY
FIC
PUS
MEL
Cucumis taxon
C
species
ANG
DIP
ZEY
FIC
PUS
MEL
Style length (mm)
3.0
2.5
2.0
1.5
a
a
a
a
a
b
ANG
DIP
ZEY
FIC
PUS
MEL
Cucumis taxon
D
species
ANG
DIP
ZEY
FIC
PUS
MEL
Ovary length (mm)
18
14
10
d
c
b
c
a
d
ANG
DIP
ZEY
FIC
PUS
MEL
Cucumis taxon

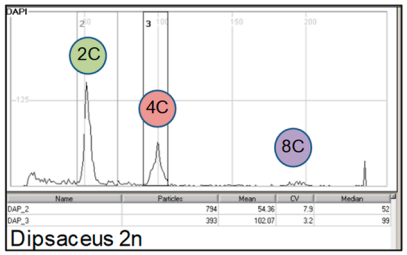
Supplementary Figure S2. Post-zygotic IRBs in *Cucumis spp*. Histograms show the estimation of four parameters: (A) fruit set (%), (B) fruit weight (g), (C) seed number/fruit and (D) average seed weight (mg) and (E) germination rate (%). Average ± standard error was estimated for fruit weight, seed number/fruit and average seed weight from >10 fruits except for F×P (4) and (D×Z)×Z (5). In addition, only one fruit was obtained from D×A and (D×Z)×D crosses. Means marked by the same non-capital letter are not statistically different (P>0.05; ANOVA test, see Methods). See also Supplementary Table S1 for taxa abbreviations. Ø symbol indicates self-pollination.


Parental taxon self-pollination
Backcross against interspecific hybrid parent
Interspecific cross
A
Fruit set (%)
B
Fruit weight (g)
C
Seed number / fruit
D
Seed weight (mg)
E
Germination %
ANGØ
ANGxDIP
ANGxZEY
DIPØ
DIPxANG
DIPxZEY
(DxZ)xZEY
(DxZ)xDIP
ZEYØ
ZEYxANG
(ZxA)xANG
FICØ
FICxANG
FICxDIP
FICxPUS
FICxZEY
PUSØ
PUSxDIP
(PxD)xDIP
(PxD)xPUS

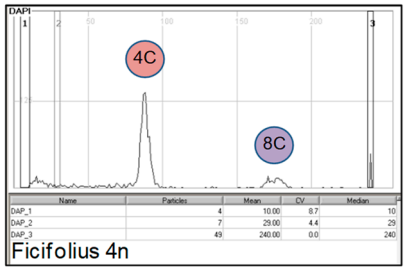
Supplementary Figure S3. Some of the diagnostic traits (leaf shape, stems and aculei) assessed in the *C. ficifolius* (FIC) and *C. prophetarum* (PRO) accessions used in this work.


C. ficifolius
Accession BGV012786/CEBAS-048
C. prophetarum
Accession PI193967
Leaf size and shape
Stem shape
Ovary aculei

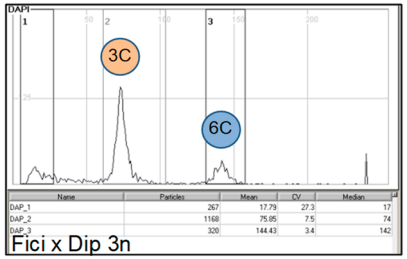
Supplementary Figure S4. Flow cytometry profiles for DIP, FIC and the F×D hybrid. Peaks corresponding to 2C, 3C, 4C, 6C and 8C nuclear DNA content are indicated. All three profiles show peaks of progressively decreasing magnitude. Typically the first one corresponds to nuclei in the G1 phase of mitosis while the second (and eventually the third) corresponds to nuclei in the G2 phase plus those under endomitotic DNA replication.



Dipsaceus 2n



Ficifolius 4n



Fici x Dip 3n

Supplementary Table S1. Plant material used for the GBS analysis. All the scientific names of taxa were assigned according to [46]. Ploidy was determined by flow cytometry (see Methods).

Species	Accessions	Seed source[a]	Origin	Ploidy	Abbr.[b]
C. anguria L.*	PI282442 * CUC27/1983	NCRPIS (IRTA) IPK (IRTA)	South Africa (Zimbabwe) South Africa	2x 2x	ANG ANG'
C. dipsaceus Ehrenb. ex Spach*	PI236468	NCRPIS (IRTA)	East Africa (Ethiopia)	2x	DIP
C. prophetarum L.	PI193967	NCRPIS (IRTA)	East Africa (Ethiopia)	4x	PRO
C. sagittatus Wawra & Peyr.	PI282441	NCRPIS (IRTA)	South Africa (Namibia)	2x	SAG
C. zeyheri Sond.*	PI282450	NCRPIS (IRTA)	South Africa (Transvaal)	2x	ZEY
C. pustulatus Naudin ex Hook.f.*	CUC44/1989[c]	IPK (IRTA)	East Africa	2x	PUS
C. globosus C.Jeffrey	CUC76/1995	IPK (IRTA)	South Africa (South Africa)	2x	GLO
C. ficifolius A.Rich.*	BGV012786 /CEBAS-048	COMAV	North-East Africa	4x	FIC
C. myriocarpus Naudin	BGV008535/CEBAS-112	COMAV	South Africa	2x	MYR
C. metuliferus E.Mey. ex Naudin	BGV011135/CUC16/1982	COMAV	South West Africa	2x	MET
C. melo L.*	Ames24297/TRI 5-4	NCRPIS (IRTA)	Pakistan	2x	MEL
C. maderaspatanus Siddarthan s.n.	322854 [*Mukia maderaspatana* (L.) M.Roem.]	Kew Garden	Mali	2x	MAD
C. zeyheri × *C. anguria*	PI282450 × PI282442	This work	----	2x	Z×A

C. dipsaceus × *C. zeyheri*	PI236468 × PI282450	This work	----	2x	D×Z
C. pustulatus × *C. dipsaceus*	CUC44 × PI236468	This work	----	2x	P×D
C. ficifolius × *C. anguria*	BGV012786× PI282442	This work	----	3x	F×A
C. ficifolius × *C. dipsaceus*	BGV012786 × PI236468	This work	----	3x	F×D
C. ficifolius × *C. pustulatus*	BGV012786 × CUC44	This work	----	3x	F×P
C. ficifolius × *C. zeyheri*	BGV012786 × PI282450	This work	----	3x	F×Z
C. dipsaceus × *C. anguria*	PI236468 × CUC27	This work	----	2x	D×A'
C. anguria × *C. anguria*	PI282442 × CUC27 / CUC27 × PI282442	This work	----	2x	A×A'/A'×A

[a] NCPRIS: North Central Region Plant Introduction Station (USA); IRTA: Institut de Recerca i Tecnologia Agroalimentàries (Spain); IPK: Institut für Pflanzengenetik und Kulturpflanzenforschung (Germany); COMAV: Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (Spain); Kew Garden: Royal Botanic Gardens, Kew (UK).

[b] Abbr.: Abbreviation.

[c] This accession is named as *C. figarei* by IPK germplasm bank but this is not an accepted name by [46]. We used instead *C. pustulatus* Naudin ex Hook.f. since phenotypic diagnostic traits correspond very well with this species.

* Accessions used to phenotype interspecific reproductive barriers.

Supplementary Table S2. Results of tests performed to determine statistical differences among means or mean ranks. Levene's test for homogeneity of variance, Shapiro-Wilk normality test, and ANOVA or Kruskal Wallis chi squared test in case normality could not be assumed, for each pre-mating variable.

	Levene's test			Shapiro-Wilk test		ANOVA								Kruskal – Wallis test		
	df	Fvalue	Pr(>F)	W	p-value	df	SS	MS	Fvalue	Res.df	Res. SS	Res. MS	Pr(>F)	χ^2	df	p-value
Pollen feret	5	4.725	<0.001	0.699	<0.001	-	-	-	-	-	-	-	-	-	-	-
Stigma length	5	1.472	0.214	0.986	0.702	5	27.16	5.43	44.96	54	6.524	0.12	<0.001	-	-	-
Style length	5	1.248	0.300	0.959	0.040	-	-	-	-	-	-	-	-	28.04	5	<0.001
Ovary length	5	2.376	0.051	0.973	0.211	5	839.1	167.8	75.79	54	119.6	2.21	<0.001	-	-	-

Supplementary Table S2a.Bonferroni-corrected pair-wise Mann-Whitney-Wilcoxon tests for non-homoscedastic and non-normal pollen feret data among *Cucumis* species.

	C. anguria	*C. dipsaceus*	*C. ficifolius*	*C. melo*	*C. pustulatus*	*C. zeyheri*
C. dipsaceus	<0.001					
C. ficifolius	<0.001	<0.001				
C. melo	<0.001	<0.001	<0.001			
C. pustulatus	<0.001	<0.001	<0.001	<0.001		
C. zeyheri	<0.001	0.014	<0.001	<0.001	<0.001	

Supplementary Table S3. Diagnostic traits measurement in leaves, male/female flowers and fruits of seed parent species and interspecific hybrids. Average ± standard deviation estimated from at least five measurements is shown for each trait.

	Leaf			
Taxon	Pedicel (L)	Leaf (L)	Leaf (W)	Leaf (L/W)
C. anguria	10.3 ± 1.3	15.7 ± 2.9	16.5 ± 1.1	0.9 ± 0.1
C. pustulatus	4.8 ± 0.6	10.3 ± 1.0	10.0 ± 0.3	1.0 ± 0.1
C. ficifolius	6.7 ± 1.0	8.5 ± 1.9	10.0 ± 1.0	0.8 ± 0.1
C. dipsaceus	10.0 ± 1.5	8.9 ± 0.9	11.6 ± 1.4	0.8 ± 0.04
C. zeyheri	4.1 ± 0.7	9.8 ± 0.2	8.8 ± 0.5	1.1 ± 0.03
C. melo	8.5 ± 1.3	9.9 ± 1.0	11.7 ± 1.3	0.8 ± 0.05
ZxA	7.6 ± 0.6	15.6 ± 2.6	16.7 ± 1.2	0.9 ± 0.1
DxZ	6.8 ± 0.8	9.6 ± 0.3	11.1 ± 0.4	0.9 ± 0.02
PxD	8.9 ± 1.0	9.6 ± 0.2	9.6 ± 0.3	1.0 ± 0.02
FxA	6.6 ± 0.9	12.5 ± 1.7	13.6 ± 1.0	0.9 ± 0.1
FxD	7.2 ± 1.1	12.5 ± 1.1	12.9 ± 0.8	1.0 ± 0.1
FxP	4.5 ± 0.5	9.8 ± 1.3	11.3 ± 0.6	0.9 ± 0.1
FxZ	4.2 ± 0.7	10.2 ± 0.8	10.8 ± 0.7	0.9 ± 0.03

	Female flower								
Taxon	Pedicel (L)	Ovary (L)	Ovary (W)	Ovary (L/W)	Sepal (L)	Petal (L)	Petal (W)	Petal (L/W)	Aculei hyaline/opaque (L)*
C. anguria	3.8 ± 1.5	0.9 ± 0.1	0.6 ± 0.1	1.5 ± 0.2	0.4 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.3 ± 0.3	0.5 ± 0.1
C. pustulatus	2.4 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	1.7 ± 0.02	0.3 ± 0.03	1.0 ± 0.1	0.9 ± 0.06	1.1 ± 0.1	0.3 ± 0.07
C. ficifolius	1.4 ± 0.5	1.2 ± 0.1	0.6 ± 0.1	2.0 ± 0.2	0.3 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	1.6 ± 0.2
C. dipsaceus	2.0 ± 0.3	1.2 ± 0.1	0.7 ± 0.1	1.8 ± 0.1	0.6 ± 0.1	1.3 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.7 ± 0.2
C. zeyheri	2.8 ± 0.7	1.3 ± 0.1	0.5 ± 0.1	2.5 ± 0.2	0.2 ± 0.04	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	0.8 ± 0.2
C. melo	1.4 ± 0.2	0.8 ± 0.1	0.5 ± 0.04	1.6 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	---
ZxA	5.2 ± 1.1	1.1 ± 0.1	0.6 ± 0.1	1.8 ± 0.2	0.3 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	1.1 ± 0.2	0.6 ± 0.05
DxZ	3.6 ± 1.5	1.7 ± 0.2	0.9 ± 0.1	1.9 ± 0.3	0.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	0.9 ± 0.1
PxD	3.7 ± 0.6	1.7 ± 0.2	1.0 ± 0.1	1.6 ± 0.1	0.6 ± 0.03	1.6 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.8 ± 0.4
FxA	2.5 ± 0.5	1.1 ± 0.1	0.5 ± 0.1	2.1 ± 0.2	0.4 ± 0.04	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.3 ± 0.2
FxD	2.0 ± 0.3	1.5 ± 0.1	0.8 ± 0.1	2.0 ± 0.2	0.5 ± 0.1	1.4 ± 0.2	1.1 ± 0.4	1.3 ± 0.5	1.1 ± 0.2
FxP	2.7 ± 1.0	1.4 ± 0.1	0.8 ± 0.1	1.9 ± 0.2	0.4 ± 0.1	1.2 ± 0.2	0.8 ± 0.2	1.6 ± 0.6	1.1 ± 0.1
FxZ	Female sterile (did not produce female flowers)								

	Male flower							
Taxon	Pedicel (L)	Calyx (L)	Calyx (W)	Calyx (L/W)	Sepal (L)	Petal (L)	Petal (W)	Petal (L/W)
C. anguria	2.0 ± 0.6	0.5 ± 0.04	0.3 ± 0.1	1.4 ± 0.2	0.2 ± 0.1	0.9 ± 0.2	0.7 ± 0.1	1.3 ± 0.1
C. pustulatus	2.2 ± 0.6	0.5 ± 0.0	0.4 ± 0.02	1.2 ± 0.1	0.2 ± 0.04	1.0 ± 0.04	0.9 ± 0.0	1.1 ± 0.05
C. ficifolius	0.9 ± 0.1	0.6 ± 0.03	0.3 ± 0.04	1.7 ± 0.2	0.2 ± 0.02	0.7 ± 0.04	0.5 ± 0.04	1.2 ± 0.1
C. dipsaceus	2.4 ± 0.3	0.5 ± 0.04	0.3 ± 0.03	1.6 ± 0.1	0.4 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.2
C. zeyheri	2.5 ± 0.6	0.5 ± 0.03	0.4 ± 0.04	1.3 ± 0.2	0.2 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	1.2 ± 0.2
C. melo	0.9 ± 0.1	0.4 ± 0.1	0.3 ± 0.02	1.4 ± 0.1	0.2 ± 0.03	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.2
ZxA	1.7 ± 0.3	0.5 ± 0.04	0.4 ± 0.0	1.4 ± 0.2	0.3 ± 0.02	0.7 ± 0.1	0.7 ± 0.1	1.2 ± 0.2
DxZ	Male sterile (did not produce male flowers)							
PxD	2.1 ± 1.0	0.7 ± 0.05	0.4 ± 0.04	1.8 ± 0.1	0.3 ± 0.06	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.1
FxA	Male sterile (did not produce male flowers)							
FxD	Male sterile (did not produce male flowers)							
FxP	0.8 ± 0.1	0.5 ± 0.04	0.3 ± 0.02	1.7 ± 0.2	0.1 ± 0.04	0.5 ± 0.1	0.5 ± 0.1	1.0 ± 0.2
FxZ	Male sterile (did not produce male flowers)							

	Fruit**						
Taxon	Peduncle (L)	Peduncle d/p (W)***	Aculei (L)	Fruit (L)	Fruit (W)	Fruit (L/W)	Seed number/ fruit
C. anguria	7.8 ± 1.9	1.9 ± 0.5	0.9 ± 0.2	4.2 ± 0.3	3.8 ± 0.2	1.1 ± 0.03	147.2 ± 34.3
C. pustulatus	10.1 ± 2.6	3.2 ± 0.6	0.6 ± 0.3	7.4 ± 0.8	5.4 ± 0.5	1.4 ± 0.1	652.8 ± 294.1
C. ficifolius	2.8 ± 0.4	1.3 ± 0.2	0.06 ± 0.03	4.7 ± 0.6	3.4 ± 0.4	1.4 ± 0.1	175.3 ± 61.7
C. dipsaceus	2.7 ± 0.6	1.4 ± 0.3	0.5 ± 0.1	5.9 ± 0.7	3.3 ± 0.5	1.8 ± 0.2	523.0 ± 202.3
C. zeyheri	5.6 ± 1.3	1.4 ± 0.2	0.5 ± 0.1	7.6 ± 0.3	4.3 ± 0.1	1.8 ± 0.1	167.7 ± 22.1
C. melo	2.6 ± 0.5	1.8 ± 0.5	---	3.9 ± 0.6	3.4 ± 0.2	1.1 ± 0.1	158.0 ± 31.1
ZxA	6.5 ± 1.2	1.8 ± 0.3	0.4 ± 0.1	4.2 ± 0.3	2.8 ± 0.3	1.5 ± 0.1	77.5 ± 9.3
DxZ	3.2 ± 1.2	0.9 ± 0.4	0.6 ± 0.1	6.3 ± 0.3	3.0 ± 0.4	2.2 ± 0.4	123.0 ± 7.1
PxD	9.1 ± 1.8	2.8 ± 0.6	0.8 ± 0.03	8.0 ± 0.8	5.7 ± 0.6	1.4 ± 0.1	452.7 ± 243.9
FxA	3.0 ± 1.0	1.3 ± 0.2	0.4 ± 0.1	3.5 ± 0.1	2.3 ± 0.2	1.5 ± 0.1	0
FxD	1.9 ± 0.2	1.5 ± 0.3	0.2 ± 0.02	4.3 ± 0.3	2.3 ± 0.4	1.9 ± 0.3	22.0 ± 13.2
FxP	Only one small fruit without seeds was obtained by backcrossing						
FxZ	Female sterile (did not produce female flowers)						

L (length in cm)
W (width in cm)
* Relative length of the apical hyaline bristle to the opaque basal portion of the aculei
** Fruits were obtained by self-fertilization in seed parent species and by backcrossing in male-sterile hybrids
***Peduncle (distal/proximal thickness ratio)

Supplementary Table S4. Crossability between *C. anguria* accessions PI282442 (ANG) and CUC27/1983 (ANG').

Cross	Fuit set	Fruit Weight	Seed number	Germination	F1 hybrid	F1 hybrid self-pollination
ANG self-pollination	80%	10,4 ± 0,7	164,7 ± 10,8	75%		
ANG X ANG'	67%	11,5 ± 0,4	170,4 ± 29,5	80%	male fertile	verified
ANG' self-pollination	50%	5,9 ± 2,4	38,3 ± 15,2	63%		
ANG' X ANG	80%	8,5 ± 2,70	59,8 ± 14,6	58%	male fertile	verified

Supplementary Table S5. Percentage of heterozygous sites detected in the *Cucumis* taxa analyzed. Proportion of heterozygous sites was estimated from the set of 10967 SNPs used to infer genetic distances among *Cucumis* species.

Taxa	Number of sites	Number heterozygous	Proportion heterozygous	%
MEL	10967	3	0,0003	0,03
LON	10967	30	0,0027	0,27
MAD	10967	37	0,0034	0,34
SAG	10967	40	0,0036	0,36
MET	10967	28	0,0026	0,26
GLO	10967	43	0,0039	0,39
ANG	10967	29	0,0026	0,26
PUS	10967	31	0,0028	0,28
DIP	10967	35	0,0032	0,32
ZEY	10967	45	0,0041	0,41
MYR	10967	29	0,0026	0,26
FIC	10967	1417	0,1292	12,92
PRO	10967	1407	0,1283	12,83
DXZ	10967	1107	0,1009	10,09
ZXA	10967	1124	0,1025	10,25
PXD	10967	1478	0,1348	13,48
LXA	10967	174	0,0159	1,59
DXL	10967	1046	0,0954	9,54
FXD	10967	1916	0,1747	17,47
FXA	10967	1998	0,1822	18,22
FXZ	10967	1980	0,1805	18,05
FXP	10967	2139	0,1950	19,50
A'XA	10967	174	0,0159	1,59

Supplementary Table S6. Pre-zygotic (pollen-pistil compatibility) RI indices for all species pairs, where RI = 1- (average success of interspecific crosses/average success of self-pollinations).

Pollen-pistil compatibility

		Pollen parent					
	♀	**ANG**	**ZEY**	**DIP**	**PUS**	**FIC**	**MELO**
Seed Parent	**ANG**	0.00	0.35	0.80	0.88	0.75	0.75
	ZEY	0.00	0.00	0.48	1.00	0.75	0.63
	DIP	0.23	0.31	0.00	1.00	0.69	0.85
	PUS	0.00	0.19	0.13	0.00	0.28	0.31
	FIC	0.04	0.00	0.00	0.20	0.00	0.41
	MEL	0.91	0.89	0.89	0.69	1.00	0.00

Supplementary Table S7. Post-zygotic (fruit set) RI indices for all species pairs, where RI = 1 - (average success of interspecific crosses/average success of self-pollinations).

Fruit set

		Pollen parent					
	♀	**ANG**	**ZEY**	**DIP**	**PUS**	**FIC**	**MEL**
Seed Parent	**ANG**	**0.00**	**0.56**	**0.52**	**1.00**	**1.00**	**1.00**
	ZEY	0.18	0.00	1.00	1.00	1.00	1.00
	DIP	0.95	0.56	0.00	1.00	1.00	1.00
	PUS	1.00	1.00	0.14	0.00	1.00	1.00
	FIC	0.00	0.00	0.00	0.59	0.00	1.00
	MEL	1.00	1.00	1.00	1.00	1.00	0.00

Supplementary Table S8. Post-zygotic (fruit weight) RI indices for all species pairs, where RI = 1 - (average success of interspecific crosses/average success of self-pollinations).

Fruit weight

		Pollen parent					
	♀	**ANG**	**ZEY**	**DIP**	**PUS**	**FIC**	**MEL**
Seed Parent	**ANG**	0.00	0.63	0.55	1.00	1.00	1.00
	ZEY	0.00	0.00	1.00	1.00	1.00	1.00
	DIP	0,48	0.40	0.00	1.00	1.00	1.00
	PUS	1.00	1.00	0.00	0.00	1.00	1.00
	FIC	0.03	0.20	0.19	0.55	0.00	1.00
	MEL	1.00	1.00	1.00	1.00	1.00	0.00

Supplementary Table S9. Post-zygotic (seed set) RI indices for all species pairs, where RI = 1 - (average success of interspecific crosses/average success of self-pollinations).

Seed set

		Pollen parent					
	♀	**ANG**	**ZEY**	**DIP**	**PUS**	**FIC**	**MEL**
Seed Parent	**ANG**	0.00	0.87	0.79	1.00	1.00	1.00
	ZEY	0.07	0.00	1.00	1.00	1.00	1.00
	DIP	0.91	0.73	0.00	1.00	1.00	1.00
	PUS	1.00	1.00	0.11	0.00	1.00	1.00
	FIC	0.00	0.00	0.00	0.46	0.00	1.00
	MEL	1.00	1.00	1.00	1.00	1.00	0.00