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Oranges and lemons: clues to the taxonomy of *Citrus* from molecular markers

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Go into any grocery store and one is confronted with an array of *Citrus* fruit: oranges, grapefruit, mandarins (tangerines), lemons and limes. This is rich bounty for the shopper, but taxonomists are perplexed as to how to classify the various kinds of *Citrus* that have existed since antiquity. Now, thanks to new genetic and molecular biological techniques, the relationships between these fruit are being unraveled and show that there are probably only three true species.

Until the mid 1970s, *Citrus* taxonomists based their conclusions solely on morphological and geographical data. This led to major disagreements on the classification of species within the *Citrus* subgenus (Box 1). One definition of a species is that when populations of two kinds occur together without interbreeding, they are different species. Thus, some 'joiners' believe that all *Citrus* types belong to one large species because they are all graft-compatible and, were it not for the reproductive barriers described below, they are all interfertile. Swingle, in 1943, devised a system for *Citrus* where ten species in the subgenus *Citrus* were recognized (Table 1)[1,2]. However, Tanaka[3] (the ultimate 'splitter') defined 147 different species in 1954. A major difference in these two systems was in how mandarins were treated; Swingle placed all mandarins except *C. tachibana*, a wild species from Japan, and *C. indica*, a wild species from India, in *C. reticulata*, whereas Tanaka separated mandarins into 36 species. The vast difference in number of species recognized in these two systems and some intermediate ones reflected opposing theories on what degree of morphological difference justified species status and whether presumed hybrids among naturally occurring forms should be given species status.

Several characteristics of the *Citrus* subgenus made taxonomic classification on the basis of morphology and geography especially difficult. First, *Citrus* is an ancient crop, with records of human cultivation extending back to at least 2100 BC (Refs 4,5). Therefore, it has been difficult to ascertain centers of origin and diversity because of natural and perhaps human-aided hybridization, wide dispersion, and the paucity of remaining wild *Citrus* stands. At the moment, the center of origin and diversity of *Citrus* and its related genera is generally considered to be Southeast Asia, especially east India, north Burma and southwest China, but possibly ranging from northeastern India eastward through the Malay archipelago, north into China and Japan, and south to Australia[3–8]. The wild relatives of *Citrus* are native to Southeast Asia, the East Indian Archipelago, New Guinea, Melanesia, New Caledonia and Australia.

The exact routes of dispersion of *Citrus* from its origin are also unknown. However, *Citrus* is mentioned in the ancient writings of many cultures[4,8]. The oldest known reference to *Citrus* appears in Sanskrit literature that dates to before 800 BC; descriptions in Chinese, Greek and Roman literature followed[8]. It is believed that some *Citrus* types, including citrons, sour oranges and lemons, spread slowly (from 500 to 1300 AD) through wide areas, including into Europe, by successive waves of invaders and travelers – Muslim armies, Arab traders, Crusaders and others moving along trade routes.

Although some *Citrus* trees were grown in Europe during these centuries, the types available were probably bitter and used mostly as condiments. Columbus and Ponce de Leon carried various *Citrus* fruits to the New World in the late 1400s and early 1500s, as did others. The Portuguese introduced one or more superior types of sweet orange into Europe, probably in the 16th century. Although mandarins had been cultivated in China and Japan from ancient times, the first mandarin varieties were brought to England in 1805 by Sir Abraham Hume, and they only subsequently spread to the Mediterranean region[7,8].

All *Citrus* types except the *Citrus* relative the trifoliate orange are highly sensitive to freezing; this limits their growth range, and the trees could not survive unprotected in most parts of Europe. Therefore, orangeries and other devices for citriculture were developed. European sailors became aware of the benefits of *Citrus* consumption in preventing scurvy during their voyages, and they carried *Citrus* fruit for consumption and seeds and trees to plant along trade routes. *Citrus* culture proliferated in Florida in the late 1700s, when the first commercial shipments were made. At about the same time, *Citrus* was introduced into California, although commercial production only began there much later[4].

A second factor that makes taxonomic classification difficult is the reproductive

Glossary

Accession: A distinct type or variety of plant, not necessarily a cultivar; e.g. a breeding line or a related wild species.

Apomixis: Reproduction of a plant without any form of sexual union. Obligate apomixis, where reproduction occurs by apomixes only; facultative apomixis, where reproduction can occur by apomixes or normal sexual reproduction.

Cultivar: A term contracted from 'cultivated variety'; a plant type that is clearly distinguished from any other type by any characters (morphological, physiological, cytological, chemical, or any others), and that, when reproduced (sexually or asexually), retains its distinguishing characters. A variant cultivar is one that differs from others in the group of cultivars for a specific characteristic, such as an isozyme genotype.

Discontinuity of traits: A distinct profile of characteristics maintained in a species by reproductive isolation.

Intraspecific affinity: The degree of similarity, measured statistically by correlation analyses, of given characters within members of a species.

Introgression: Hybridization between donor and recipient parents, followed by multiple crosses to the recipient parent such that only a relatively small part of the genome of the donor parent is ultimately transferred.

Jaccard coefficient: A statistical measurement of similarity of attributes between samples.

Rootstock: The root system, the bottom part of a plant propagated by grafting.

Trihybrid intergeneric cross: A cross involving three original parental types, with at least one of the parents from a different genus than the others.

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Box. 1. Taxonomic classification

The hierarchical groupings used by taxonomists, using an example from *Citrus*, that of *Citrus sinensis*, the sweet orange. Most taxonomists include six closely related genera in the subtribe of the 'true citrus fruit trees' – *Citrus*, *Pocirus* (trifolate orange), *Fortunella* (kumquat), *Microcitrus* (Australian wild lime), *Eremocitrus* (Australian desert lime) and *Clymenia*. The genus *Citrus* is divided into two subgenera. The common cultivated types of this fruit are placed in the subgenus *Citrus* (*Eucitrus*). Species of the subgenus *Papeda* do not bear edible fruit.

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biology of the genus. Many *Citrus* types reproduce asexually by seed through a process called nucellar embryony[7]. The nucellus, a temporary nutritive tissue in developing seeds, gives rise to 'nucellar embryos' that are genetically identical to the plant on which the fruit is developing. Many nucellar embryos can be present, although usually only two or three develop to maturity; *Citrus* types that display this trait are referred to as polyembryonic. Very frequently, the nucellar embryos out-compete the single zygotic (sexual) embryo for space or nutrients in the ovule. Thus, when a *Citrus* seed is planted, the resulting seedling often has the same genetic composition as the tree from which it arose.

This aspect of *Citrus* reproduction greatly complicates taxonomic analyses of the genus. One the one hand, *Citrus* contains an enormous degree of variation, with abundant natural hybridization giving rise to a wide range of phenotypes, suggesting one species with many subspecies. On the other hand, because this type of facultative apomixis (see Glossary) is so widespread in the genus, exchange of genes is often prevented, leading to reproductive isolation, a requirement for species designation. Furthermore, there is an extremely high rate of bud and limb mutations ('sports') in the genus. Beneficial ones can be vegetatively propagated, and might, in fact, be ancient in origin. Thus, the biological concept of speciation where there is exchange of genes between members of a species and barriers to such an exchange between species is difficult to apply to the *Citrus* genus[9]. Because of these conflicts, the term *Citrus* 'biotype' will be used in this discussion when proper designation as a species is not clear[10]. A biotype is all individuals that have the same genotype, except for mutations. Because the offspring of an apomictally perpetuated biotype are asexually produced, these individuals are collectively a clone.

Fortunately, modern techniques have been instrumental in deciphering the taxonomic situation in *Citrus*. In the mid 1970s, Barrett and Rhodes performed a comprehensive phylogenetic study that evaluated 146 morphological and biochemical tree, leaf, flower and fruit characteristics[10]. This study, and a second one[8], suggested that only three *Citrus* types, citron (*C. medica*), mandarin (*C. reticulata*) and pummelo (*C. grandis*; now *C. maxima*) constituted valid species. How, then, did the other *Citrus* types arise and maintain their integrity? How closely related are the various *Citrus* types? The development of various biochemical and molecular markers has provided some answers. In particular, DNA markers, with their phenotypic neutrality, abundance and imperviousness to environmental conditions, have been most useful (Box 2).

C. aurantifolia Christm. (limes)

The limes include both acid and sweet varieties[1]. In addition, the sour limes consist of two kinds, the small-fruited Mexican (West Indian, Key) type, and the large-fruited Tahiti (Persian) lime, which is triploid and therefore seedless. Limes are one of the *Citrus* biotypes that are believed to be apomictically perpetuated[10]. There are very few varieties, and these are fairly homogenous; seven cultivars have invariant isozyme genotypes at four loci[11]. However, limes are also relatively heterozygous; they have heterozygous genotypes at seven of ten isozyme loci evaluated[11,12]. Barrett and Rhodes[10] suggested that the lime probably arose from a trihybrid intergeneric cross involving *C. medica* (citron), *C. grandis* (pummelo) and a *Microcitrus* species. Nicolosi *et al.*[13] found that all RAPD and SCAR markers present in Mexican lime are also present in citron or in *C. micrantha* (a small-flowered papeda, a *Citrus* relative that produces inedible fruit), suggesting

Table 1. Citrus types designated as species by Swingle[3]

Swingle taxonomy[a]	Common name	Examples of varieties
C. aurantifolia Christm.	Lime	Mexican, Key West Indian, Key, Bearss
C. aurantium L.	Sour orange	Chinotto, Seville
C. indica Tan.	An Indian species that is not cultivated	
C. maxima Merril; formerly *C. grandis* Osbeck	Pummelo, shaddock	Acidless, Thong Dee
C. limon (L.) Burm. f.	Lemon	Femminello, Lisbon
C. medica L.	Citron	Etrog, Corsican
C. paradisi Macf.	Grapefruit	Marsh, Redblush, Flame
C. reticulata Blanco	Mandarin, tangerine	Clementine, Dancy, Satsuma
C. sinensis Osbeck	Sweet orange	Valencia, Washington navel, Shamouti
C. tachibana (Mak.) Tan.	A Japanese species that is not cultivated	

[a] In this binomial classification, *Citrus* is the genus; *aurantifolia*, *aurantium*, etc. is the species; and Chistm., L., etc. is the authority.

Box 2. Types of DNA marker used in the analysis of *Citrus* taxonomy

Inter-simple sequence repeat (ISSR): PCR amplification of DNA using a single primer composed of a microsatellite sequence such as $(CA)_8$ anchored at the 5′ or 3′ end by two to four arbitrary nucleotides.

Microsatellite probes: Short repetitive nucleotide sequences (e.g. GTG_5) used either as radiolabelled probes for hybridization with restricted citrus DNA or as single primers in PCR reactions.

Random amplified polymorphic DNA (RAPD): Generation of a typically dominant marker through the use of a single, small, random-sequenced primer in a PCR reaction.

Restriction fragment length polymorphism (RFLP): Variation in band size following cutting of DNA with a specific restriction enzyme, electrophoresis and hybridization with a specific probe.

Sequence characterized amplified region (SCAR): A polymorphic band, e.g. from a RAPD reaction, that is sequenced, so that specific PCR primers for the band can be designed.

that these two species were the parents. That citron is a parent of limes is supported by RFLP data[14].

C. aurantium L. (sour oranges)

There are three types of fruit grouped within the highly apomictic sour oranges: the common type that is used principally as a ROOTSTOCK for sweet oranges and other *Citrus* biotypes, and for the preparation of marmalade (the well-known Seville oranges); the bittersweet orange, whose fruit is similar to the common type, but less acidic; and the variant bitter oranges that are grown primarily as ornamentals and for their flowers, from which neroli oil is extracted[1]. Barrett and Rhodes[10] found INTRASPECIFIC AFFINITY (correlation coefficients of traits measured) to be quite high within common sour oranges, although the biotype appeared highly heterozygous. This condition was believed to be maintained by facultative apomixis; thus, sour orange is also not a true species. Barrett and Rhodes[10] believed sour oranges to be of predominantly *C. reticulata* (mandarin) genotype INTROGRESSED with genes from *C. grandis* (pummelo). This is supported by SCAR and RAPD analyses[13]. Chloroplast DNA analyses (Southern blots probed with labeled tobacco chloroplast DNA or PCR amplification of chloroplast sequences using 'universal' primers) revealed that *C. aurantium*, *C. limon*, *C. paradisi*, *C. sinensis* and *C. grandis* had the same Southern hybridization pattern, whereas *C. reticulata* and *C. medica* each had a unique pattern[13,15]. Pummelo appeared to be the maternal parent, which is not unexpected because it is monoembryonic and produces zygotic seedlings[13].

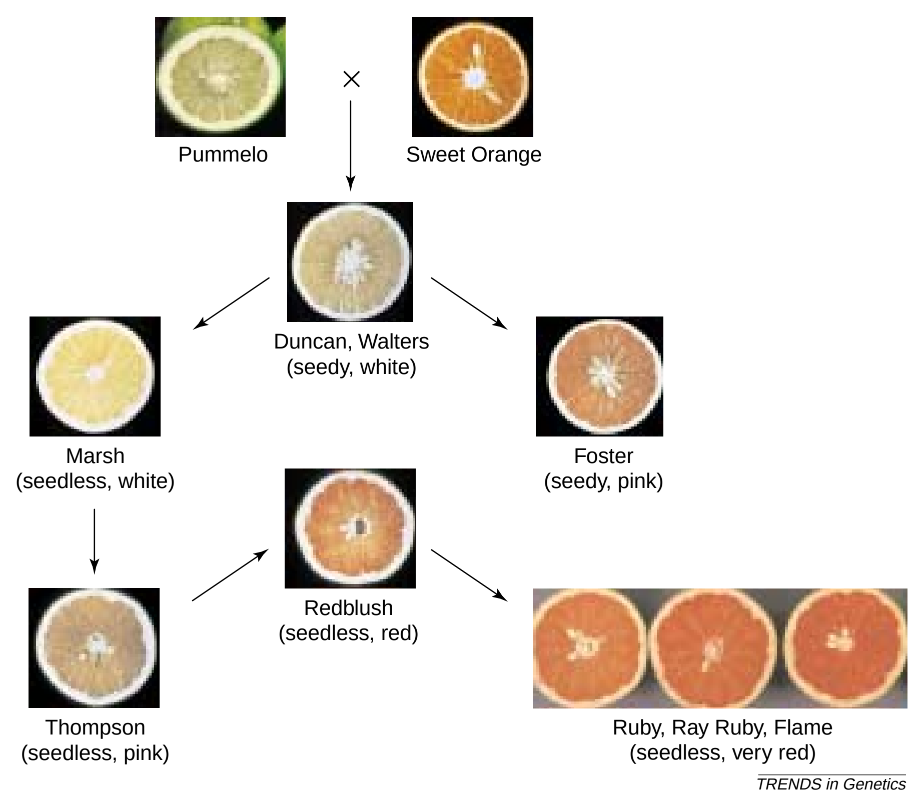

Fig. 1. Grapefruit is unusual in the genus *Citrus* because its history is mostly documented. The original grapefruit biotype originated in the Caribbean, most probably by a natural hybridization between pummelo and sweet orange, perhaps followed by introgression back to pummelo[13,17]. The fruit of this type were white-fleshed and very seedy. All other grapefruit cultivars arose as mutations, selected either for being seedless or for having increasingly red fruit color.

Barrett and Rhodes[10] point out that the kind and number of differences among clones of cultivated *Citrus* types might be partially explained by the needs of the people who domesticated and cultivated them. Sour oranges, for example, were used in ancient times for flavoring, perfumery and medicine. Although these uses continue in a minor way today, the primary modern use of sour oranges is as a rootstock, where uniformity of type is required. This would militate against selection of variants. Supporting this theory, of 15 ACCESSIONS examined, two had a variant isozyme genotype; the others were identical[11].

The bergamot is a hybrid grown for its distinctive rind oil used in Earl Grey tea and in perfume. According to RAPD and SCAR markers, bergamot is a hybrid of citron and sour orange[13].

C. grandis (L.) Osbeck; *C. maxima* Merril (pummelos)

The pummelo is a dessert fruit in Southeast Asia, but is grown elsewhere[1] mostly as a curiosity. This largest fruit of the *Citrus* genus can be pigmented or unpigmented, and acid or acidless. Pummelos are one of the three *Citrus* types Barrett and Rhodes[10] proposed as a true species. This was because pummelos are monoembryonic, rather than being facultative apomicts, yet have a well-developed DISCONTINUITY OF TRAITS from other *Citrus* types (i.e. reproductive isolation). Although pummelo is primarily cross-pollinating, some clones produce relatively vigorous selfed progeny. Barrett and Rhodes[10] found intraspecific affinity to be quite high in this *Citrus* type, and this, along with the propensity for self-fertilization, was believed to be because the species was relatively homozygous. Isozyme data supports this; pummelo was homozygous at all ten loci examined[11,12]. *C. grandis* could be separated from other *Citrus* types by its unique ISSR banding patterns[16]. Of 14 accessions, only three had variant isozyme patterns[11].

C. limon (L.) Burm. f. (lemons)

The lemons consist of the common, acid varieties and a few sweet or acidless types[1]. Lemons were used medicinally in ancient times; presently, they are mostly used for juice and flavoring. Barrett and Rhodes[10] found intraspecific affinity to be quite high in this species. They speculate that because present needs are met by a uniform product, selection in this biotype has favored the unique, original apomictic type, with only minor mutational variations. Isozyme data supports this; of 16 accessions, only two had a variant isozyme genotype[11].

Barrett and Rhodes[10] speculated that lemons are a complex hybrid similar to limes but carrying a greater proportion of citron genes. Molecular marker data indicates that lemon originated from citron and sour orange, with sour orange being the maternal parent[13]. When ISSR markers were examined, a total of 12 polymorphic fragments generated by seven primers were detected among six lemon cultivars, suggesting a possible polyphyletic origin (development from more than one ancestral type) for lemon[16]. Six of ten isozyme loci examined were heterozygous[11,12].

C. medica L. (citrons)

The citrons also fall into acid and sweet classes, with several varieties extant for each class[1]. Citrons are grown mostly for their peel, which is then candied. Historically, the fruit had great religious significance for some cultures. Citron is the second type Barrett and Rhodes[10] advanced as a true species. It is monoembryonic. It is also relatively homozygous; eight of ten isozyme loci were homozygous, and of six cultivars, one was variant[11,12]. Molecular marker data support species status[13]. Citron had a unique chloroplast hybridization pattern[13,15], also supporting species status. Interestingly, the chloroplast data[13] indicate that citron always acted as the male parent – unexpected given the monoembryonic nature of this species.

C. paradisi Macf. (grapefruit)

Grapefruit is the one *Citrus* biotype in which a hybrid origin and subsequent selection for mutants is well documented (Fig. 1). RAPD and SCAR marker data indicate that grapefruit was derived from a backcross between sweet orange and pummelo, as do historical and morphological data[13,17]. Not unexpectedly, intraspecific affinity was very high in this biotype[10]. When isozymes were used to examine 13 cultivars, no variation was detected[11]; when 1230 ISSR markers were used to characterize seven cultivars, one was different from the norm, which was attributed to mutation[18]. Surprisingly, grapefruit might be rather homozygous; eight of ten isozyme loci were homozygous in this biotype[11].

C. reticulata Blanco (mandarins)

Mandarins are the most phenotypically heterogeneous group in *Citrus*; both monoembryonic and polyembryonic clones exist, as do self-fertile and self-incompatible types[1]. This suggested to Barrett and Rhodes[10] that a broader-based, more-complex heterozygosity was present than was found in the other facultatively apomictic types. Therefore, this was the third *Citrus* type assigned species status. Sweet mandarin types have been used for dessert fruit since ancient times, although sour types have been used as rootstocks, and for flavorings and medicine. Thus, it is difficult to assess the relative importance of genetic versus mutational variation in the complex history of this species.

SCAR and RAPD analyses did not allow the identification of progenitor type[13]. Although the molecular marker data reveal great heterogeneity within this group, it supports species status[11,13,16,19,20]. For example, when RAPD markers were used to evaluate genetic similarity among 35 mandarin accessions, the minimum JACCARD COEFFICIENT was 0.77, which indicates a high genetic similarity in this group. The researchers proposed that the mandarin group is a single species, composed of several genetically different individuals and a great number of hybrids[20]. Finally, *C. reticulata* has a unique chloroplast banding pattern, again suggesting that it should have species status[15].

C. sinensis Osbeck (sweet oranges)

Sweet orange, the most widely grown and consumed *Citrus* type, presents something of a mystery. Four kinds of sweet oranges are recognized: the common, or blond, orange, which is the most important and of which there are many varieties; the acidless orange, of minor importance; the blood orange, which has a red pigmentation in the flesh due to the accumulation of anthocyanins; and the navel orange, grown for fresh consumption[1]. They can also be categorized on the basis of season of maturity as early, mid-season, or late. Barrett and Rhodes[10] observed much lower intraspecific affinity in this type than in any of the other types they considered facultative apomicts (sour orange, lemon and grapefruit); in other words, there was a relatively great diversity in phenotype. Some studies indicate that genetically, sweet oranges are a biotype. Chromosome banding patterns of ten clones were heterozygous and invariant[21]. When three microsatellite probes were assayed, no differences were detected among ten cultivars[19]. This points to a monophyletic origin for sweet orange followed by somatic mutation and selection of

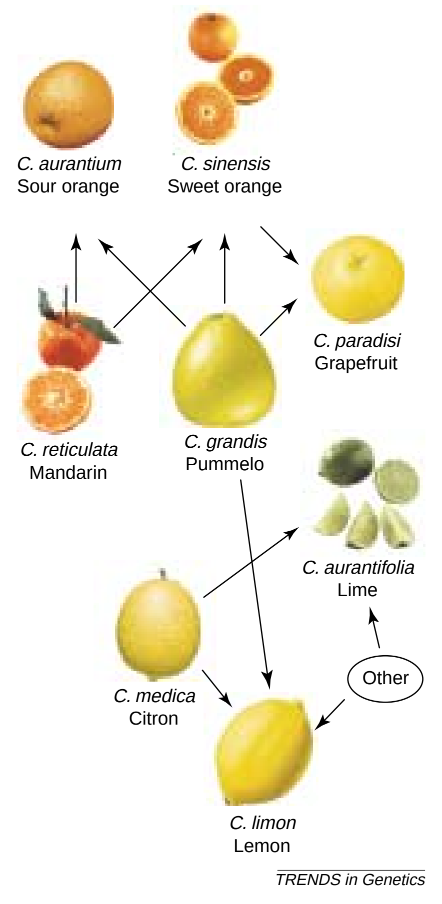

Fig. 2. The most probable relationships among the different *Citrus* types.

desirable clones[19]. However, when four isozyme loci were used to examine 21 cultivars, there were seven variants at one locus[11]. Further, of 31 cultivars, 14 differed from the basic ISSR profile of about 1230 fragments by one to four ISSR bands[18]. Fang and Roose[18] believed these differences originated by mutation. However, it seems unusual that small mutations could be revealed at such a frequency using molecular markers.

Like sour oranges, sweet oranges are thought to be predominantly of *C. reticulata* (mandarin) genotype introgressed with *C. grandis*[10,13]. The sweet orange and sour orange biotypes are thought to have a parallel but separate origin, with their differences stemming from parentage from separate subspecies from within the polytypic *C. reticulata*. Similar microsattelite patterns observed with sweet oranges and mandarins agree with the close phylogenetic relationships of these species[19].

Conclusions

Historical records and genetic analyses lead to the conclusion that there are three true species in the genus *Citrus* (citron, mandarin and pummelo) and additional cultivated biotypes (Fig. 2). Oranges, lemons, limes and grapefruit, although disseminated throughout the world by men in both ancient and modern times, each have a narrow genetic base. Cultivated types were selected by men and propagated by grafting or nucellar seedlings. The variation within these biotypes is due primarily to somatic mutations. This genetic homogeneity is reinforced by consumer expectations and industry regulations. Within the genus, and in related genera, there is genetic variability that a breeder can exploit; for instance, to create resistance to both biotic and abiotic stresses and for horticultural improvements such as increased fruit quality. However, breeders might then face many generations of crossing and selection to return to an edible type or one that fits industry standards. This makes modern genetic techniques, such as genetic transformation, attractive for the improvement of these economically important and aesthetically pleasing fruit.

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Rosa Beddington

Rosa Beddington, who served on the editorial board of *Trends in Genetics* from 1993, died on May 18th 2001 following a prolonged battle against breast cancer. She was just 45. A distinguished embryologist, she made a great contribution to the understanding of anterior–posterior axis formation and of the function of mammalian extra-embryonic tissues in head formation.

Full obituaries can be found in *Cell* (2001) 105, 709–710 and *Nature* (2001) 412, 138. Photograph courtesy of The Medical Research Council National Institute for Medical Research