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R. Hertel

Institut für Biologie III der Universität
Schänzlestr. 1
D-79104 Freiburg
Germany

E-mail: hertel@uni-freiburg.de

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Phylogenetic Analysis of the C-Terminal Sequence of *rbcL*[1]

G. F. Wildner, J. Schlitter, and T. Stützel

Department of Biology, Ruhr-Universität Bochum, Germany

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Abstract: At the C-terminal end of Rubisco's large subunit major differences in sequence length and in charges of the amino acid residues occur in unicellular organisms and in plants. This C-terminal segment of the large subunit participates in large movements during the catalytic cycle. It participates in the closing mechanism of the binding niche for the substrate RuBP, changing from an ordered structure in the "open" enzyme conformation to a position, stretched over the protein surface, in a "closed" conformation. We analyzed the sequence variability in the C terminus in *rbcL* to investigate whether this structurally important entity evolved in an ordered process. Cyanobacteria and chlorophytes show similar C-terminal sequences (DXX), whereby D-473 is the last strictly conserved amino acid residue for all *rbcLs*. Contrary to the gymnosperms (D + 2 residues), the C termini of the angiosperms show variable lengths from D + 2 to D + 17 residues. The plant orders of Asterales, Batales, Capparales, Caryophyllales, Fabales, Gentianales, Lamiales, Rubiales, Myrtales, Scrophulariales, and Solanales contain species with particularly elongated C termini. Recent studies regarding enzyme kinetics demonstrated that molecules with longer C termini are better adapted for a wider temperature range. We speculate that longer C termini confer properties to the enzyme that modulate the success of different species in different environments. This is supported by the fact that "modern" (e.g., phylogenetically young taxa in an actual radiation process) generally display a long C terminus, while conservative taxa have a relatively short C terminus.

Key words: Rubisco, *rbcL*, evolution, phylogenetic analysis, C terminus.

Abbreviations:

Rubisco:	Ribulose 1,5-bisphosphate carboxylase/oxygenase
RuBP:	Ribulose 1,5-bisphosphate
SF:	specificity factor

Introduction

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) is a key enzyme of carbon assimilation at the interface between biosphere and atmosphere. This important role explains the special attention that plant biochemists and physiologists have given to the enzyme to understand the interactions between protein properties and plant productivity in different environments. Several enzyme structures with or without bound ligands in the open and closed enzyme conformations have been published (Entrez March 1999: 18 structure sets, http:www.ncbi.nlm.nih.gov). Rubisco catalyzes not only the carboxylation of RuBP in the reductive pentose phosphate cycle but also the oxygenation of RuBP to yield phosphoglycolate and 3-PGA. The enzyme specificity (as defined as the ratio of carboxylase to oxygenase activities at a given $[CO_2]/[O_2]$) varied in the course of evolution, in that Rubisco isolated from cyanobacteria has a low enzyme specificity, the enzymes from chlorophytes have an intermediate value, and those from higher plants have higher values (Jordan and Ogren, 1981[13]). It is evident that an adaptation process occurred optimizing the enzyme in a changing atmosphere with increasing oxygen and decreasing carbon dioxide concentrations.

Rubisco has developed in the last decade as a leading object for phylogenetic and taxonomic studies. The enzyme consists of two types of subunits (L, large and S, small), which form a hexadecameric structure. In most eukaryotes, except a few unicellular organisms, the genetic information for the large subunit (*rbcL*) resides in the chloroplast genome, and that for the small subunit *(rbcS)* in the nuclear genome. The polyploidic occurrence of *rbcL* in plastids and the slow mutagenesis rate (1.3×10^{-9} substitution per synonymous site per year, Clegg, 1993[6]) explains the high homology between *rbcL* genes from bacterial to higher plants. A large body of sequence data has been gathered (Entrez March 1999: 5200 sequences of *rbcL*), but the vast majority of those are only partial sequences of PCR products. Approx. 300 complete sequences are available for comparison of the C-terminal ends.

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We undertook a comparative approach for all available *rbcL* sequences from monocots and dicots, and evaluated the different orders taxonomically. We also considered the question whether the process of elongation of the C termini seen in some orders occurred randomly or specifically, depending on the time scale of appearance in evolution. In the latter case, the structure of the C terminus might reflect the evolutionary history of individual plant families.

Sequence Analysis

The cladistic method of sequence analysis is a powerful tool to study phylogenetic relationships of taxa at all levels. In addition to 18S rRNA genes, the *rbcL* gene is widely used for phylogenetic classification (Chase et al., 1993[4]; Clegg, 1993[6]; Freshwater et al., 1994[8]; Delwiche and Palmer, 1996[7]; Rodman et al., 1996[21]). The reliability of this analytical approach is widely accepted (Soltis and Soltis, 1995[25]).

Two branches of *rbcL* genes arose from a eubacterial ancestor gene (Shimada et al., 1995[23]); these branches differ in the C-terminal sequence of *rbcL* (Table **1**). One branch includes the Rhodophyta, Chrysophyta, Bacillariophyta, Cryptophyta, Chromophyta, and Haptophyta, whereas the other branch includes the Chlorophyta, Glaucophyta, and Euglenophyta. The prokaryotic cyanobacteria and all the Metaphyta also belong to the latter group. Both groups have characteristic C termini which are different in length and composition.

The algae of the first group have longer *rbcL* sequences throughout (485 amino acid residues) with a common pattern at the C terminus. Y T S T **D** T A/P D F V/A E T/V X T X X X (D-473 in bold, compiled in Table **1**). This group occurs predominantly in the marine environment of tropical, moderate and cold-water zones under mainly isothermic conditions. The C-terminal part of the enzyme was recognized to play a significant role in determining enzyme kinetics (Portis, 1990[17]; Zhu et al., 1998[30]). Assuming a complementarity of the structure and function of Rubisco, the properties of the enzymes from rhodophytes should differ significantly from enzymes of chlorophytes. Indeed, the enzyme specificity of the former group is at least double that of the latter, i.e., the ratio of carboxylase to oxygenase activity is significantly higher for rhodophytes (SF = 129, *Porphyridium cruentum,* Read and Tabita, 1994[19]) compared to chlorophytes (SF = 62, *Chlamydomonas reinhardtii,* Chen et al., 1988[5]).

The second group varies considerably in length and charges. The C terminus of *rbcL* of cyanobacteria is short (475 amino acid residues), although, a marine species of *Synechoccocus* WH 7803 has a C terminus that is 7 residues longer (Watson and Tabita, 1996[28]). The short C terminus prevailed during the evolution of the Chlorophyta (Table **1**). The last strictly conserved amino acid residue is D-473 (numbering of spinach *rbcL*), which is extended by two additional residues. This type of enzyme (D + 2) can also be found in the Euglenophytes. Even in the non-photosynthetic, permanently bleached *Astasia longa* the C terminus is quite homologous to *Euglena gracilis.*

The conservation of the short form can be noticed in the embryophytes (Table **2**). The short form (D + 2) can be detected in the phylum of mosses, liverworts and ferns. The same form can be found in the gymnosperms of the spermatophyta (Table **3**). The consensus sequence for the conifers is DRL, for the cycadophytes DVL, and for the gnetophytes DTL.

Table 1 C-Terminal sequences of rbcL of eucaryotic algae

Chlorophyta	
Chlorella vulgaris	FE**T**I**D**TL
Chlamydomonas reinhardtii	F**D**TI**D***K*L
Glaucophyta	
Cyanophora paradoxa	FE**T**I**D**TI
Euglenophyta	
Euglena gracilis	FE**T**I**D***K*L
Astasia longa	F**D**TV**D***K*L
Rhodophyta	
Gelidium pulchellum	YTST**D**TA**D**FV**E**TPTA
Sarcodia dentata	YTST**D**TA**D**FV**E**TPTANV
Porphyra sp.	YTST**D**TA**D**FV**E**TPTANI
Galdiera sulphuria	YTST**D**TS**D**FV**E**TPTANI
Porphyridium sp.	YTST**D**TA**D**FVQTPTANV
Cyanidium caldarum	FTST**D**TA**D**FV**E**TPTANV
Antithamnion sp.	YTST**D**TA**D**FV**E**TPTANV
Trematocarpus dichotomus	YTST**D**TA**D**FV**E**TPTTSA
Polyneura latissima	YTST**D**TA**D**FV**E**TPTANV
Chrysophyta	
Pylaiella littoralis	YTST**D**TP**D**FV**E**VATE*S*R
Ectocarpus fasciculatus	YTST**D**TP**D**FV**E**VATG*S*R
Bacillariophyta	
Cylindrotheca sp.	YTST**D**TA**D**FAATSTANV
Cryptophyta	
Cryptomonas phi	YAST**D**TA**D**FV**E**TATAN*K*
Chromophyta	
Olisthodiscus luteus	YTST**D**TA**D**FV**E**TATSNP
Haptophyta	
Pleurochrysis carterae	YAST**D**TA**D**FV**E**TPTAN*R*

Contrary to the uniformity of the C termini as presented above, the Angiosperms show a greater variability in the *rbcL* genes (Table **4**). The shortest C terminus was recognized for the Fabaceae, *Medicago sativa* DN, only one residue past the conserved D, and the longest for the Rubiaceae, *Hofmannia,* with 16 residues past D-473. This length is only surpassed by the heterotrophic holoparasite *Cuscuta reflexa* which has a *rbcL* gene of 499 residues.

The analysis of the Angiosperms is aimed at two questions: (1) Was the elongation of the *rbcL* gene a random process which occurred in many families? and (2) Was the elongation process the result of selection that can be correlated to ecological factors? The length and composition (with emphasis on the number of charged amino acid residues) are listed in Table **4** for several families of monocotyledons and dicotyledons.

We investigated the C-terminal composition of the following orders (alphabetical listing): Apiales, Arales, Asterales, Batales, Bromeliales, Campanulales, Capparales, Caryophyllales, Celastrales, Commelinales, Cyperales, Dioscoreales, Dipsacales, Fabales, Gentianales, Geraniales, Lamiales, Myrtales, Pandanales,

Table 2 C-Terminal sequence of rbcL of Embryophyta

Bryopsida (mosses)		
Physcomitrella patens	Funariaceae	**D** TL
Sphagnum palustre	Sphagnaceae	**D** TV
Marchantiopsida (liverworts)		
Marchantia polymorpha	Marchantiaceae	**D** TL
Bazzania trilobata	Lepidoziaceae	**X** TL
Tracheophyta (vascular plants)		
Euphyllophyta		
Equisetum avense	Equisetaceae	**D** TL
Adiantum capillus-veneris	Adiantaceae	**D** TL
Lycopodiophyta		
Isoetes melanopoda	Isoetaceae	**D** TL
Selaginella sp.	Selaginellaceae	**D** TL
Lycopodium digitatum	Lycopodiaceae	**D** TL

Table 3 C-Terminal sequences of rbcL of Spermatophyta (Gymnosperms)

Coniferophytina		
Pinus thunbergii	Pinaceae	**D** RL
Pinus wallachiana	Pinaceae	**D** RL
Cathaya argyrophylla	Pinaceae	**D** TL
Amentotaxus argotaenia	Taxaceae	**D** RL
Taxodium distichum	Taxodiaceae	**D** RL
Cupressus sempervirens	Cupressaceae	**D** RL
Chamaecyparis obtusa	Cupressaceae	**D** RL
Diselma archeri	Cupressaceae	**D** RL
Juniperus conferta	Cupressaceae	**D** RL
Libocedrus plumosa	Cupressaceae	**D** RL
Platycladus orientalis	Cupressaceae	**D** RL
Fokienia hodginsii	Cupressaceae	**D** RL
Cycadophytina		
Zamia inermis	Zamiaceae	**D** VL
Encephalartos arenarius	Zamiaceae	**D** VL
Cycas circinalis	Cycadaceae	**D** VL
Bowenia serrulata	Cycadaceae	**D** VL
Gnetophytina		
Welwitschia mirabilis	Welwitschiaceae	**D** TL
Ephedra tweediana	Ephedraceae	**D** TL

Poales, Rubiales, Saxifragales, Scrophulariales, Solanales, Typhales and Velloziales (Table **4**). The number of representative species is, unfortunately, very diverse, i.e., some orders are represented by only one species, whereas the list for the order of Rubiales, Scrophulariales and Solanales are significantly longer.

The alignment of the 3′ sequences of the Asteraceae reveals the closely related nature of those species with the common sequence: **D** X L **D** X **D/E K D K** X **K** X **R.** Eleven of the thirteen amino acid residues are charged. Especially interesting is the repetition of the DK motif, which forms the predominant amino acid residues at the variable part of the terminal region. Despite the wide distribution of Asteraceae, ranging from the polar regions to tropical forests, a similarity of the *rbcL* end motif was observed. Two representatives of the Campanulales, *Sphenoclea zeylanica* (Campanulaceae) and *Scaevola frutescens* (Goodeniaceae), show significantly different termini. The vagueness between the boundaries of Asterales/Campanulales was recently reviewed (Wagenitz, 1997[27]).

The investigation is extended to an interesting order, the Caryophyllales, to determine whether the uniformity which was seen in the family of Asteraceae can be observed in this order as well. The phylogenetic analysis of the Caryophyllales is complicated by differing taxonomic perspectives (Rettig et al., 1992[20]). The classification of that order as a monophyletic unit that contains two suborders, Caryophyllineae (Caryophyllaceae and Molluginaceae) and Chenopodiineae, is based on the cladistic analysis of nucleotide changes in the *rbcL* gene (Rodman et al., 1984[21]). As revealed in Table **4**, the Chenopodiaceae (*Spinacea oleracea* and *Atriplex rosea*) show a short form of the C terminus (D T V) whereas other members of the Caryophyllales are provided with longer termini (D + 7 to D + 9).

The largest number (73 species) of sequencing data for the 3′ ends was accumulated in the family Rubiaceae. Their variability with respect to length and composition ranged from D + 4 to D + 17. The consensus sequence for this group shows that the first residues are quite conserved (D K/T L D K/P), with random variations in the following residues. Charged amino acid residues, like the DK motif, were found predominantly in species with longer C termini.

Similar extensions of the C termini were observed in the orders Lamiales and Capparales. The length of the C termini in Lamiales varied between D + 4 and D + 13, and for the Capparales between D + 4 and D + 10. It can be noticed that, in the order of Gentianales (as also in other orders), the increase in length occurred in representatives of several families, like the Loganiaceae and the Gentianaceae. The very large order Saxifragales, with its ecological and taxonomic complexity, contained mainly the short form of the terminus (DTL), with one exception that is listed in Table **4**.

Most of the species of the orders (in alphabetical order) Balanopales, Cornales, Euphorbiales, Hamamelidales, Malvales, Proteales, Rhamnales, Rosales, Theales, Urticales and Violales have termini like DTL or DTI (data not shown). In the subclass of the Ranunculidae, the three families for which data were found (Papaveraceae, Berberidaceae, and Ranunculaceae) showed only short sequences (DTL, DTI, and DAI).

In the class of the Monocotyledoneae members of the orders of Asparagales, Bromeliales, Commelinales, Cyperales, Liliales, and Poales were compared. The C termini of the Poaceae have species with short (D + 2; *Zea mays, Saccharum officinarum*) to intermediate length (D + 4; *Oryza sativa, Triticum aestivum, Avena sativa;* D + 5, *Neurachne,* and D + 6 *Thurnia macrocephala*), whereas all the completely sequenced Bromeliales possess a uniform C terminus (D K L D K T/A K). Liliales are very similar, with a sequence (D K L D) that was also found in one species of Orchidaceae (*Epipactis helleborine*); Asparagales show some variations (D X X D).

Table 4 C-Terminal sequences of rbcL of Spermatophyta (Angiosperms)

Liliopsida (Monocotyledonae)		
Dioscoreales		
Dioscorea bulbifera	Dioscoreaceae	**D** TL**D**L
Arales		
Zantedeschia aethiopica	Araceae	**D** *K*I**D***R*VS
Gymnostachys anceps	Araceae	**D** P**D**V*KK*IQ
Liliales		
Veratrum palviflorum	Melanthiaceae	**D** *K*L**D**
Tulipa kolpakoskiana	Liliaceae	**D** *K*L**D**
Lilium superbum	Liliaceae	**D** *K*L**D**
Burchardia umbellata	Colchicaceae	**D** *K*L**D**
Pandanales		
Pandanus veitchii	Pandanaceae	**D** *K*L**D**NT
Typhales		
Typha latifolia	Typhaceae	**D** *K*L**D***K*
Commelinales		
Cartonema phylidroides	Cartonemataceae	**D** *K*L**D**VT
Stegolepsis allenii	Rapateaceae	**D** *K*L**D***K*V
Tradescantia sp.	Commelinaceae	**D** TV**D***K*V
Xyris involucrata	Xyridaceae	**D** P**DE**SPGA
Poales		
Zea mays	Poaceae	**D** TI
Saccharum officinarum	Poaceae	**D** TL
Avena sativa	Poaceae	**D** TI**DE**
Bromus inermis	Poaceae	**D** TI**D***K*
Oryza sativa	Poaceae	**D** *K*L**D**S
Puccinellia distans	Poaceae	**D** TI**D**N
Triticum aestivum	Poaceae	**D** TI**D***K*
Neurachne munroi	Poaceae	**D** TV**D***K*V
Neurachne tennifolia	Poaceae	**D** TV**D***K*V
Thurnia macrocephala	Thurniaceae	**D** *K*L**D***K*S*K*
Cyperales		
Gahnia deusta	Cyperaceae	**D** *K*L**D***R*
Rhynchospora fascicularis	Cyperaceae	**D** TI**D***K*
Eriophorum viridi-carinatum	Cyperaceae	**D** *K*L**D***K*A*K*
Kobresia simpliciscula	Cyperaceae	**D** *K*L**D***K*A*K*
Oxychloe andina	Cyperaceae	**D** *K*L**D***K*A*K*
Prionium serratum	Cyperaceae	**D** *K*L**D***K*S*K*
Bromeliales		
Aechmea chantinii	Bromeliaceae	**D** *K*L**D***K*T*K*
Ananas comosus	Bromeliaceae	**D** *K*L**D***K*T*K*
Catopsis montana	Bromeliaceae	**D** *K*L**D***K*T*K*
Hechtia montana	Bromeliaceae	**D** *K*L**D***K*A*K*
Puya dyckioides	Bromeliaceae	**D** *K*L**D***K*A*K*
Tillandsia elisabethae	Bromeliaceae	**D** *K*L**D***K*A*K*
Velloziales		
Vellozia sp.	Velloziaceae	**D** *K*L**D**P**E***KK*

Magnoliopsida (Dicotyledonae)		
Caryophyllales		
Spinacea olearia	Chenopodiaceae	**D** TV
Atriplex rosea	Chenopodiaceae	**D** TV
Alluaudia procera	Didieraceae	**D** TL**D***KKK*G
Basella alba	Basellaceae	**D** TL**D***KKK*G
Gisekia pharnacioides	Phytolaccaceae	**D** IL**D***KKK*S
Rivina humilis	Phytolaccaceae	**D** IL**D***KKK*S
Mollugo verticillata	Molluginaceae	**D** VL**D***KA*K*K*
Pereskia aculeata	Cactaceae	**D** TL**D***KKK*G
Schlumbergera truncata	Cactaceae	**D** TL**D***KKK*G
Mirabilis jalapa	Nyctaginaceae	**D** VL**D***KKK*N
Mesembryanthemum crystallinum	Aizoaceae	**D** VL**D***KKKK*
Trianthema portulacastrum	Aizoaceae	**D** VL**D***KKKK*
Portulaca grandiflora	Portulacaceae	**D** VL**D***KK*N*KK*
Bougainvillea glabra	Nyctaginaceae	**D** VL**D***K***E***K*V**E***K*
Phytolacca americana	Phytolaccaceae	**D** VFH*KG*K*KK*N
Stegnosperma halmifolium	Stegnospermataceae	**D** TL**D***K***D***KKK*G
Saxifragales		
Saxifraga cernua	Saxifragaceae	**D** TL
Saxifraga intergrifolia	Saxifragaceae	**D** TL
Saxifraga mertensiana	Saxifragaceae	**D** TL
Saxifraga oppositifolia	Saxifragaceae	**D** TL
Lepuropetalon spathulatum	Saxifragaceae	**D** TL
Pterostemon rotundifolius	Grossulariaceae	**D** TL
Tetracarpaea tasmanica	Grossulariaceae	**D** TL
Eremosyne pectinata	Saxifragaceae	**D** TL**D***K*
Myrtales		
Montinia caryophyllacea	Onagraceae	**D** *K*L**D**P*KK***D**AI*K*
Fabales		
Medicago sativa	Fabaceae	**D** N
Pisum sativum	Fabaceae	**D** TL
Maackia floribunda	Fabaceae	**D** *K*L**D***K*L
Sophora bhutanica	Fabaceae	**D** *K*L**D***K*L
Tephrosia heck-manniana	Fabaceae	**D** TFVIQ
Baphia massaiensis	Fabaceae	**D** TF**E**SNNY*R*SIL
Amorpha fructicosa	Fabaceae	**D** TLNPVITV*R*FINCN
Angylocalyx braunii	Fabaceae	**D** TCNPVITV*R*SINCN
Geraniales		
Floerkea proserpinaco	Limnanthaceae	**D** **E**L**D**V**E***VK*
Limnanthes douglasii	Limnanthaceae	**D** **E**L**D**V**E***VK*
Celastrales		
Azima tetracantha	Salvadoraceae	**D** *K*L**D**LT*K*SN
Capparales		
Capparis hastata	Capparaceae	**D** *K*L**D**V
Koeberlinia spinosa	Capparaceae	**D** *K*L**D**V

continued next page

Table 4 continued

Arabidopsis thaliana	Brassicaceae	**D** *K*L**D**GG**D**
Brassica oleracea	Brassicaceae	**D** *K*L**D**GQ**D**
Setchellanthus caeruleus	Capparaceae	**D** *K*L**D**QV*K*
Reseda alba	Resedaceae	**D** *K*L**D**VFAA
Cleome hassleriana	Capparaceae	**D** *K*L**D**VAAAYIN
Batales		
Gyrostemon sp.	Gyrostemonaceae	**D** *K*L**D**GPV**E***K*F**D**
Gyrostemon tepperi	Gyrostemonaceae	**D** *K*L**D**GPV**E***K*F**D**
Tersonia cyathiflora	Gyrostemonaceae	**D** *K*L**D**GPV**E***K*F**D**
Batis maritima	Batidaceae	**D** TI**D***K*L**D**PT*K*S*K*
Apiales		
Mackinlaya macrosciadia	Araliaceae	**D** TL**D***K*
Centella erecta	Apiaceae	**D** TL**D***KK*
Micropleura renifolia	Apiaceae	**D** TL**D***VK*
Panax quinquefolius	Araliaceae	**D** IL**D**VV
Gentianales		
Nicodemia diversifolia	Loganiaceae	**D** TL**D***K*
Kopsia fructicosa	Apocynaceae	**D** TL**D**T
Gentianella rapunculoides	Gentianaceae	**D** TL**D**V
Spigelia anthelmia	Loganiaceae	**D** TL**D**PS
Gelsemium sempervirens	Loganiaceae	**D** TL**D**CA
Mostuea brunonis	Loganiaceae	**D** TL**D**LV*K*
Plocosperma buxifolium	Loganiaceae	**D** TL**D***KVK*
Anthocleista grandiflora	Loganiaceae	**D** TL**D**PL*K*S
Exacum affine	Gentianaceae	**D** TL**D**PL*K*S
Fagrae sp.	Loganiaceae	**D** TL**D**PL*K*S
Strychnos mux-vomica	Strychnaceae	**D** TL**D**PL*K*S
Gentiana procera	Gentianaceae	**D** *K*L**D**PL*K*S
Rubiales		
Burchellia bubalina	Rubiaceae	**D** TL**D***K*
Cubanola domingensis	Rubiaceae	**D** TL**D**L
Oldenlandia corymbosa	Rubiaceae	**D** TL**D***K*
Catesbaea spinosa	Rubiaceae	**D** TL**D**P
Tamridaea capsulifera	Rubiaceae	**D** *K*L**D***K*
Aidia micrantha	Rubiaceae	**D** TL**D***K*A
Antirhea lucida	Rubiaceae	**D** TL**D**PS
Aoranthe penduliflora	Rubiaceae	**D** TL**D***K*G
Calycophyllum candidissimum	Rubiaceae	**D** TL**D**PS
Canthium coromandelicum	Rubiaceae	**D** TL**D**PT
Cephalanthus occidentalis	Rubiaceae	**D** TL**D**PS
Chomelia sp.	Rubiaceae	**D** TL**D**PS
Coprosma pumila	Rubiaceae	**D** TL**D**VV
Declieuxia fructicosa	Rubiaceae	**D** TL**D**PQ
Euclinia longiflora	Rubiaceae	**D** NL**D***K*G
Gardenia thunbergia	Rubiaceae	**D** *K*L**D***K*V
Hallea rubrostipulata	Rubiaceae	**D** TL**D**PS
Hippotis sp.	Rubiaceae	**D** TL**D**PS
Hydnophytum formicarum	Rubiaceae	**D** TL**D**PQ
Ixora hookeri	Rubiaceae	**D** TX**D**PQ
Meyna tetraphylla	Rubiaceae	**D** TL**D**PA
Mussaenda arcuata	Rubiaceae	**D** TL**D**PA
Mussaenda erythrophylla	Rubiaceae	**D** TL**D**PS
Nauclea orientalis	Rubiaceae	**D** TL**D**PS
Neurocalyx zeylanicus	Rubiaceae	**D** TL**D**PS
Opercularia vaginata	Rubiaceae	**D** TL**D**RV
Ophiorrhiza mungos	Rubiaceae	**D** TL**D**VL
Pinckneya pubens	Rubiaceae	**D** TL**D**PS
Posoqueria latifolia	Rubiaceae	**D** TL**D**PS
Pseudomussaenda flava	Rubiaceae	**D** TL**D**PS
Sarcocephalus latifolius	Rubiaceae	**D** TL**D**PS
Bertiera breviflora	Rubiaceae	**D** TL**D**PTA
Feretia aeruginescens	Rubiaceae	**D** *K*L**D***K*I*K*
Genipa americana	Rubiaceae	**D** *K*L**D***K*VS
Glossostipula concinna	Rubiaceae	**D** *K*L**D**PV*K*
Heinsia crinita	Rubiaceae	**D** TL**D**PSA
Keetia zanzibarica	Rubiaceae	**D** TL**D**PE*K*
Kraussia floribunda	Rubiaceae	**D** *K*L**D***K*V*K*
Massularia acuminata	Rubiaceae	**D** *K*L**D***K*L*K*
Oxyanthus zanquebarius	Rubiaceae	**D** TX**D**PT*K*
Palicourea sp.	Rubiaceae	**D** TL**D**PE**G**
Pentanisia longituba	Rubiaceae	**D** TL**D**ES*K*
Pouchetia gilletii	Rubiaceae	**D** *K*L**D***K*V*K*
Pseudosabicea arborea	Rubiaceae	**D** TL**D***K*V*K*
Sukunia longipes	Rubiaceae	**D** TL**D***K***E**A
Virectaria major	Rubiaceae	**D** *K*L**D***K*VA
Calochone redingii	Rubiaceae	**D** *K*L**D***K***E**T*K*
Casasia clusiifolia	Rubiaceae	**D** *K*L**D***K*TTP
Chiococca alba	Rubiaceae	**D** TL**D***K*PSS
Cremaspora triflora	Rubiaceae	**D** *K*L**D***K*VI*K*
Deppea grandiflora	Rubiaceae	**D** TL**D**PE**R****E**
Enterospermum coriaceum	Rubiaceae	**D** *K*L**D***K*LV*K*
Erithalis fructicosa	Rubiaceae	**D** TL**D***K*AAS
Exostema caribaeum	Rubiaceae	**D** TL**D***R*PAS
Hillia triflora	Rubiaceae	**D** TL**D**PNPS
Kailarsenia ochreata	Rubiaceae	**D***K*L**D***K*VSS
Luculia grandifolia	Rubiaceae	**D** TL**D**PLAS
Rhachicallis americana	Rubiaceae	**D** TL**D**PQT*K*
Rogiera suffrutescens	Rubiaceae	**D** TL**D**P**D**PV
Rondeletia odorata	Rubiaceae	**D** TL**D**PQT*K*
Tarenna neurophylla	Rubiaceae	**D** *K*L**D***K*VI*K*
Aliberta edulis	Rubiaceae	**D** *K*L**D***K***E***K*G*K*
Coffea arabica	Rubiaceae	**D** *K*L**D***K***E***K***D**L
Lepectina platyphylla	Rubiaceae	**D** *K*L**D***K*P*K*P*K*
Paracoffea melanocarpa	Rubiaceae	**D** *K*L**D***K***E***K***E**L
Porterandia crosbyi	Rubiaceae	**D** *K*L**D***K*P*K***E**A
Amphidasya ambigua	Rubiaceae	**D** TL**D**T**D**L*K*P*K*
Didymosalpinx norae	Rubiaceae	**D** *K*L**D***K*VAP*KK*

continued next page

Table 4 continued

Pavetta lanceolata	Rubiaceae	**D** *K*LD*K*VID*KVK*
Tricalysia ovalifolia	Rubiaceae	**D** *K*LD*K*I*K*ID*K*A
Hoffmannia sp.	Rubiaceae	**D** LLDN*R*S*R*LCHNYLLFS
Psilanthus mannii	Rubiaceae	**D** *K*LD*K*E*K*D*K*E*K* **D** *K*E*K*EL
Hamelia cuprea	Rubiaceae	**D** TLDPESDSIIITFCFLS
Dipsacales		
Valeriana officinalis	Valerianaceae	**D** TCNQ
Scrophulariales		
Arrabidea pubescens	Bignoniaceae	**D** VLD*K*
Collinsia grandiflora	Scrophulariaceae	**D** TLDQ
Cycnium racemosum	Scrophulariaceae	**D** TLD*K*
Digitalis purpurea	Scrophulariaceae	**D** VLD*K*
Eccremocarpus scaber	Bignoniaceae	**D** TLDV
Antirrhinum majus	Scrophulariaceae	**D** TLDV
Martinella martinii	Bignoniaceae	**D** TLDM
Nematanthus hirsutus	Gesneriaceae	**D** TLD*K*
Halleria lucida	Scrophulariaceae	**D** TLDV
Callitriche heterophylla	Callitrichaceae	**D** TLD*K*
Celsia arturus	Scrophulariaceae	**D** TLD*K*
Orobanche corymbosa	Orobanchaceae	**D** TLD*K*
Orobanche fasciculata	Orobanchaceae	**D** TLD*K*
Verbascum thapsus	Scrophulariaceae	**D** TLD*K*
Lathraea clandestina	Scrophulariaceae	**D** VLD*K*
Tecoma stans	Bignoniaceae	**D** TLD*R*
Campsis radicans	Bignoniaceae	**D** TLD*RK*
Schlegelia parviflora	Bignoniaceae	**D** *K*LD*K*H
Tabebuia heterophylla	Bignoniaceae	**D** TLDPE
Pandorea jasminoides	Bignoniaceae	**D** TLD*R*E
Alonsoa unilabiata	Scrophulariaceae	**D** TLD*K*EG
Amphitecna apiculata	Bignoniaceae	**D** TLD*RK*S
Crescentia portoricensis	Bignoniaceae	**D** TLDPE*K*
Kigelia africana	Bignoniaceae	**D** TLDPE*K*
Macfadyena unguis-cati	Bignoniaceae	**D** TLDPES
Oroxylon indicum	Bignoniaceae	**D** TLDPQ*K*
Streptocarpus holsti	Gesneriaceae	**D** TLDEE*KK*
Physostegia virginiana	Bignoniaceae	**D** TLDEE*KK*N
Ligustrum vulgare	Oleaceae	**D** TLDPSSD*K*
Justicia americana	Acanthaceae	**D** TLDPE*K*AE
Solanales		
Retzia capensis	Solanaceae	**D** TLDI
Borago officinalis	Boraginaceae	**D** *K*LDY
Nicotiana tabacum	Solanaceae	**D** VLD*K*
Petunia hybrid.	Solanaceae	**D** VLD*K*
Physalis alkekengi	Solanaceae	**D** VLD*K*
Solandra grandiflora	Solanaceae	**D** VLD*K*
Solanum lycopersicum	Solanaceae	**D** VLD*K*
Solanum tuberosum	Solanaceae	**D** VLD*K*
Datura stramonium	Solanaceae	**D** VLD*K*
Capsicum baccatum	Solanaceae	**D** VLD*K*
Goetzea elegans	Solanaceae	**D** TLD*K*E
Metternichia princeps	Solanaceae	**D** TLD*K*G
Nicandra physalodes	Solanaceae	**D** ILDN*K*
Schizanthus pinnatus	Solanaceae	**D** TLD*RK*
Schwenckia laterifolia	Solanaceae	**D** *K*LD*K*P
Heliotropium arborescens	Boraginaceae	**D** TLDPE*K*
Hydrolea ovata	Hydrophyllaceae	**D** TLDVS*K*
Ipomoea purpurea	Convolvulaceae	**D** TLDPDGN
Convolvulus tricolor	Convolvulaceae	**D** TLDPDE*KK*
Lamiales		
Glechoma hederacea	Lamiaceae	**D** TLD*K*
Verbena bonariensis	Verbenaceae	**D** TLD*K*
Salvia divinorum	Lamiaceae	**D** TLD*K*
Teucrium fructicans	Lamiaceae	**D** TXDPE
Euthystachys abbreviata	Stilbaceae/ Verbenaceae	**D** TLDT*R*
Callicarpa dichotama	Verbenaceae	**D** TLDPE*K*
Clerodendrum fragrans	Verbenaceae	**D** TLDPN*R*
Stilbe vestita	Verbenaceae	**D** TLD*KGR*
Pogostemon heyneanus	Lamiaceae	**D** TLDEE*K*DEE*KAEK*
Campanulales		
Sphenoclea zeylanica	Campanulaceae	**D** TLDP*K*
Scaevola frutescens	Goodeniaceae	**D** TLDVA*K*
Asterales		
Senecio mikanioides	Asteraceae	**D** TLDDD*K*D*K*D*KKR*
Felicia bergeriana	Asteraceae	**D** TLDDD*K*D*KTKLR*
Eupatorium atrorubens	Asteraceae	**D** TLDTD*K*D*K*D*KKR*
Helianthus annuus	Asteraceae	**D** PLDTD*K*D*K*D*KKR*
Chrysanthemum maximum	Asteraceae	**D** TLDDD*K*D*K*D*KKR*
Blennosperma nana	Asteraceae	**D** TLDGD*K*D*K*D*KKR*
Achillea millefolium	Asteraceae	**D** TLDGD*K*D*K*D*KKR*
Dimorphotheca pluvialis	Asteraceae	**D** TLDTD*K*D*K*D*KKR*
Flaveria bidentis	Asteraceae	**D** TLDTD*K*D*K*D*KKR*
Flaveria pringlei	Asteraceae	**D** TLDTD*K*D*K*D*KKR*
Chromolaena sp.	Asteraceae	**D** VLDTE*K*D*K*D*KKR*
Tagetes erecta	Asteraceae	**D** TLDTD*K*D*K*D*KKR*

The data of Table **3** and **4** are presented in Fig. **1** to visualize the connections between the extension of *rbcL* and the relations and classifications of the Angiosperms, adopting and modifying the scheme of Barthlott (1991[3]). The numbers in the fields correspond to the number of amino acid residues beyond D-473. The orders in the centre of the scheme represent the "oldest", and at the periphery, the "younger" ones. As discussed below, the data in Fig. **1** suggest that the longer forms developed from the ancient D + 2 form and are more distinct at the periphery. The critical assessment of this suggestion will be discussed in detail below.

Conclusions

The proposed hypothesis concerning the evolutionary adaptation of the C terminus of *rbcL* is based on structural and functional evidence of Rubisco. The enzyme mechanism for the RuBP carboxylase and for the oxygenase reactions are de-

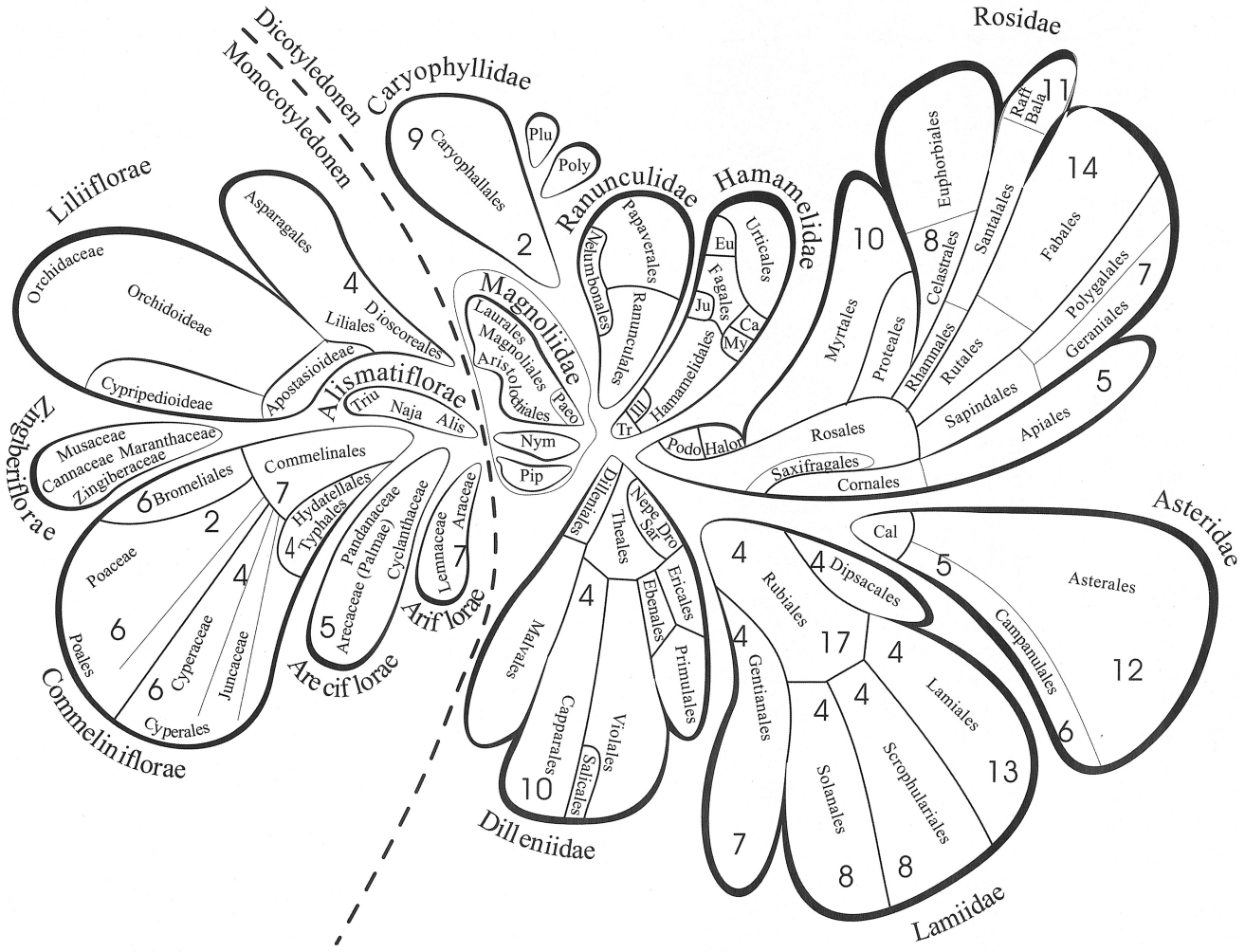

Fig. 1 Classification of Angiosperms according to Barthlott (1991). In more recent schemes the Rosidae and Dilleniidae are merged, and the Hamamelidae are split and combined to different groups. The basic information presented in this scheme is the arrangement of the more primitive groups in the centre and the more derived ones at the periphery. The numbers in the fields represent the length of the C terminus as D-473 plus additional residues. It is striking that the longer C termini are confined to more derived orders, with the only exception being the large and heterogenous Rubiales.

scribed and the multiple steps during the catalytic reactions are well documented (Hartman and Harpel, 1994[10]; Gutteridge and Gatenby, 1995[9]). The catalytic cycle starts with the enzyme molecules in the open conformation. A rearrangement of structural entities occurs after the binding of the substrate RuBP in the active centre, and proceeds with the activation of substrate in the formation of the enediol compound. Fig. **2** shows a steric view of the protomeric unit of Rubisco: the loop 6 segment (medium grey) folds over the binding site and the C-terminal segment (dark grey) stretches over the protein surface to keep loop 6 in the closed position (Newman and Gutteridge, 1993[16]). After the reaction has proceeded to form 3-PGA, only or in the case of photorespiration 3-PGA with 2-phosphoglycolate, the C-terminal segment rolls up to form an α helix and loop 6 returns into the open position to release the enzyme products (Schreuder et al., 1993[22]). The movements of the protein segments are rather drastic, and the terminal residue moves by 35 Å. The C-terminal segment is anchored onto the protein surface by salt bridges, such as D-473 – R-134, and by several hydrogen bonds (Knight et al., 1990[14]). The formation and release of the contacts are parts of the energetics of the enzyme reactions. Furthermore, the temperature dependence of the enzyme reaction kinetics is partially determined by those contacts. The activation enthalpy for the carboxylase reaction was measured in the range of 10 to 35° for spinach (short end: **D**TV) and for Rubisco isolated from a member of the Asteraceae, *Flaveria pringlei* (long end: **DTLDTDKDKDKKR**). The $\Delta H^{\ddagger}$ value was equivalent to between 16.4 kcal mol^{-1} (spinach) and 26 kcal mol^{-1} (*F. pringlei*), i.e., the relative activity for *F. pringlei* at 35° was two-fold better compared to the spinach enzyme (Zhu et al., 1998[30]). These authors suggested that the enzyme with a longer C terminus arose as an adaptive improvement for carboxylation at higher temperatures, and therefore, the high temperature-adapted Rubisco contributed to this evolutionary process (Zhu et al., 1998[30]).

However, it should be noted that species in the genus *Flaveria* can fix carbon dioxide by three pathways: C_3, C_3–C_4 intermediate, and C_4 (Wessinger et al., 1989[29]). *Flaveria pringlei* (C_3 species) and *Flaveria bidentis* (C_4 species) have identical C termini and similar k_{cat}/K_M (CO_2) ratios (Hudson et al., 1990[11]). It was suggested that C_4 plants evolved from C_3 plants and that C_3–C_4 species are evolutionary intermediates

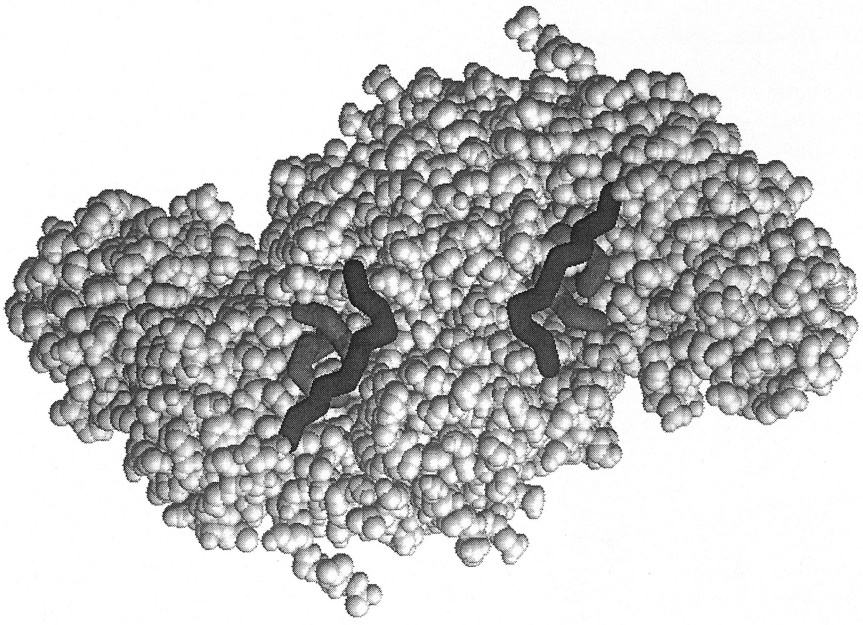

Fig. 2 Structure of the *Synechococcus* PCC6301 Rubisco dimer (L_2). The protein surface was modelled by RASMOL using the file 1 rbl.pdb (Brookhaven database). The loop 6 segment which covers the binding niche for RuBP (shaded in medium grey) consists of the residues 325–340; the C-terminal region is presented in dark grey and consists of the residues 465–475, the C-terminal residue V-475 is located on the short side of the L-shaped tail.

(Powell, 1978[18]; Apel and Maass, 1981[1]; Moore, 1982[15]). The homology between the C termini of the Asteraceae indicates that Rubisco kept the elongated C terminus as an advantage despite the divergent development of the carbon fixation pathways. During the divergence of the species, three changes in the *rbcL* sequence of *F. pringlei* and *F. bidentis* occurred (E 149 A, V 265 I, and M 309 I) which are neither close to the active centre nor to the contact area of the protein surface and the C terminus.

We will address two questions to discuss the evolutionary processes in the development of *rbcL*: first, splitting of the evolutionary tree into two branches, and second, the possible interpretation of the C-terminal elongation as an optimization process.

As already mentioned above, two different types of C termini evolved in eukaryotic organisms: One group, including the Rhodophytes, is characterized by mainly hydrophobic amino acids and several threonine residues; and the other group, including the Metaphytes, contain mainly the DK motif with alternating negatively and positively charged amino acid residues (Table **1** and **4**). It is interesting to compare the two types of organisms with respect to the kinetic properties of their Rubiscos. The SF value is higher in red algae compared to higher plants, in contrast, the k_{cat} values are higher in higher plants (Uemura et al., 1996[26]; Read and Tabita, 1994[19]). The higher specificity was achieved at the cost of the maximum carboxylation rate, since both are negatively correlated (Bainbridge et al., 1995[2]). Marine algae live under isothermic conditions and, therefore, the free energy requirement of the movement of the C termini would be mainly entropic and temperature independent, whereas the movement of C termini of higher plant Rubisco would be rather enthalpic and, therefore, strongly temperature dependent (Zhu et al., 1998[30]). The evolutionary optimization of Rubisco in the red algae occurred with respect to the enzyme specificity, whereas the land plants have a wider temperature range with reasonable carboxylation rates. The advantage of a longer C terminus is manifested by higher carbon fixation rates at higher temperatures. The younger families, such as the Asteraceae, have most of their species in the flora of temperate zones (approx. 12% in Central Europe, Jacob et al., 1981[12]).

We conclude that the elongation of *rbcL* occurred step by step. The ancient form of *rbcL*, as D(473)XX in cyanobacteria, was passed on to green algae and lower plants. The elongation event started in higher plants and was developed fully in the youngest families, such as the Asteraceae (Asteridae), Scrophulariales and Lamiales (Lamiidae), and Fabales and Batales (Rosidae). It is tempting to speculate that the length of the *rbcL* gene correlates with the evolutionary appearance and successful establishment of a family.

A pertinent question arises, why not all Rubisco molecules developed during evolution to include the best properties, like the Rhodophytes. A trivial answer might be that there was less time for development in the Angiosperms compared to the Rhodophytes. However, this argument does not hold up in comparing all angiosperms. The more ancient orders had more time during their evolution to optimize than the younger orders. The problem is more complicated in the sense that the extension of the C terminus must be linked to a double adjustment process, i.e., the C terminus itself and the protein surface with which it interacts. A comparison of the adjacent protein surface area to the C terminus in the Rhodophytes and in the Angiosperms reveals that the charge density at the protein surface is considerably higher in the latter group.

The hypothesis which we put forward is based on several new results of phylogenetic analysis and biochemical data on Rubisco. The sequencing data are rather limited to a small number of complete sequences, despite the large number of PCR amplified DNA sequences. A more comprehensive assessment should be possible when larger quantities of sequencing material are available. The argument that the C terminus is too variable and therefore should be ignored for similarity analysis should be turned around to indicate that the sequence of this domain offers a new and powerful tool to examine the development of phylogenetic divergence.

We acknowledge that the sequences that were picked were chosen because they were available from the data bank, and no attempt was made to add additional material. The published material is mainly the result of particular taxonomic problems with rearrangements of diverse families to orders. With the presentation of these ideas, we encourage other workers to extend *rbcL* sequence analysis to the 3′end to obtain more data for critical assessment.

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G. F. Wildner

Lehrstuhl für Biochemie der Pflanzen
der Ruhr-Universität Bochum
Universitätstr. 150
D-44780 Bochum
Germany

E-mail: Guenter.Wildner@ruhr-uni-bochum.de

Section Editor: F. Salamini